Fetuin A Level in Type 2 Diabetic patients, Gezira State-Sudan

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Fetuin A Level in Type 2 Diabetic Patients, Gezira State-Sudan

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Dedication

To my Dear Father

To my Beloved Mother

To my Sisters and Brothers
AKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor, Dr. Hani Yousif Zaki for his valuable advice and great guidance. Thanks is also extended to all members of the Biochemistry Department, Faculty of Medicine, University of Gezira. I would like to offer my special thanks to my colleagues for their help. A lot of thanks is extended to the staff of Abo Aagla Diabetic Center for helping in collection of sample.

I will never forget the very valuable assistance that offered to me from my aunt Nada Abed Alla. My deepest gratitude also goes to my parents, sisters, and brothers for their unequivocal support and love throughout my study.
Fetuin A Level in Type 2 Diabetic Patients, Gezira State-Sudan

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Abstract

Diabetes is one of the prevalent diseases in Sudan. It is heterogeneous group of symptoms characterized by elevation of fasting blood glucose (≥126 mg/dl). Diabetes is classified into two types, type 1, which is characterized by absolute deficiency of insulin and type 2, which is characterized by combination of β cells dysfunction and insulin resistance. Insulin resistance play key role in pathogenesis of type 2 diabetes. Serum fetuin A level were strongly and independently associated with metabolic syndrome and its associated complications. The aim of this study is to examine the relation between Fetuin A and insulin resistance in type 2 diabetic Sudanese patients attending Abo Aagla diabetic care centre in Wad Medani, Gezira State, Sudan. The study included 90 type 2 diabetic patients. Blood specimens were collected from participants and biochemical parameters were analyzed according to standard laboratory methods. Our findings could not demonstrate an association of fetuin-A with IR in patients with T2DM and the mean levels of serum fetuin A were (485.082 ± 27.280) mg/L and no difference between males and females. Fetuin A showed positive significant correlation with HbA1C (r = 0.237, p = 0.024). In stepwise multivariate regression analysis, Only HbA1c remained independent from other contributing factors for the fetuin-A levels analysis (β = 0.318, p = 0.009). The association between high fetuin-A and metabolic syndrome has been established. Overall, 43.3 % (n=39) of participants met criteria for the metabolic syndrome. T2DM patients are more likely to develop metabolic syndrome when the fetuin A level greater than 500 mg/l (OR = 9.75  p value = 0.003). To sum the fetuin A level is significantly associated with increased BMI and poor glycemic control in type 2 diabetic patients. Properly designed prospective studies are required to confirm the association of serum fetuin-A and the development of T2DM in order to explicate possible guidelines to treat or prevent diabetes and its associated complication.
مستوى فتوين-أ في مرضى السكري من النوع الثاني، ولاية الجهراء، السودان

أيناس يعقوب على إبراهيم

ملخص الدراسة

مرض السكري أحد الأمراض المنتشرة في السودان ويتميز بارتفاع معدل سكر الدم أكثر من أو يساوي (126 mg/dl) يقسم مرضى السكري إلى نوعين النوع الأول يتميز بنقص حاد في هرمون الإنسولين النوع الثاني و الذي ينتج أما من اضطرابات وظيفية في خلايا بيتا أو مقاومة الإنسولين. مقاومة الإنسولين تلعب دوراً رئيسيًا في حدوث مرض السكري من النوع 2. الهدف من هذا البحث هو دراسة العلاقة بين الجلوكوسيتر. ومقاومة الإنسولين لمرضى السكري المرضى (النوع الثاني) السودان الذين يعانون على مركز أبو عاعلة لرعاية مرضى السكري. ضمت الدراسة 90 مريضاً (ذكور و إناث). جمعت عينات دم من المشاركون وجرت تحليل القياسات الكيميائية وفقاً للطرق المختبرية المناسبة. أظهرت النتائج عدم وجود علاقة بين فتوين-أ و مقاومة الإنسولين في المرضى الذين يعانون من مرض السكري النوع الثاني و لا يوجد فرق بين الذكور والإناث في مستوى فتوين-أ. أظهرت الدراسة وجود علاقة إيجابية مع نسبة خضاب الدم السكري (r = -0.237, p = 0.024). وجد أن 33% من المشاركون تتوافق مع معايير متلازمة الأبيض. وكانت المستويات الوسطى من فتوين-أ أعلى بكثير في المرضى الذين يعانون من متلازمة التمثيل الغذائي بالمقارنة مع المرضى الذين لا يعانون من متلازمة التمثيل الغذائي. ونوصي بدراسة مستقبليه لتوضيح العلاقة بين فتوين-أ ومرض السكري النوع الثاني واستخدامه في مجالات علاجية وعلاج مرض السكري ومضاعفات.
### LIST OF ABBREVIATIONS

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<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<td>HbA1c</td>
<td>Glycated Hemoglobin</td>
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<td>IR</td>
<td>Insulin Resistance</td>
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<tr>
<td>HOMA – IR</td>
<td>Homeostasis Model Assessment – Insulin Resistance</td>
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<td>MS</td>
<td>Metabolic Syndrome</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
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<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidyl Inositol 3-Kinase</td>
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<tr>
<td>IRS -1</td>
<td>Insulin Receptor Substrate -1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Ras – Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>IL -1</td>
<td>Inter Leukin -1 beta</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immune Sorbent Assay</td>
</tr>
<tr>
<td>IEC</td>
<td>International Expert Committee</td>
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<tr>
<td>ESRD</td>
<td>End Stage Renal Disease</td>
</tr>
<tr>
<td>ATP III</td>
<td>Adult Treatment Panel III</td>
</tr>
<tr>
<td>BP</td>
<td>Blood Pressure</td>
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<tr>
<td>ADA</td>
<td>American Diabetic Association</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

Background

Diabetes Mellitus (DM) is a metabolic disorder characterized by chronic hyperglycaemia and disturbances of carbohydrate, fat and protein metabolism (Ahmed, 2002). The vast majority of cases of DM falls into two broad etiopathogenetic categories: type 1 and type 2 DM (T1DM and T2DM, respectively). T1DM, previously named insulin-dependent diabetes or juvenile-onset diabetes, results from cellular-mediated autoimmune destruction of pancreatic β cells; therefore, patients are dependent on exogenous insulin. Individuals with T1DM are considered to have a genetic predisposition, although environmental factors, such as dietary components, also contribute to T1DM development (Dib and Gomes, 2009). T1DM appears predominately in children and young adults and affects 5%–10% of diabetic patients (L’Heveder and Nolan, 2013).

T2DM is chronic disorder caused by insulin secretion deficiency and insulin resistance. T2DM is a complex trait that results from the contribution of many genes (Hansen and Pedersen, 2005), many environmental factors, including diet (Schulze and Hu, 2005) and the interactions among these genes and environmental factors. T2DM is more common among individuals aged 40 to 60 years and accounts for most cases of DM (more than 90%) (L’Heveder and Nolan, 2013). T2DM represents a major global public health hazard and, together with obesity, constitutes an important contributor to the expected decline in life expectancy (Olshansky, et al., 2005).

1.1.1. Epidemiology of T2DM

The number of people with T2DM is increasing in every country with 80% of people with DM living in low- and middle-income countries. Mortality of 4.6 million was reported in 2011. It is estimated that 439 million people would have T2DM by the year 2030 (Chamnan, et al., 2011). In 2011, about 14 million individuals were estimated to have diabetes in Africa, and this is expected to rise to 28 million by 2030 (Bos and Agyemang, 2013).
The incidence of T2DM varies substantially from one geographical region to the other as a result of genetic diversity, environmental and lifestyle risk factors. (Zimmet, et al., 2001).

1.1.2. Pathophysiology of T2DM

The pathophysiology of T2DM is complex: In addition to impaired insulin secretion from β-cells, reduced insulin sensitivity was found to play a predominant role in the pathogenesis of the disease (Stumvoll, et al., 2005). Although insulin resistance shows little variation among patients with T2DM, pancreatic β-cell function declines progressively over time. The United Kingdom Prospective Diabetes Study (UKPDS) and the Belfast Diet Study have shown that progressive loss of β-cell function is a major cause of hyperglycemia and is also related to treatment failure of diabetes (Lee, et al., 2014). Available data suggest that glucose regulation and the pathophysiology of diabetes show sex differences that may be particularly relevant in older adults (Regitz-Zagrosek, et al., 2006). For example, a markedly higher prevalence of post challenge hyperglycemia has been reported in older women compared with that in older men, whereas fasting hyperglycemia is more common in older men than in older women (Williams, et al., 2003).

Ongoing Studies documented the role of mitochondrial dysfunction in the development of insulin resistance and etiology of T2DM (Garcia-Roves, 2011). Further, adipocytokines from adipose tissue (i.e., leptin, TNF alpha, resistin, and adiponectin) are implicated in insulin resistance and possibly beta-cell dysfunction (Fujiocka, 2007, Park, et al., 2004). Adiponectin is known as an adipose tissue-derived insulin sensitizer, and it modifies glucose homeostasis and exhibits anti-inflammatory and anti-atherogenic effects (Kadowaki, et al., 2006). Insulin resistance is a major pathophysiological factor of diabetes, and adiponectin, given its strong association with insulin sensitivity and its underlying mechanism to sensitize insulin, is hypothesized to be centrally involved in the events leading to diabetes (Kadowaki, et al., 2006).

In contrast to adipokines which come from fat, fetuin-A is secreted from the liver (Ix, et al., 2012). In vitro, fetuin-A reversibly binds the insulin receptor tyrosine kinase in peripheral tissues thereby inhibiting the insulin-induced intracellular signal cascade, producing peripheral insulin resistance (Ix, et al., 2012).
Consistent with this function, fetuin-A knock-out mice are insulin sensitive (Mathews, et al., 2006) whereas wild-type mice treated with exogenous fetuin-A acutely develop insulin resistance (Hennige, et al., 2008).

1.1.3. Screening and Diagnosis of Diabetes Mellitus

Tests for screening and diagnosis of DM are readily available. Although about 25% of patients with T2DM already have microvascular complications at the time of diagnosis suggesting that they have had the disease for more than 5 years at the time of diagnosis (Olokoba, et al., 2012). It is still based on the American Diabetic Association (ADA) guidelines of 1997 or World Health Organization (WHO) National diabetic group criteria of 2006, which is for a single raised glucose reading with symptoms (polyuria, polydipsia, polyphagia and weight loss), otherwise raised values on two occasions, of either fasting plasma glucose (FPG) ≥7.0 mmol/L (126 mg/dL) or with an oral glucose tolerance test (OGTT), two hours after the oral dose a plasma glucose ≥11.1 mmol/L (200 mg/dL) (Cox EM, 2009).

The 1997 ADA recommendations for diagnosis of DM focus on the FPG, while WHO focuses on the OGTT (Cox EM, 2009). The glycated hemoglobin (HbA1c) is also still useful for determining blood sugar control over time. However, practicing physicians frequently employ other measures in addition to those recommended. In July 2009, the International Expert Committee (IEC) recommended the additional diagnostic criteria of an HbA1c result ≥6.5% for DM. This committee suggested that the use of the term pre-diabetes may be phased out but identified the range of HbA1c levels ≥6.0% and <6.5% to identify those at high risk of developing DM (International Expert, 2009).

1.1.4. Management of DM

Studies have shown that there was significant reduction in the incidence of T2DM with a combination of maintenance of body mass index of 25 kg/m², eating high fiber and unsaturated fat and diet low in saturated and trans-fats and glycemic index, regular exercise, abstinence from smoking and moderate consumption of alcohol (Olokoba, et al., 2012). Suggesting that majority of T2DM can be prevented by lifestyle modification. Patients with T2DM should receive a medical nutrition evaluation; lifestyle recommendations should be tailored according to physical and functional ability (Chiniwala and Jabbour, 2011).
1.1.5. Pharmacological Agents

Biguanides

Biguanides, of which metformin is the most commonly used in overweight and obese patients, suppresses hepatic glucose production, increases insulin sensitivity, enhances glucose uptake by phosphorylating GLUT-enhancer factor, increases fatty acid oxidation, and decreases the absorption of glucose from the gastrointestinal tract (Collier, et al., 2006).

Sulfonylureas

These generally well tolerated but because they stimulate endogenous insulin secretion, they carry a risk of hypoglycemia (Chiniwala and Jabbour, 2011). Elderly patients, with DM who are treated with sulfonylurea have a 36% increased risk of hypoglycemia compared to younger patients (Olokoba, et al., 2012).

Meglitinides

Repaglinide and nateglinide are non-sulfonylurea secretagogues which act on the ATP dependent K-channel in the pancreatic beta cells thereby stimulating the release of insulin from the beta cells, similar to sulfonylurea, though the binding site is different (Olokoba, et al., 2012). Meglitinides have a rapid onset and a short duration of action (4-6 hrs) and thus lower risk of hypoglycemia.

Emerging evidence has shed light on the role of Fetuin A as marker of insulin resistance and study of this protein have provided new insights to the biology of glucose regulation, and may also lead to identification of novel candidate therapeutic targets.

1.2. Fetuin A

Fetuin-A, also called Alpha 2-Heremans Schmid Glycoprotein (AHSG), is a multifunctional plasma agent with a molecular weight of approximately 64 kDa and half-life of several days (Ketteler, et al., 2003). It was first discovered in 1944 by Kai O. Pedersen in calf serum (Xu, et al., 2011). Several years later, Heremans (in 1960) and Schmid with Burgi (in 1961), in the independent studies, isolated it in humans (Jahren-Dechent, et al., 2011). During fetal development fetuin-A is abundantly synthesized by multiple tissues.
In adults it is secreted predominantly by the liver (>95%) (Stefan, et al., 2006). Serum fetuin-A concentration is a good indicator of liver cell function and it ranges from approximately 450–600 µg/ml in healthy individuals (Kalabay, et al., 2007).

1.2.1. Structure of Fetuin-A

Fetuin-A was isolated from fetal calf serum. It has three carbohydrate units, which are present on a peptide chain linked with threonine and serine residues (Yang, et al., 2008). It belongs to the class of cysteine proteinase inhibitors, which are responsible for bone resorption (Park, et al., 2007). Two chains A and B are encoded by a single mRNA transcript forming its structure (Olivier, et al., 2000). The A chain consists of 282 amino acid residues and it is a long chain. It contains 24% β-pleated sheet, 29% a-helix and 26% reverse turns (Osawa, et al., 2001). The B chain consists of 27 amino acid residues, and these amino acid residues are distributed unequally into the neutral and charged portion (Sato, et al., 2007).

1.2.2. Physiological effects of Fetuin-A

Fetuin-A is a physiological inhibitor of insulin receptor tyrosine kinase and thus associated with insulin resistance, metabolic syndrome (MS) and an increased risk for type 2 diabetes (Kaushik, et al., 2009). Fetuin-A has adipogenic properties, reduces expression of the atheroprotective adipokine adiponectin and deteriorates free fatty acid uptake and storage in adipocytes (Song, et al., 2011). Therefore fat accumulation in the liver may be associated with higher levels of fetuin-A (Singh, et al., 2012). There is a significant association among a higher level of serum fetuin and type-2 diabetes, serum albumin, body mass index and hypertriglyceridaemia (Burke, et al., 2007).

1.2.3. Clinical context of fetuin-A

Fetuin-A is associated with impaired glucose tolerance, fatty liver, metabolic syndrome and an atherogenic lipid profile. These conditions are demonstrated by hypoadiponectinaemia and subclinical inflammation (Hennige, et al., 2008). Insulin resistance is the primary abnormality leading to both metabolic syndrome and fatty liver disease. In insulin resistance the level of fetuin-A elevates and its level also increases in morbidly obese patients (Brix, et al., 2010). Deficiency of fetuin-A has been directly linked with uraemic vascular calcification (Ketteler, et al., 2003), its low level increases
the death rate in patients on dialysis and a high level is linked with cardiovascular complications in the non-renal patient (Lorant, et al., 2011). An elevated serum fetuin-A level is associated with the components of MS, such as high blood pressure, central obesity, high blood glucose and high triglycerides (Xu, et al., 2011).

1.2.4. Fetuin-A as a natural inhibitor of insulin receptor

Fetuin-A belongs with the cystatin family of the proteinase inhibitors and natural inhibitors of insulin receptor (Cintron, et al., 2001). Insulin signaling is controlled by complex processes as shown in Figure 1

**Figure 1** The action of insulin receptor. Two main pathways: the phosphatyl inositol 3-kinase (PI3-K)-AKT/protein kinase B (PKB) and the Ras-mitogen-activated protein kinase (MAPK) pathway. Fetuin-A could inhibit insulin receptor tyrosine kinase activity by inhibiting the autophosphorylation of tyrosine kinase and IRS-1, which leads to insulin resistance and acts as a natural inhibitor of insulin receptor.
The insulin receptor in the presence of insulin phosphorylates the insulin receptor substrate proteins (IRS proteins). These IRS proteins are linked to the two main pathways: the phosphatidyl inositol 3-kinase (PI3-K)–AKT/protein kinase-B (PKB) and the Ras-mitogen activated protein kinase (MAPK) pathway as shown in Figure 1 (Taniguchi, et al., 2006). Fetuin-A inhibits insulin receptor tyrosine kinase activity by inhibiting the autophosphorylation of tyrosine kinase and IRS-1 (Song, et al., 2011).

IRS protein plays an important role in regulating the insulin signaling pathway and regulates various cellular functions like glucose storage/transport, protein/fat metabolism, differentiation and growth of cells. Defects in the IRS protein causes metabolic disorders like insulin resistance and type-2 diabetes (Wilson, et al., 2006).

1.2.5. Role of Fetuin-A in diabetes

Fetuin-A is increased in insulin resistance and it is an independent predictor of type-2 diabetes, and is related to atherosclerosis (Brix, et al., 2010). Fetuin-A is associated with type-2 diabetes because in humans a higher concentration of it causes insulin resistance (Ix, et al., 2008). Susceptibility to T2DM is strongly related to the position of the gene encoding for fetuin-A. This gene is present on the 3q27 chromosome and this location is responsible for metabolic syndrome and type-2 diabetes (Ix, et al., 2008).

The association between elevated levels of fetuin-A and high risk of T2DM development is explained by mechanisms of insulin and fetuin-A actions (Kaushik, et al., 2009). Various circulating proteins like fetuin-A and adiponectin regulate insulin sensitivity and the gene for fetuin-A and human adiponectin is present on chromosome 3q27, almost next to one another. Previously, this location mapped as a metabolic syndrome and type-2 diabetes susceptibility locus (Hennige, et al., 2008). Adiponectin polymorphism showed to modulate the risk of T2DM, because the gene for adiponectin is located on chromosome 3q27, which is the susceptibility locus for T2DM and MS. Furthermore, the direct correlation of fetuin-A with visceral adiposity, observed in many diabetics, may lie on causal pathway between fetuin-A and incident diabetes (Ix, et al., 2008). It is proved
that some drugs-insulin-sensitizing medications such as metformin, pioglitazone and niacin may affect on the level of Fetuin-A in serum (Kaushik, et al., 2009).

Treatment with pioglitazone and metformin results in a decline in Fetuin-A levels (Kaushik, et al., 2009). Niacin treatment lowers serum Fetuin-A concentration as well and these change correlates with the beneficial changes in serum lipids (Kaushik, et al., 2009). Furthermore, a short-term diet and exercise interventions result in reduction of serum fetuin-A concentrations (Ix and Sharma, 2010).

1.2.5.1. Insulin resistance (IR)

Insulin resistance a physiological condition is marked by hyperglycemia and failure of cells to respond to normal action thus hyperinsulinemia. It is prevalent in individuals having genetic predisposition and family history of type 2 diabetes mellitus (Kaur, et al., 2014). Insulin resistance (IR) is one of the key pathophysiological mechanisms of T2DM, which may contribute to the development of T2DM and T2DM-associated complications. IR is also considered to be the common cause of dyslipidemia and hypertensive diseases (Yin, et al., 2014). The condition of insulin resistance or pre diabetes can be detected (Kaur, et al., 2014) and if managed with dietary, life style modifications and or pharmacological intervention (Matthaei, et al., 2000) can delay if not prevent the onset of type 2 diabetes mellitus in the population.

1.2.5.2. Measurement of IR

There are a number of methods to assess insulin sensitivity in the dynamic versus static state. Estimates of IR based on fasting insulin concentration may not be adequate in patients with Chronic Kidney Disease (CKD) as it largely reflects hepatic defects, whereas CKD impairs insulin catabolism (Pham, et al., 2011). Hyperinsulinemic euglycemic clamp is the gold standard for IR determination because it provides a direct measure of whole body sensitivity to insulin, primarily skeletal muscle. At lower doses of insulin infusion, the addition of labeled glucose can allow for specific assessment of the ability of insulin to suppress endogenous glucose production. This method can differentiate between peripheral IR and hepatic IR and provides a direct and precise IR measurement (Pham, et al., 2011). The IR status was evaluated by the homeostasis model
assessment–insulin resistance (HOMA-IR) index. The HOMA-IR score was available only in patients not receiving exogeneous insulin.

1.2.5.3. Homeostatic model assessment (HOMA)

Homeostatic model assessment of β-cell function and insulin resistance (IR) was first described in 1985. The technique is a method for assessing β-cell function and IR from basal glucose and insulin or C-peptide concentration. The HOMA model is derived from a mathematical assessment of the interaction between β-cell function and IR in an idealized model that is then used to compute steady-state insulin and glucose concentrations.

Insulin resistance was estimated by homeostasis model assessment of insulin resistance (HOMA-IR) as described by Mathews et al.: 
\[ \text{HOMA-IR} = \frac{\text{fasting insulin concentration (mU/mL) } \times \text{FPG (mmol/L)}}{22.5} \]

Homeostatic model assessment insulin resistance (HOMA-IR) value was calculated by 
\[ \text{HOMA-IR} = \frac{\text{Fasting Glucose (mg/dL) } \times \text{Fasting Insulin (uIU/mL)}}{405} \]

The insulin resistance was accepted as positive for patients with HOMA score ≥ 2.5 (Kaviani, et al., 2012).

1.2.5.4. HOMA Modeling by Using Fasting C peptide

C-peptide is a single chain 31 amino acid (AA 33-63) connecting (C) polypeptide with a molecular weight of approximately 3021 daltons. C-peptide, which is cleaved from insulin in secretory granules, is a well-known marker for β-cell function (Meier, et al., 2009). In contrast to other indices for insulin secretion, C-peptide evaluation is able to assess β-cell function even in patients undergoing insulin therapy. The modified HOMA formula were: 
\[ \text{HOMA IR (CP)} = 1.5 + \frac{\text{fasting blood glucose } \times \text{fasting C-peptide}}{2800} \]

(Li, et al., 2004).

1.2.6. Fetuin-A as a marker of cardiovascular disease

Due to its multiple functions, elevated or decreased serum Fetuin A concentrations may be tightly linked to the progression of various diseases, atherosclerosis, for instance (Lorant, et al., 2011). Two of the primary physiologic functions of Fetuin-A may be critically important to cardiovascular health. First, Fetuin-A acts as an inhibitor of
calcification by increasing the blood solubility of calcium and phosphorus, and preventing spontaneous mineral precipitation in the vasculature (Ketteler, et al., 2006). In end stage renal disease (ESRD) populations, lower plasma Fetuin-A levels are associated with greater prevalence and severity of vascular calcification and increased risk of CVD events and mortality (Wang, et al., 2005) independent of traditional CVD and kidney disease risk factors. On the other hand, decreased serum Fetuin A concentrations may directly limit cardiac functions by effectively promoting cardiac fibrosis and calcification and thus influence CVD progression (Lim, et al., 2013).

Fetuin-A excess may lead to insulin resistance and metabolic dysregulation which leads to atherosclerosis (Creager, et al., 2003). Insulin resistance, hyperglycaemia and dyslipidaemia are abnormalities associated with diabetes, and all these factors impair the normal function of endothelium and platelets. Due to these factors, arteries become susceptible to atherosclerosis (Beckman, et al., 2002). Normally, nitric oxide production increases due to stimulation of the nitric oxide synthetase of endothelial cell via the PI3K pathway, and hyperglycaemia decreases endothelium derived nitric oxide production. Thus, in insulin resistance vasodilation is reduced due to impairment of endothelium function, and is characterized by a decrease in the production of nitric oxide as well as an increase in the synthesis of vasoconstrictors like endothelin and prostanoids (Creager, et al., 2003). Some clinical studies have indicated the opposite trends high serum Fetuin-A level may be a sensitive marker of macrovascular complications in diabetics (Xu, et al., 2011). Both the MS and T2DM increase the risk of CVD (Singh, et al., 2012).

1.2.7. Fetuin-A in Metabolic Syndrome

The metabolic syndrome (MS) is defined as a constellation of metabolic risk factors that are associated with cardiovascular events and all-cause mortality (Klein, et al., 2002). Metabolic syndrome is estimated to affect 47 million Americans, including 40% of adults ≥60 years of age (Ford, et al., 2002). Insulin resistance is thought to be the primary underlying abnormality leading to metabolic syndrome (Deedwania, 2004). Several clinical studies proposed that high-serum Fetuin-A levels are associated with MS and that Fetuin-A therefore may present a risk factor for MS (Ix, et al., 2006).

The presence of MS was defined using a modified version of the Adult Treatment Panel III (ATP III) criteria. Briefly, four of the five MS components were defined using the
following ATP III categorisations: (1) high BP: ≥130/85 mmHg or the patients using anti-hypertensive agents, (2) hypertriglyceridaemia: ≥150 mg/dL, (3) low high-density lipoprotein cholesterol (HDL-C): <40 mg/dL in men and <50 mg/dL in women and (4) fasting blood glucose ≥ 100 mg/dl or the patients using hypoglycaemic agents.

The fifth component was defined based on the body mass index (BMI) because waist circumference measurements were not available for all the subjects. Classifying the subjects with a BMI ≥ 25 (kg/m^2) as having high central obesity.

The subjects with three or more of the above-mentioned criteria defined as having MS (Expert Panel on Detection and Treatment of High Blood Cholesterol in, 2001). Several arguments support the role of Fetuin-A in MS, the human Fetuin-A gene located on chromosome 3q27 which has been mapped as MS quantitative trait locus. It enhances insulin resistance which is thought to be the mechanism leading to the MS phenotype, and suppresses adiponectin that potentiates low-grade inflammation and atherosclerosis (Dabrowska, et al., 2015, Hennige, et al., 2008, Ix, et al., 2006, Kaushik, et al., 2009, Mathews, et al., 2002). Fetuin-A knockout mice showed resistance to weight gain when fed with high-fat diet (Mathews, et al., 2002).

Previous studies have demonstrated that Fetuin A has emerged as a biomarker for risk of T2DM and T2DM associated complications. Moreover, Fetuin-A are known to inhibit arterial calcification, and lower levels have been associated with subclinical CVD in some, but not all studies. On the other hand serum Fetuin A is absolutely related with visceral obesity and dyslipidemia also serum Fetuin A levels were strongly and independently associated with MS and its components result in CVD.
1.3. Justification of the Study
DM is a global health problem and one of the most common non communicable diseases of the twenty-first century. DM is a major cause of disability and reduced quality of life, and among the top five leading causes of mortality in affluent societies (Christos, et al., 2014). Poor glycemic control in diabetic patients accelerates the development of different diabetic complications such as microvascular complications (retinopathy and nephropathy) and cardiovascular disease. Vascular complications of T2DM account for the majority of social and economic burden among patients and societies (Salminen, et al., 2013). The existence of these complications leads to most of the diabetic death. Fetuin-A is a physiological inhibitor of insulin receptor tyrosine kinase and thus associated with insulin resistance, metabolic syndrome (MS) and an increased risk for type 2 diabetes (Kaushik, et al., 2009). So this study has shed light on the role of Fetuin A as marker of insulin resistance in T2DM Sudanese patients.

1.4. Study Objectives

General Objective
The aim of this study is to measure the level of fetuin A in Sudanese with type 2 diabetes mellitus and to examine the relation between serum Fetuin A and insulin resistance.

Specific objectives:
The specific objectives of this study were:

1. To determine the body mass index (BMI) of T2DM patients.
2. To measure fasting plasma glucose, glycated hemoglobin HbA1c, and serum C peptide in T2DM patients.
3. To assess the insulin resistance by using HOMA IR index.
4. To estimate the serum Fetuin-A level.
5. To correlate serum Fetuin A with the studied parameters.
6. To assess the level of serum Fetuin A in MS patients.
CHAPTER TWO
SUBJECTS, MATERIALS AND METHODS

2.1 Study Subjects, Area and Design
This study is cross-sectional study that included 90 Sudanese patients diagnosed with T2DM attending Abo Agla Diabetic Center, Wed Madeni, Gezira State, central Sudan. Patients who had renal dysfunction, severe cardiac problems, uncontrolled hypertension, or type1 diabetes were excluded.

2.1.2 Ethical approval
The study was approved by the Ethics Committee, Faculty of Medicine, University of Gezira. All the study patients were informed about the study objectives and acclaimed to participate.

2.2 Methods
2.2.1 Collection of Blood Samples
Venous blood samples were collected from each patient after > 8 h of overnight fasting. Two ml was put in a container with lithium heparin as anticoagulant, whole blood was used for measurement of HbA1c. Plasma sample was prepared and used for the measurement of glucose. Four ml was put in a plain container and samples were centrifuged for 20 minutes at 1000 rpm after clotting for 30 minutes at room temperature. Serum samples were subsequently stored at -20°C until further analysis of C-peptide and Fetuin-A levels.

2.2.2 Data collection
Weight and height were measured for the calculation of body mass index (BMI) for each participant. Questionnaire was used for collection of relevant medical history.
2.2.3 Biochemical Measurements

2.2.3.1 Fasting plasma glucose

Method: Glucose oxidase / peroxidase (code 12503)

Principle of the method: Glucose in the sample originates, by means of the coupled reactions described below, a coloured complex that can be measured by Spectrophotometer.

\[
\text{Glucose} + \frac{1}{2} \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Gluconate} + \text{H}_2\text{O}_2
\]

\[
2 \text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{Phenol} \rightarrow \text{Quinoneimine} + 4 \text{H}_2\text{O}
\]

2.2.3.2 Fasting C peptide

Method: The electrochemiluminescence immunoassay “ECLIA” was used cobas e immunoassay analyzers.

1st incubation: 20 μL of sample, a biotinylated monoclonal C peptide specific antibody, and a monoclonal C peptide specific antibody labeled with a ruthenium complex a react to form a sandwich complex. 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.

Results are determined via a calibration curve which is instrument specifically generated by 2- point calibration and a master curve provided via the reagent barcode.
2.2.3.3 Fasting Fetuin A

**Method:** Fetuin A level was measured using a commercially available enzyme-linked immunosorbent assay (ELISA)

**Principle of the method:** The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human Fetuin A (FETU-A) in samples. Fetuin A (FETU-A) is added to monoclonal antibody enzyme well which is pre-coated with Human Fetuin A (FETU-A) monoclonal antibody, incubation; then, added Fetuin A (FETU-A) antibodies labeled with biotin, and combined with Streptavidin-HRP to form immune complex; then carried out incubation and washing again to remove the uncombined enzyme. Then Chromogen Solution A, B is added, the color of the liquid changes into the blue, and at the effect of acid, the color finally becomes yellow. The chroma of color and the concentration of the Human Substance Fetuin A (FETU-A) of sample were positively correlated.

2.2.3.4 Glycated hemoglobin (HbA1c)

**Method:** The Labonacheck A1c is a boronate affinity assay.

**Principle of the test:** The labonacheck A1c HbA1c test kit consists of the cartridge. The R1/Reagent contains the agents that lyse erythrocytes and precipitate hemoglobin specifically as well as a blue boronic acid conjugate that binds cis–diol of glycated hemoglobin.

When blood is added to the R1/Reagent, the erythrocytes are lysed and all hemoglobin precipitates as well as the boronic conjugate bind to the cis–diol configuration of glycated hemoglobin. An aliquot of the reaction mixture is added to the cartridge and all the precipitate is evaluated by measuring the blue (glycated hemoglobin) and the red (total hemoglobin) color intensity respectively with labonacheck A1c HbA1c Analyzer the ratio between them being proportional to the percentage of glycated hemoglobin in the sample.
HOMA–IR calculation:
The insulin resistance status was evaluated by the homeostasis model assessment-insulin resistance (HOMA-IR) index. HOMA-IR was calculated by:
The modified HOMA formula were: HOMA IR (CP) = 1.5 + fasting blood glucose × fasting C-peptide / 2800 (Li, et al., 2004). the insulin resistance was accepted as positive for patients with HOMA score ≥ 2.5 (Kaviani, et al., 2012).

2.2.4 Statistical Analysis
Statistical analysis was carried-out using statistical package for social science (SPSS version 20). Data were reported as mean ± Standard Error of means or as number of participants (percentages). Differences in means of variables were evaluated using independent t test. Associations of Fetuin-A were tested by correlation and multivariate linear regression analysis. P value < 0.05 were considered to be statistically significant. One-way analysis of variance (ANOVA) was used to evaluate differences of means among tertiles of fetuin-A groups.
CHAPTER THREE
RESULTS

3.1 Characteristics of the study group
A total of 90 patients with T2DM were included in this study. The general characteristics of the study participants are presented in Table (3-1). The mean age of the participants was 50 years, 35.5% (n = 32) were males and 65.5% (n = 58) were females. The mean BMI was 26.4 Kg/m². The mean levels of serum Fetuin A were 485.082 ± 27.280 mg/L and no difference between males and females 484.01 ± 47.93 and 488.73 ± 33.82 p = 0.935 respectively.

Table 3-1: General Characteristics of the Participants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>50.25 ± 0.493</td>
</tr>
<tr>
<td>Male /Female (%)</td>
<td>35.5% / 64.5%</td>
</tr>
<tr>
<td>Duration of Diabetes Mellitus (Years)</td>
<td>5.70 ± 0.361</td>
</tr>
<tr>
<td>Body Mass Index (BMI) (Kg/m²)</td>
<td>26.36 ± 0.459</td>
</tr>
<tr>
<td>Fetuin A (mg/l)</td>
<td>485.082 ± 27.280</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mg/dl)</td>
<td>189.56 ± 7.132</td>
</tr>
<tr>
<td>Fasting C–peptide (ng/ml)</td>
<td>3.022 ± 0.155</td>
</tr>
<tr>
<td>HOMA -IR</td>
<td>5.273 ± 0.243</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.370 ± 0.123</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± Std. Error of means
Figure 2: Serum Fetuin A level in males and females diabetic patients

The participants were stratified according to the BMI index ≤ 25 as group 1 and > 25 as group 2. The characteristics of clinical parameters according to the BMI are shown in Table 3-2. In-between group differences were analyzed by a two-tailed independent t test. The levels of glucose, C peptide, HbA1c and HOMA did not show significant differences. The levels of fetuin A demonstrated an increasing significant trend as the BMI increases (427.469 vs 553.938, p = 0.02).

Table 3-2: The characteristics of clinical parameters according to the BMI groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1 (n=49)</th>
<th>Group 2 (n=41)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 25 kg/m²</td>
<td>&gt; 25 kg/m²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetuin A</td>
<td>427.45</td>
<td>553.94</td>
<td>0.02</td>
</tr>
<tr>
<td>HOMA</td>
<td>5.07</td>
<td>5.52</td>
<td>0.35</td>
</tr>
<tr>
<td>FBG</td>
<td>189.14</td>
<td>190.05</td>
<td>0.95</td>
</tr>
<tr>
<td>HbA1c</td>
<td>6.35</td>
<td>6.39</td>
<td>0.85</td>
</tr>
<tr>
<td>C- peptide</td>
<td>2.89</td>
<td>3.19</td>
<td>0.33</td>
</tr>
</tbody>
</table>
3.3 Correlation Analysis:
Pearson’s correlation analysis were performed to assess relationships between serum Fetuin-A levels and clinical parameters. Bivariate correlation analyses between serum fetuin-A and various clinical parameters are shown in Table 3-3. Serum fetuin-A levels showed significant positive correlations with HbA1c (r = 0.237, p = 0.024).

Table 3-3: Correlation of Serum Fetuin A with other Clinical Variables

<table>
<thead>
<tr>
<th>Parameter</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.148</td>
<td>0.165</td>
</tr>
<tr>
<td>Duration of DM</td>
<td>-0.034</td>
<td>0.753</td>
</tr>
<tr>
<td>BMI</td>
<td>0.133</td>
<td>0.211</td>
</tr>
<tr>
<td>Fasting Blood Glucose</td>
<td>-0.045</td>
<td>0.674</td>
</tr>
<tr>
<td>C peptide</td>
<td>-0.08</td>
<td>0.451</td>
</tr>
<tr>
<td>HOMA –IR</td>
<td>-0.091</td>
<td>0.395</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.237</td>
<td><strong>0.024</strong></td>
</tr>
</tbody>
</table>

r : correlation coefficient

3.4 Regression Analysis:
Only HbA1c remained independent from other contributing factors for the fetuin-A levels in stepwise multivariate regression analysis (β = 0.318, p = 0.009).

Table 3-4 Multiple linear regression analysis of serum fetuin A

<table>
<thead>
<tr>
<th>Model</th>
<th>Beta</th>
<th>t</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMA –IR</td>
<td>0.043</td>
<td>0.095</td>
<td>0.924</td>
</tr>
<tr>
<td>FPG</td>
<td>-0.209</td>
<td>-0.736</td>
<td>0.464</td>
</tr>
<tr>
<td>C peptide</td>
<td>-0.041</td>
<td>-0.115</td>
<td>0.909</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.318</td>
<td>2.656</td>
<td><strong>0.009</strong></td>
</tr>
</tbody>
</table>
**Fetuin A Tertiles**:  
The participants were divided into three groups according to the levels of serum Fetuin-A. The characteristics of clinical parameters according to the Fetuin-A tertile are shown in Table 3-5.  

**Table 3-5: Comparisons of clinical variables according to the tertile of Fetuin-A levels.**

<table>
<thead>
<tr>
<th></th>
<th>First tertile</th>
<th>Second tertile</th>
<th>Third tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 19</td>
<td>n = 53</td>
<td>n = 18</td>
</tr>
<tr>
<td></td>
<td>less than 350 mg/l</td>
<td>(&gt; 350 &lt; 500) mg/l</td>
<td>(&gt; 500) mg/l</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>5.244±0.440</td>
<td>5.582±0.354</td>
<td>4.393±0.370</td>
</tr>
<tr>
<td>C peptide</td>
<td>2.934±0.190</td>
<td>3.226±0.238</td>
<td>2.516±0.235</td>
</tr>
<tr>
<td>FBG</td>
<td>195.632±18.381</td>
<td>191.793±9.483</td>
<td>176.556±11.441</td>
</tr>
<tr>
<td>HbA1c</td>
<td>6.058±0.219</td>
<td>6.359±0.167</td>
<td>6.733±0.280</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE

The mean levels of Fetuin A among first, second, third tertiles 304.579 ± 10.433, 409.650 ± 5.304, 897.716 ± 77.983 mg/l respectively. One-way analysis of variance (ANOVA) was used to evaluate differences of means among tertiles of Fetuin-A groups. The levels of HOMA, C peptide, FBG and HbA1c did not show significant differences according to the Fetuin-A tertiles P > 0.05.

**3.5 Comparison of Serum Fetuin–A Levels and the Presence of MS:**

We also examined whether the serum Fetuin A levels were associated with MS in this population. The presence of MS was defined using a modified version of the defined by modified IDF criteria: elevated fasting glucose ≥110 mg/dL, BMI ≥30 kg/m² and HbA1c ≥7%. Overall, 43.3 % (n = 39) of participants met the criteria for the metabolic syndrome. The mean levels of serum Fetuin A were significantly higher in patients with MS compared to patients without MS 557.093 ± 310.983 vs 409.820 ± 150.650 mg/l, P = 0.004.
The highest Fetuin-A tertiles (third) was associated with MS. The comparison between group with MS and group without MS among Fetuin A tertiles were assessed by using pearson chi-square test ($P = 0.007$).

**Table 3.6: Metabolic Syndrome among Fetuin A Tertiles**

<table>
<thead>
<tr>
<th>Fetuin A tertiles</th>
<th>-MS</th>
<th>+ MS</th>
<th>$X^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>First tertile</td>
<td>15</td>
<td>04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second tertile</td>
<td>31</td>
<td>22</td>
<td>10.031</td>
<td>0.007</td>
</tr>
<tr>
<td>Third tertile</td>
<td>05</td>
<td>13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MS: metabolic syndrome  
$X^2$: pearson chi square

Comparing the number of patients having fetuin A level and metabolic syndrome, the patients were more likely to develop metabolic syndrome when the fetuin A level greater than 500 mg/l (OR = 9.75  $p$ value = 0.003) in the logistic regression analysis.
CHAPTER FOUR
DISCUSSION

The aim of this study is to examine the relation between serum Fetuin A and insulin resistance in Sudanese patients with T2DM. Insulin resistance (IR) is one of the key pathophysiological mechanisms of T2DM, which may contribute to the development of T2DM and T2DM-associated complications. Fetuin-A is a multifunctional molecule secreted by the liver (Denecke, et al., 2003). Fetuin-A resulted in insulin resistance through binding insulin receptor tyrosine kinase in adipocytes and skeletal muscle (Mathews, et al., 2000). Previous studies have demonstrated that Fetuin-A has emerged as a biomarker for risk of type 2 diabetes (Sun, et al., 2013).

Our findings could not demonstrate an association of Fetuin-A with IR in patients with T2DM. In contrast with our study result, Song et al. reported that HOMA-IR increased across Fetuin-A tertiles in Chinese T2DM patients (Song, et al., 2011). Also serum fetuin-A levels showed significant positive correlations with HOMA-IR and the mean levels of HOMA-IR were significantly increased progressively across fetuin-A tertiles (Jung, et al., 2013). Our results and published studies have been inconsistent concerning the effects of Fetuin-A on insulin resistance. Some possible explanations can be suggested: differences in the study population, small numbers of subjects, and type of treatment.

Obesity is the most important predictor of T2DM among individuals aged 40–79 years (Biggs, et al., 2010, Laaksonen, et al., 2010). Serum Fetuin-A levels is absolutely related with visceral obesity and dyslipidemia (Chen, et al., 2009). Our study showed that serum Fetuin-A levels are significantly increased in patients with higher BMI. This is in accordance with previous studies, which suggest that weight control would apparently be the most effective strategy in T2DM prevention among an adult population (Mozaffarian, et al., 2009, Schulze, et al., 2007). As it is suggested that Fetuin-A may have a role in triggering the processes leading to IR in T2DM patients.
In the present study we also found that serum Fetuin A was positively and significantly correlated with the glycated hemoglobin (HbA1c) which is considered the first line of screening and diagnosis of diabetes (Mannarino, et al., 2013). In agreement with our study, Yin, et al reported positive correlation with plasma Fetuin A and HbA1c (Yin, et al., 2015). However, in contrast, Jung, et al reported that Fetuin A showed no significant correlation with HbA1c (Jung, et al., 2013).

Epidemiological studies have demonstrated a significant positive relation between serum Fetuin-A and MS in different populations. Study findings revealed that a significant association of Fetuin-A with MS and we found 43.5 % of the patients in our study having MS defined by modified IDF criteria. Data from the study in china by Xu et al. revealed that serum Fetuin-A levels were strongly and independently associated with MS (Xu, et al., 2011). Inconsistent with our study, Ishibashi et al. reported that no significant association of Fetuin-A with MS was detected in Japanese men (Ishibashi, et al., 2010). Also, Roos et al. could not demonstrate an association of Fetuin-A with MS in patients with T2DM (Roos, et al., 2010). The contradictory results have been reported regarding the association of serum Fetuin-A levels with the presence of MS. One of the possible explanations is that the patients with T2DM may already control the components of MS via lifestyle modification, anti-diabetic, anti-hypertensive or anti-lipidaemic medications such as statin medication more strictly than general population. Also, the definitions of MS are different between the studies.

Several limitations of our study should be addressed. First, due to the cross-sectional design, we cannot determine the relationship between Fetuin-A and IR, prospective studies are required to address this important issue. Second, the present study included a small numbers of subjects a larger number of patients should be analyzed for the confirmation of our results. Third, we used HOMA-IR as index of IR, HOMA-IR is less useful in insulin-deficient conditions such as diabetes and may not give appropriate results in subjects with severely impaired or absent beta-cell function. However, HOMA-IR is likely to be the most simple repeatable index in diabetic clinics, and log (HOMA-IR) may be useful for evaluation of IR in individuals with mild-to-moderate diabetes (Muniyappa, et al., 2008).
CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions
Study findings revealed that:

- Higher serum Fetuin-A concentrations were independently associated with BMI in type 2 diabetic patients.
- Fetuin A was positively correlated with HbA1c
- A significant association of Fetuin-A with metabolic syndrome.

5.2 Recommendations

- Future prospective studies with larger numbers of patients are required to confirm the relationship between serum Fetuin-A levels and insulin resistance.
- Measuring of Fetuin A level in Sudanese healthy individuals is needed to obtain base line data to compare and correlate.
- Controlling the confounding factors such as type of treatment, newly diagnosed diabetic patients could be considered.
- Future studies are required to test the association of serum Fetuin-A and the development of T2DM in order to explicate possible guidelines to treat or prevent diabetes and its associated complications.
REFERENCES


Appendix 1
Questionnaire

ID#:……………………………………… Date:………………………………………………

Gender:……………………………… Age:………………………………………………

Height:…………………………………m   Weight:………………………………………

Body mass index
(weight/height²):……………………………………………………………………………kg/m²

Duration of the disease:
……………………………………………………………………………………….years.

Treatment status
yes ( )      No ( )

Risk factors:

Family history of diabetes
yes ( )      No ( )

Abdominal obesity
yes ( )      No ( )

Other disease:

Hypertension
yes ( )      No ( )

Liver disease
yes ( )      No ( )
Appendix 2

Biochemical Analysis Result Form

ID #:……………………………
Data:…………………………

Age:……………………………
Gender:…………………………

<table>
<thead>
<tr>
<th>Parameter</th>
<th>value</th>
<th>unite</th>
<th>Normal Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma Glucose</td>
<td></td>
<td>mg/dl</td>
<td>&lt; 126</td>
</tr>
<tr>
<td>Glycated hemoglobin (HbA1c)</td>
<td></td>
<td>%</td>
<td>4 – 6.5</td>
</tr>
<tr>
<td>Fetuin A</td>
<td></td>
<td>mg/l</td>
<td>400 - 600</td>
</tr>
<tr>
<td>C- Peptide</td>
<td></td>
<td>ng/ml</td>
<td>1.1- 4.4</td>
</tr>
</tbody>
</table>