



بسم الله الرحمن الرحيم

Sudan University for Science and Technology

College of Graduate studies



## Assessment of Insulin Resistance Index (HOMA-IR) among Gallstone Patients in Khartoum State

تقويم مؤشر مقاومة الانسولين لدى المرضى المصابين بحصاوي المرارة  
في ولاية الخرطوم

*A dissertation submitted in partial fulfillment for the requirement of M.Sc  
Degree in Medical Laboratory Science – Clinical Chemistry*

By:

**Hafsa Ahmed Elrheima Ahmed**

B.Sc in Clinical Chemistry Sudan University of Science & Technology

Supervisor:

**Associ. Prof. Dr. Amar Mohamed Ismail**

B.Sc, M.Sc, Ph.D in Clinical Chemistry

2017

# الآية

قال تعالى:

{أَمَّنْ هُوَ قَانِتٌ آنَاءَ اللَّيْلِ سَاجِدًا وَقَائِمًا يَحْذَرُ الْآخِرَةَ وَيَرْجُو رَحْمَةَ رَبِّهِ قُلْ هَلْ يَسْتَوِي الَّذِينَ يَعْلَمُونَ وَالَّذِينَ لَا يَعْلَمُونَ إِنَّمَا يَتَذَكَّرُ أُولُو الْأَلْبَابِ }

سورة الزمر الآية (9)

# *Dedication*

*This research is lovingly dedicated to*

*The fountain that does not tired of giving, for my  
happiness made strings woven from her heart*

**MOM**

*The candle that burning to illuminate my way to success  
and who taught me patience*

**DAD**

*To whom we grew up together and with them I walked  
the path step by step*

**BROTHERS AND SISTERS**

**HAFSA**

# Acknowledgements

In the name of ALLAH, the beneficent, the merciful, praise is to our god and the lord of the world who made me strong and gave me health to do this work.

I would like to express my sincere thanks and appreciations to all those helped me during this study especially my supervisor **Dr. Amar Mohamed Ismail** for his excellent guidance, kind encouragement and valuable advices during this study.

My thanks for **U. Ismail Mohammed Ismail** the biostatistician for all helps in data analysis

Thanks intended to all staff of clinical chemistry department in Sudan University of sciences & technology.

Special thanks to all staff of **Ibn Sina teaching hospital** for their valuable and kind cooperation.

My deepest gratitude and everlasting appreciations are to **patients** from whom samples were taken.

Furthermore, I highly acknowledge and recognize all individuals who participate in this study.

And lastly my thanks intended to all my friends and colleagues.

## Abstract

**Background:** Gall stones formation is a multifactorial process and many studies have shown that insulin resistance is one of the associated factors; previous studies have been established to link between insulin resistance and gall stones disease. This study aims to evaluate insulin resistance index (HOMA-IR) among gall stones patients in Khartoum state.

**Materials and methods:** sixty individuals were enrolled in this study and divided into two groups, thirty patients with gall stones and thirty apparently healthy individuals as control group. Fasting plasma glucose and serum insulin were measured using fully automated devices, and then HOMA-IR was calculated.

**Results:** results showed that the insulin resistance index (HOMA-IR) is significantly increased in gall stones patients when compared to control group  $p$ -value =0.001

The study showed insignificant difference in mean concentration of glucose and insulin resistance index among sex with  $p$  -value =0.611 and 0.623 respectively. But the study showed that there is significant increase in mean concentration of insulin among males with  $p$ - value=0.045.

Also the study showed that there were insignificant correlation between age and HOMA-IR and between BMI and HOMA-IR, but there is strong positive correlation between HOMA-IR and glucose and insulin with  $p$ -value =0.000 for both.

**Conclusion:** The data suggested that the risk of gallstone formation increased in female, obese, postmenopausal women and was associated with changes in insulin resistance index.

## المستخلص

**الخلفية:** تكوين حصاوي المرارة هي عملية متعددة العوامل، وقد أظهرت العديد من الدراسات أن مقاومة الأنسولين هي واحدة من العوامل المرتبطة بها. وقد تم إنشاء دراسات سابقة للربط بين مقاومة الأنسولين وحصوات المرارة. تهدف هذه الدراسة إلى تقييم مؤشر مقاومة الأنسولين بين مرضى المرارة في ولاية الخرطوم.

**طرق ومواد الدراسة:** تم تسجيل ستين فردا في هذه الدراسة، وقسموا إلى مجموعتين، ثلاثين مريضا مصابين بحصوة المرارة وثلاثين من الأفراد الأصحاء على ما يبدو (مجموعة السيطرة). تم قياس الجلوكوز والأنسولين في بلازما الدم (بعد التأكد من صوم الافراد المعينين) باستخدام الاجهزة ، ومن ثم تم حساب مؤشر مقاومة الانسولين.

**النتائج:** أظهرت النتائج أن مؤشر مقاومة الانسولين يزداد بشكل ملحوظ في المرضى الذين يعانون من حصوات المرارة عند المقارنة مع مجموعة السيطرة (القيمة المطلقة = 0.000). أظهرت الدراسة عدم وجود فروقات ظاهرة في متوسط تركيز الجلوكوز ومؤشر مقاومة الانسولين بين الجنسين (القيمة المطلقة = 0.611 و 0.623 على التوالي). لكن الدراسة بينت ان مستوى الاتسولين يختلف بطريقة واضحة بين الجنسين (القيمة المطلقة = 0.045) حيث ان مستوى الانسولين اعلى لدى الرجال.

كما أظهرت الدراسة عدم وجود علاقة ارتباط بين العمر و مؤشر مقاومة الانسولين وبين مؤشر كتلة الجسم و مؤشر مقاومة الانسولين، ولكن هناك ارتباط إيجابي قوي بين مؤشر مقاومة الانسولين مع الجلوكوز والأنسولين (القيمة المطلقة = 0.000 لكليهما).

**خاتمة الدراسة:** اشارت البيانات الى ان خطورة تكون حصوة المرارة تزداد عند النساء اللواتي يئسن من المحيض كما ان البدنيين هم اكثر عرضة للاصابة بحصوات المرارة ويصاحب تكونها تغير في مؤشر مقاومة الانسولين.

## List of tables

|           |  |
|-----------|--|
| Table 3.1 | The frequency and percentage of patients BMI   |
| Table 3.2 | Mean concentration of glucose, insulin , insulin resistance index and BMI in case VS control group |
| Table 3.3 | Mean concentration of glucose, insulin and insulin resistance index in males VS females            |

## List of figures

|            |  |
|------------|--|
| Figure 3.1 | Sex distribution among patients                                |
| Figure 3.2 | Distribution of age among patients                             |
| Figure 3.3 | Correlation between age and insulin resistance index           |
| Figure 3.4 | Correlation between BMI and insulin resistance index           |
| Figure 3.5 | Correlation between insulin level and glucose level            |
| Figure 3.6 | Correlation between insulin resistance index and glucose level |
| Figure 3.7 | Correlation between insulin resistance index and insulin level |



## List of abbreviations

|              |   |
|--------------|---|
| ADA          | American Diabetes Association                         |
| ADP          | Adenosine di phosphate                                |
| ATP          | Adenosine tri phosphate                               |
| BMI          | Body mass index                                       |
| CCK          | Cholecystokinin                                       |
| CIGMA        | Continuous infusion of glucose with model assessment  |
| CSI          | Cholesterol saturation index                          |
| ER           | Endoplasmic reticulum                                 |
| FBG          | Fasting blood glucose                                 |
| FSIVGTT      | Frequently sampled intravenous glucose tolerance test |
| GLUT4        | Glucose transporter4                                  |
| GSD          | Gall stones disease                                   |
| HOMA         | Homeostatic model assessment                          |
| IL-6         | Inter luekin-6  |
| IR           | Insulin resistance                                    |
| IR-index     | Insulin resistance index                              |
| IST          | Insulin suppression test                              |
| ITT          | Insulin tolerance test                                |
| IVGTT        | Intra venous glucose tolerance test                   |
| KATP         | ATP-sensitive K <sup>+</sup> channel                  |
| SPSS         | Statistical package of social sciences                |
| TNF $\alpha$ | Tumor necrosis factor alpha                           |
| UPR          | Unfolded protein response                             |
| VDCC         | Voltage-dependent calcium channels                    |

## Table of content

|   | <b>Topic</b>                          | <b>Page</b> |
|---|---------------------------------------|-------------|
|   | Dedication                            | I           |
|   | Acknowledgement                       | II          |
|   | Abstract in English                   | III         |
|   | المستخلص                              | IV          |
|   | List of figures                       | V           |
|   | List of tables                        | VI          |
|   | List of abbreviations                 | VII         |
|   | Table of contents                     | VIII        |
| <b>Chapter one</b>                        |                                       |             |
| <b>Introduction and literature review</b> |                                       |             |
| 1.1                                       | Introduction                          | 1           |
| 1.2                                       | Definition of gall stones             | 2           |
| 1.2.1                                     | Types of gall stones                  | 2           |
| 1.2.2                                     | Pathogenesis                          | 2           |
| 1.2.3                                     | Cholesterol saturation index          | 3           |
| 1.2.4                                     | Risk factors for gall stones          | 3           |
| 1.2.5                                     | Symptoms of gall stones               | 5           |
| 1.2.6                                     | Management                            | 6           |
| 1.3                                       | Insulin definition                    | 6           |
| 1.3.1                                     | Insulin secretion                     | 6           |
| 1.3.2                                     | Insulin action                        | 7           |
| 1.3.3                                     | Mechanism of insulin resistance       | 10          |
| 1.3.4                                     | Factors influence insulin sensitivity | 11          |
| 1.3.5                                     | Causes of insulin resistance          | 11          |

|                              |  |    |
|------------------------------|--|----|
| 1.3.6                        | Diagnosis                                | 12 |
| 1.3.7                        | Management                               | 13 |
| 1.4                          | Hyper insulinemia and insulin resistance | 13 |
| 1.5                          | Back ground studies                      | 13 |
| 1.6                          | Rationale                                | 15 |
| 1.7                          | General objectives                       | 16 |
| 1.8                          | Specific objectives                      | 16 |
| <b>Chapter two</b>           |  |    |
| <b>Materials and methods</b> |  |    |
| 2.1                          | Study design                             | 17 |
| 2.2                          | Study area                               | 17 |
| 2.3                          | Study population                         | 17 |
| 2.4                          | Inclusion criteria                       | 17 |
| 2.5                          | Exclusion criteria                       | 17 |
| 2.6                          | Collection of sample                     | 17 |
| 2.7                          | Ethical considerations                   | 17 |
| 2.8                          | Estimation of glucose                    | 18 |
| 2.9                          | Estimation of insulin                    | 18 |
| 2.10                         | Calculation of insulin resistance index  | 19 |
| 2.11                         | Statistical analysis                     | 19 |
| <b>Chapter three</b>         |  |    |
| <b>Results</b>               |  |    |
| 3                            | Results                                  | 20 |

| <b>Chapter four</b>                                |                 |    |
|--|-----------------|----|
| <b>Discussion, conclusions and recommendations</b> |                 |    |
| 4.1  | Discussion      | 32 |
| 4.2  | Conclusions     | 35 |
| 4.3  | Recommendations | 35 |
|  | References      | 36 |
|  | Appendices      | 45 |

# ***CHAPTER ONE***

## ***INTRODUCTION AND LITERATURE REVIEW***

## 1.1 Introduction

A gallstone is a solid crystal deposit that forms in the gallbladder, which is a pear-shaped organ that stores bile salts until they are needed to help digest fatty foods. Gallstones can migrate to other parts of the digestive tract and cause severe pain with life-threatening complications (Hale *et al.*, 2005).

Cholelithiasis is common with incidence ranging from 10 – 20% of world population. The incidence is a four times higher in women than in men (Rosai, 1996).

Prevalence rate is difficult to determine because calculous disease may often be asymptomatic. There is great variability regarding the worldwide prevalence of gallstone disease. High rates of incidence occur in the United States, Chile, Sweden, Germany, and Austria. The prevalence among the Masai peoples of East Africa is 0% whereas it approaches 70% in Pima Indian women. Asian populations appear to have the lowest incidence of gallstone disease. In the United States, approximately 10–15% of the adult population has gallstones, with approximately one Million cases presenting each year. Gallstones are the most common gastrointestinal disorder requiring hospitalization (Rosai, 1996).

In Sudan no literature was found.

Insulin resistance is a state in which there are impaired biological and physiological responses to insulin in tissues (Hunter and Garvey, 1998).

Researcher associate between insulin resistance and pathogenesis of gall stones (Sang *et al.*, 2011). Therefore the present study hypothesizes that insulin resistance associated with gall stones in Sudanese population.

## **1.2 Definition of gallstones**

A gallstone is a solid crystal deposit that forms in the gallbladder, which is a pear-shaped organ that stores bile salts until they are needed to help digest fatty foods. Gallstones can migrate to other parts of the digestive tract and cause severe pain with life-threatening complications (Hale *et al.*, 2005).

### **1.2.1 Types of gallstones**

Mixed cholesterol gall stones consist of 50 – 80% cholesterol and are slightly more common than pure cholesterol stones. Black pigment stones are either composed of pure calcium bilirubinate or polymetalite complexes with calcium, copper and large amount of mucin glycoproteins. A regular crystalline structure is not present; they are usually small, multiple with rough surfaces. Brown pigment stones composed of calcium salts of fatty acids and unconjugated bilirubin with varying amount of cholesterol and proteins .these stones are usually found in bile ducts (Mahtoob, 2009).

### **1.2.2 Pathogenesis**

Three defects involved in gall stones formation, cholesterol super saturation, accelerated nucleation and gall bladder hypomobility. The molar proportion of cholesterol, phospholipids and bile acids in bile are often represented on triangular coordinates each side of triangle shows the molar fraction represented by its constituents .Cholesterol is secreted into bile by hepatocytes with the help of rate limiting enzyme HMG.CO A reductase. It is virtually insoluble in bile by association with bile salts and phospholipids in form of mixed micelles and vesicles. Cholesterol gall stones forms when cholesterol concentration in bile exceeds the ability of bile to hold it in solution so that crystals form and grow as stones (Mahtoob, 2009).

### **1.2.3 Cholesterol saturation index (CSI)**

CSI is the ratio of cholesterol content to the maximum micellar solubility of bile. A CSI of more than 1 indicates that the bile is supersaturated with cholesterol and given other precipitating factors could lead to formation of stones (lithogenics) (John, 2009).

### **1.2.4 Risk factors for gall stones**

Risk factors for gall stones formation include:

#### **1.2.4.1 Age**

The incidence of gallstone disease increases with age. Symptomatic calculous disease of the biliary tract is rare before the age of 20, and when found in this group, is commonly among patients with chronic predisposing conditions such as cystic fibrosis or hemolytic anemia. (John, 2009)

#### **1.2.4.2 Drugs**

Many drugs have been implicated in gallstone disease. The most common offenders include ceftriaxone, clofibrate, oral contraceptives, estrogen replacement, progestogens, and octreotide. Some drugs are secreted into bile and may complex, precipitate, and form stones. Other compounds can produce gallbladder stasis with concomitant increases in cholesterol secretion into bile (John, 2009).

#### **1.2.4.3 Gender**

The prevalence of gallstones is higher in women than men. Studies have shown that gallstone disease is common in young women but rare in young men. However, the difference narrows with increasing age. It is presumed that the reason for this gender difference is hormonal. Serum estrogen increases (especially during pregnancy) promotes biliary cholesterol saturation and increased progesterone may lead to inhibition of the contraction of the gallbladder (John, 2009).



#### **1.2.4.4 Geography and Ethnicity**

The Pima Indians of Arizona have the highest prevalence of gallstones worldwide. Ninety percent of Pima women over the age of 65 have gallstone disease. The Micmac Indians of Canada, Hispanics, and Mexican American women also have a higher prevalence of gallstone disease, as do both men and women in Norway and Chile. The risk appears to be lower in blacks. No genes have been identified in humans that are conclusively linked to gallstone development (John, 2009).

#### **1.2.4.5 Obesity**

Obesity is a significant risk factor for gallstone disease, especially in women. Studies have demonstrated that overweight women with a body mass index (BMI) greater than or equal to 30 kg/m<sup>2</sup> have double the risk of gallstone disease when compared with normal weight women with less than 25 kg/m<sup>2</sup>. Cholesterol hypersecretion (associated with obesity) is a major pathogenic factor. Gallstone disease has also been associated with the regional distribution of fat. High central or truncal adiposity has been positively correlated with risk of gallstone disease. Physical activity and diet have been studied independently as risk factors for gallstone disease. Increased vigorous physical activity and recreational activity appear to reduce gallstone disease risk (John, 2009).

#### **1.2.4.6 Weight Loss**

An increased risk of gallstone disease may be found among individuals who undergo rapid weight loss on very low calorie diets. Gallstone formation is one of the most significant complications of voluntary weight loss plans. In these instances, cholesterol is activated from adipose tissue and secreted into the bile. This leads to cholesterol super saturation and diminishes gallbladder contraction, producing stasis. Studies have shown that individuals on weight loss plans, either dramatically reduced calorie

diets or surgical weight-loss procedures, have a higher incidence of development of gallstone disease when compared to those who are not dieting. Weight fluctuation may also be a risk factor (John, 2009).

### **1.2.5 Symptoms of gall stones**

Most cases of gallstones don't cause any symptoms. But if a gallstone blocks one of the bile ducts, it can cause sudden, severe abdominal pain, known as biliary colic. Other symptoms may develop if the blockage is more severe or develops in another part of the digestive system (Warting *et al.*, 2016).

#### **1.2.5.1 Abdominal pain (biliary colic)**

Gallstones can cause sudden, severe abdominal pain that usually lasts one to five hours (although it can sometimes last just a few minutes). The pain can be felt in the center of abdomen (tummy) or just under the ribs on right-hand side it may spread from here to side or shoulder blade. When gallstones cause episodes of biliary colic, it is known as 'uncomplicated gallstone disease (Warting *et al.*, 2016).

#### **1.2.5.2 Other symptoms**

In a small number of people, gallstones can cause more serious problems if they obstruct the flow of bile for longer periods or move into other organs (such as the pancreas or small bowel). If this happens, you may develop a high temperature of 38C (100.4F) or above, more persistent pain, a rapid heartbeat, yellowing of the skin and whites of the eyes (jaundice), itchy skin, diarrhea, chills or shivering attacks, confusion and loss of appetite (Warting *et al.*, 2016).

### **1.2.6 Management**

Although the natural history of gallstones is generally benign, the physician must decide whether treatment is needed. When considering gallstones, it is helpful to categorize patients into the following groups:

those with incidentally detected, asymptomatic gallstones; with symptomatic gallstones; with atypical symptoms and gallstones on imaging; and with typical symptoms but no gallstones on imaging. Expectant management is the best approach for patients with incidentally detected, asymptomatic gallstones. However, even in these patients, cholecystectomy may be indicated in certain circumstances, such as in patients planning to have a transplant and patients with hemolytic anemia (Sherly *et al.*, 2005).

### **1.3 Insulin definition**

Insulin is a peptide hormone produced by beta cells of the pancreatic islets, It regulates the metabolism of carbohydrates, fats and protein by promoting the absorption of glucose from the blood into fat, liver and skeletal muscle cells (Stryer and Lubert, 1995).

#### **1.3.1 Insulin secretion**

B-cells are excitable endocrine cells that secrete insulin. Glucose-stimulated, KATP channel dependent pathway starts with intracellular transport of extracellular glucose by glucose transporter (GLUT2). Intracellular glucose undergoes cytosolic glycolysis catalyzed by glucokinase. Pyruvate, a product of glycolysis, is shuttled into mitochondria as a substrate of the tricarboxylic acid cycle with production of adenosine triphosphate (ATP). As a result, cytosolic ATP levels are elevated and adenosine 5'-diphosphate (ADP) levels reduced. Increased cytosolic ATP/ADP ratio closes an ATP-sensitive K<sup>+</sup> channel, KATP, which results in discontinued K<sup>+</sup> outflow, thus depolarizing the B.cell membrane. Voltage-dependent calcium channels (VDCCs) on the cell membrane are opened by depolarization, and calcium influx increases intracellular calcium (phase1), which, in turn, activates calcium-dependent calcium release from the endoplasmic reticulum (phase 2). The resultant

biphasic increase in intracellular calcium triggers fusion of secretory vesicles with the plasma membrane and insulin is released. The KATP channel plays the crucial role of converting metabolic to electric signals. All components of glucose- stimulated, KATP channel-dependent insulin secretion are found in several other cell types, except for the KATP channel, which is unique for B. cells (a similar channel is also found in muscle and brain) (Bonner-Weir, 2000).

### **1.3.2 Insulin action**

Insulin play a role in metabolism as follow:

#### **1.3.2.1 Insulin and carbohydrate metabolism**

Glucose is liberated from dietary carbohydrate such as starch or sucrose by hydrolysis within the small intestine, and is then absorbed into the blood. Elevated concentrations of glucose in blood stimulate release of insulin, and insulin acts on cells throughout the body to stimulate uptake, utilization and storage of glucose. The effects of insulin on glucose metabolism vary depending on the target tissue. Two important effects are: Insulin facilitates entry of glucose into muscle, adipose and several other tissues. The only mechanism by which cells can take up glucose is by facilitated diffusion through a family of hexose transporters. In many tissues (muscle being a prime example) the major transporter used for uptake of glucose (called GLUT4) is made available in the plasma membrane through the action of insulin. When insulin concentrations are low, GLUT4 glucose transporters are present in cytoplasmic vesicles, where they are useless for transporting glucose. Binding of insulin to receptors on such cells leads rapidly to fusion of those vesicles with the plasma membrane and insertion of the glucose transporters, thereby giving the cell an ability to efficiently take up glucose. When blood levels of insulin decrease and insulin receptors are no longer occupied, the glucose

transporters are recycled back into the cytoplasm. It should be noted here that there are some tissues that do not require insulin for efficient uptake of glucose: important examples are brain and the liver. This is because these cells don't use GLUT4 for importing glucose, but rather, another transporter that is not insulin-dependent. The second effect is that Insulin stimulates the liver to store glucose in the form of glycogen. A large fraction of glucose absorbed from the small intestine is immediately taken up by hepatocytes, which convert it into the storage polymer glycogen. Insulin has several effects in liver which stimulate glycogen synthesis. First, it activates the enzyme hexokinase, which phosphorylates glucose, trapping it within the cell. Coincidentally, insulin acts to inhibit the activity of glucose-6-phosphatase. Insulin also activates several of the enzymes that are directly involved in glycogen synthesis, including phosphofruktokinase and glycogen synthase. The net effect is clear: when the supply of glucose is abundant, insulin "tells" the liver to bank as much of it as possible for use later. A well-known effect of insulin is to decrease the concentration of glucose in blood, which should make sense considering the mechanisms described above. Another important consideration is that, as blood glucose concentrations fall, insulin secretion ceases. In the absence of insulin, a bulk of the cells in the body become unable to take up glucose, and begin a switch to using alternative fuels like fatty acids for energy. Neurons, however, require a constant supply of glucose, which in the short term, is provided from glycogen reserves. When insulin levels in blood fall, glycogen synthesis in the liver diminishes and enzymes responsible for breakdown of glycogen become active. Glycogen breakdown is stimulated not only by the absence of insulin but by the presence of glucagon, which is secreted when blood glucose levels fall below the normal range (Ahima and Lazar, 2008).

### **1.3.2.2 Insulin and lipid metabolism**

The metabolic pathways for utilization of fats and carbohydrates are deeply and intricately intertwined. Considering insulin's profound effects on carbohydrate metabolism, it stands to reason that insulin also has important effects on lipid metabolism, include: Insulin promotes synthesis of fatty acids in the liver. Insulin is stimulator to synthesis of glycogen in the liver. However, as glycogen accumulates to high levels (roughly 5% of liver mass), further synthesis is strongly suppressed. When the liver is saturated with glycogen, any additional glucose taken up by hepatocytes is shunted into pathways leading to synthesis of fatty acids, which are exported from the liver as lipoproteins. The lipoproteins are ripped apart in the circulation, providing free fatty acids for use in other tissues, including adipocytes, which use them to synthesize triglyceride. Insulin inhibits breakdown of fat in adipose tissue by inhibiting the intracellular lipase that hydrolyzes triglycerides to release fatty acids. Insulin facilitates entry of glucose into adipocytes, and within those cells, glucose can be used to synthesize glycerol. These glycerols, along with the fatty acids delivered from the liver, are used to synthesize triglyceride within the adipocyte. By these mechanisms, insulin is involved in further accumulation of triglyceride in fat cells. From a whole body perspective, insulin has a fat-sparing effect. Not only does it drive most cells to preferentially oxidize carbohydrates instead of fatty acids for energy, insulin indirectly stimulates accumulation of fat in adipose tissue (Ahima and Lazar, 2008).

### **1.3.2.3 Other notable effects of insulin**

In addition to insulin's effect on entry of glucose into cells, it also stimulates the uptake of amino acids, again contributing to its overall anabolic effect. When insulin levels are low, as in the fasting state, the

balance is pushed toward intracellular protein degradation. Insulin also increases the permeability of many cells to potassium, magnesium and phosphate ions. The effect on potassium is clinically important. Insulin activates sodium-potassium ATPases in many cells, causing a flux of potassium into cells. Under certain circumstances, injection of insulin can kill patients because of its ability to acutely suppress plasma potassium concentrations (Ahima and Lazar, 2008).

### **1.3.3 Mechanism of Insulin resistance**

Insulin resistance pathologically grows through many interactions of genotype lifestyle change mainly sedentary life and over eating (Pratley RE, 1998). Physiologically, many circulating factors regulate insulin sensitivity in target tissue such as adipokines, plasma lipid and circulating hormones plus their signaling pathways (Ahima and Lazar, 2008).

There is neuroendocrine axis involve adipose tissue with brain and gut which regulate Insulin metabolism by adjusting insulin sensitivity in target tissues (Zac and Tan, 2010).

Adipokines are hormone secreted by adipocytes store where some stimulate and other inhibit insulin sensitivity. In peripheral tissue insulin action is stimulated by Leptin and adiponectin. On the other hand, TNF $\alpha$ , resistin, IL-6, and retinol binding protein 4 suppress insulin sensitivity (Beale, 2015).

Although metabolism and nutrition are regulated by adipocyte derived factors pattern of life, overweight and genetic can disturb this balance (Romao and Roth, 2008). It is found that during obesity there is increase in level of inhibitory adipokines and circulating fatty acids (Ahima and Lazar, 2008).

### **1.3.4 Factors influence insulin sensitivity**

Obesity and fat distribution, centrally (abdomen and waist) placed adipose tissue especially in the viscera are implicated in insulin resistance, also two molecular peptides are suspected: Leptin and Adiponectin ,increase in age reduces insulin sensitivity, Insulin resistance has been attributed to multiple gene mutations which are not well understood, exercise and physical fitness, Dietary nutrients ,medications like growth hormones, steroids and nicotinic acid reduces insulin sensitivity (Nolan *et al.*, 2013).

### **1.3.5 Causes of insulin resistance**

There are many factors lead to development of insulin resistance. The major leading causes are overweight, sedentary life style and genetic factors. Some other factors contribute in some ways of development of insulin resistance. Most important are obesity, physical inactivity, and genetic factors. Other factors that may affect the degree of insulin resistance are diet composition, aging, and hormones (particularly glucocorticoids and androgens). High-carbohydrate diets reproduce some of the features of the metabolic syndrome. There are several factors that are postulated after several studies that cause insulin resistance. There are three main ones that converge on common pathways that inhibit insulin action, they are: the accumulation of ectopic lipids and its metabolites, the development of ER stresses and the activation of the unfolded Protein response (Shulman and Gerald, 2000).

The association of ectopic lipid accumulation and insulin resistance has been universally established. It acts at the glucose transport level GLUT4 at the cell membrane that responds to insulin signaling thereby impairing insulin signaling. The activation of the unfolded protein response (UPR) also known as endoplasm reticulum stress which positively gives cells the



capacity to adapt to changes especially the b-cells of islet (Hirosumi,*et al.*, 2002).

### **1.3.6 Diagnosis**

It is inapplicable to measure insulin resistance in isolation. The choice of the test or method used depends on type and size of study undertaken. Insulin resistance different assessment methods Include: Euglycaemic clamp (gold standard),short insulin tolerance test( ITT), homeostatic model assessment( HOMA), continuous infusion of glucose with model assessment (CIGMA), frequently sampled intravenous glucose tolerance test (FSIVGTT) and insulin suppression rest (IST) (Wallace and Matthews, 2002).

The standards ways for measurement of insulin resistance in field of research are intravenous glucose tolerance test (IVGTT) plus euglycemic insulin clamp on the other hand they are unpractical in clinical daily practice and are uneasy to carry out in population-based research studies (Ferrannini and Mari, 1998).

### **1.3.7 Management**

The main step in management of insulin resistance is nutrition which include low caloric intake to reverse over weight and obesity plus adjustment of carbs in diet to avoid aggravation of excess Insulin (Geor, 2013).

American Diabetes Association (ADA) recommends to start with lifestyle change including diet modification and exercise as first line for management of type 2 diabetes mellitus. If no achievement was notice within 3 month; drugs will be the second choice. There are around six hypoglycemic drugs used such as Insulin, Sulfonylureas, Meglitinides, Biguanides, Alpha glucosidase inhibitors, Thiazolidinediones (Barrett, 2006).

It is unluckily that no perfect drugs are available for treating insulin resistance per sea. While metformin is the only drug which reduces insulin resistance (Barrett, 2006).

#### **1.4 Hyper insulinemia and gall stone disease**

Hyperinsulinemia has been shown to interfere with cholecystokinin (CCK) action at the gallbladder. Normally, CCK binds to receptors on gallbladder muscle cells to begin contraction and emptying of bile from the gallbladder. Excessive insulin interferes with CCK action at the receptor level, which leads to impaired contraction (Barrett, 2006).

#### **1.5 Background studies**

Cynthia *et al.* performed a case control study to determine if the insulin resistance is associated with gall stones during pregnancy. Cases were 205 women with gall stones during pregnancy and 443 randomly selected women without gall stones during pregnancy. Fasting serum glucose and insulin were measured at 26–28 weeks gestation. Insulin resistance was measured by the homeostasis model. They found that Insulin resistance may represent a causal link between obesity and overweight and gallstones (Cynthia *et al.*, 2008).

Kim *et al.* conducted a case-control study for the evaluation of the association between gallstone diseases and fasting serum insulin level, insulin resistance in non-diabetic Korean general population. 118 Korean subjects were enrolled in the study. Serum fasting insulin levels were determined by radioimmunoassay and concentrations of glucose by standard enzymatic colorimetric method. Insulin resistance was determined by the homeostasis model assessment (HOMA-IR). Body mass index (BMI) was also measured. They suggested that hyperinsulinemia and insulin resistance could be associated with gallstone formation in individuals without clinical

diagnosis of diabetes mellitus and with normal serum glucose level (Kim *et al.*, 2007).

Alireza –Ansari *et al.* carried out cross sectional study on a total of 1522 males and females aged  $\geq 30$  year to evaluate the prevalence and possible risk factors of gallstone disease in the general population (Alireza –Ansari *et al.*, 2015).

## **1.6 Rationale**

In Sudan gall stone disease is in increase especially in obese females who are over 40 years old, it can cause many complications if left untreated. Insulin action is affected largely by cholesterol gall stones. In this study insulin is considered because insulin is a vital hormone which regulates blood glucose level and any disturbances in insulin level or insulin action will cause serious problems that require a medical intervention.

Several studies hypothesise that insulin resistance index increase in patients with gall stones.

### **1.7 General objectives**

To assess insulin resistance index (HOMA-IR) among gall stones patients

### **1.8 Specific objectives**

- 1- To measure fasting blood glucose and fasting blood insulin.
- 2- To calculate insulin resistance index (HOMA-IR).
- 3- To measure incidence of disease among pre-menopausal and post-menopausal women.
- 4- To correlate BMI with GSD and HOMA-IR with age, BMI, glucose and insulin.

# ***CHAPTER TWO***

## ***MATERIALS AND METHODS***

## **2.1 Study design**

Comparative cross sectional study conducted during the period from March to May 2017.

## **2.2 Study area**

This study was carried out in IBN SINA hospital in Khartoum state

## **2.3 Study population**

Thirty patients with gall stone disease and thirty apparently healthy individuals were enrolled in this study.

## **2.4 Inclusion criteria**

Overnight fasting specimens were collected from patients with gall stone disease and from apparently healthy individuals.

## **2.5 Exclusion criteria**

Diabetic patients were excluded.

## **2.6 Collection of sample**

Fasting blood samples were collected by using dry, plastic syringes, tourniquet was used, and 5ml of blood was withdrawn, equal volume of blood was dispensed in a fluoride oxalate and plain containers. Fluoride oxalated blood separated as soon as possible by centrifugation at 4000rpm to obtain plasma and stored at 20°C.

Blood in Plain containers were allowed to clot at room temperature then they were centrifuged at 4000rpm to obtain serum and stored at 20°C.

## **2.7 Ethical considerations**

Study was approved from ethical committee of the Sudan University of science and technology. All patients were verbally approved to participate.

## **2.8 Estimation of glucose**

There are many methods used for estimation of glucose, one of these methods which is common is glucose oxidase method.

### **2.8.1 Principle**

Glucose oxidase (GOD) catalyzes the oxidation of glucose to gluconic acid. The formed hydrogen peroxide ( $H_2O_2$ ) is detected by a chromogenic oxygen acceptor, phenol; 4-aminophenazone (4-AP) in the presence of peroxidase (POD). The intensity of the color formed is proportional to the glucose concentration in the sample.

### **2.8.2 Procedures**

Fully automated device is used (COBUS).

## **2.9 Estimation of insulin**

Insulin measurement achieved mainly depending on immunoassays.

### **2.9.1 Principle**

The Merocodia Insulin ELISA is a two-site enzyme immunoassay utilizing the direct sandwich technique with two monoclonal antibodies directed against separate antigenic determinants of the insulin molecule. Specimen, control, or standard is pipetted into the sample well, and then followed by the addition of peroxidase-conjugated anti-insulin antibodies. Insulin present in the sample will bind to anti-insulin antibodies bound to the sample well, while the peroxidase-conjugated anti-insulin antibodies will also bind to the insulin at the same time. After washing to remove unbound enzyme-labeled antibodies, TMB-labeled substrate is added and binds to the conjugated antibodies. Acid is added to the sample well to stop the reaction, and the colorimetric endpoint is read on a microplate spectrophotometer set to the appropriate light wavelength.



### **2.9.2 Procedures**

Fully automated device (Tosoh) is used.

### **2.10 Calculation of insulin resistance index**

Insulin resistance index is calculated using HOMA equation which state that:

$$\text{IR} = \frac{\text{FBG (mmol/l)} \times \text{fasting insulin } (\mu\text{U/ml})}{22.5}$$

### **2.11 Statistical analysis**

All data were analyzed using SPSS version 21:

- Descriptive statistics (percentage, mean and SD) were obtained.
- Independent T-test was performed for comparison between study variables.
- Person correlation test was done to correlate between results of variables, results expressed as mean +/- SD, significant difference considered as *P-value* less than 0.05.

# ***CHAPTER THREE***

## ***RESULTS***

### 3. Results

Thirty blood samples were collected from patients with gall stones (cases) from Ibn Sina Teaching hospital during the period from March to May 2017. Another thirty samples were collected from apparently healthy individuals (control). The level of glucose and insulin was measured for both groups then insulin resistance index was calculated and data obtained was analyzed using SPSS 20.

73% of patients were females and 27% of them were males as shown in **figure (3.1)**.

63.3% of patients were post-menopausal women and 36.7% of them were pre-menopausal women as shown in **figure (3.2)**.

36.7% of patients had a normal weight, 30% of them were over weighted and 33.3% of them were obese as shown in **table (3.1)**.

There was a significant increase in the mean concentration of insulin resistance index of cases in comparison to control ( $5.57\pm 4.19$ ), ( $2.81\pm 1.61$ ) respectively with  $p$ -value being =0.001 as shown in **table (3.2)**.

The mean of body mass index is higher in cases ( $28.2\pm 6.98$ ) than in control ( $25.6\pm 3.84$ ) as shown in **table (3.2)**.

There was no significant difference in glucose level between males ( $166\pm 33.3$ ) and females ( $159\pm 35.8$ ) with  $p$ -value being =0.611, also there was significant increase in insulin level of males ( $18.4\pm 8.14$ ) in comparison to females ( $12.5\pm 7.71$ ) with  $p$ -value being =0.045 and there was insignificant difference in insulin resistance index between males ( $5.57\pm 4.19$ ) and females ( $5.34\pm 3.99$ ) with  $p$ -value being =0.623 as shown in **table (3.3)**.

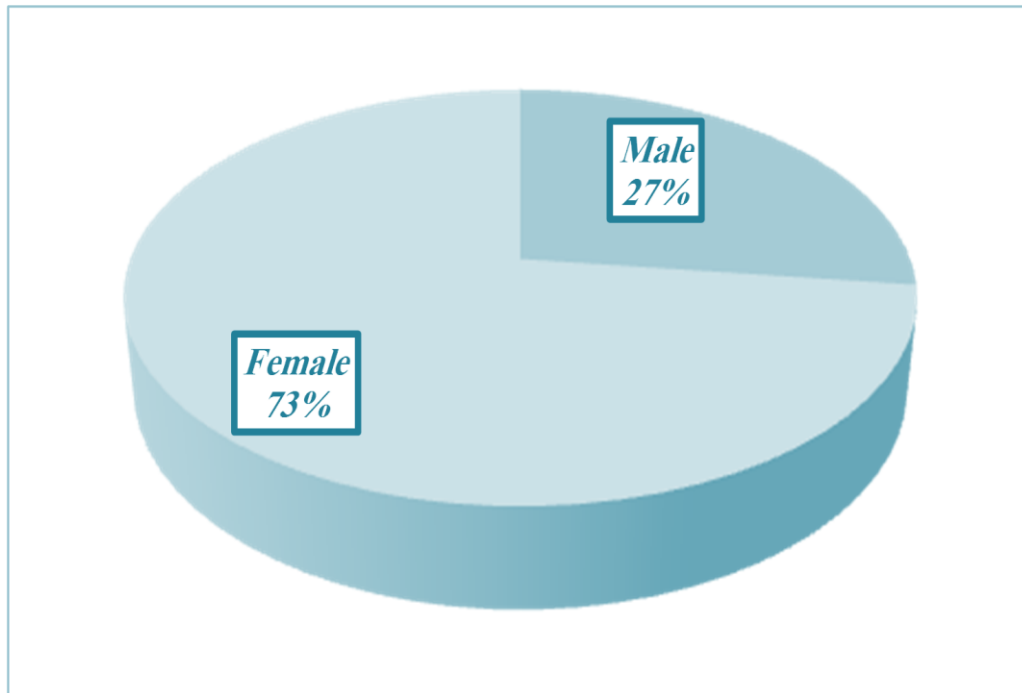
There was no correlation between age and insulin resistance index ( $r = 0.179$ ,  $p = 0.343$ ) as shown in **figure (3.3)**.

There was no correlation between BMI and insulin resistance index ( $r = -0.104$ ,  $p = 0.586$ ) as shown in **figure (3.4)**.

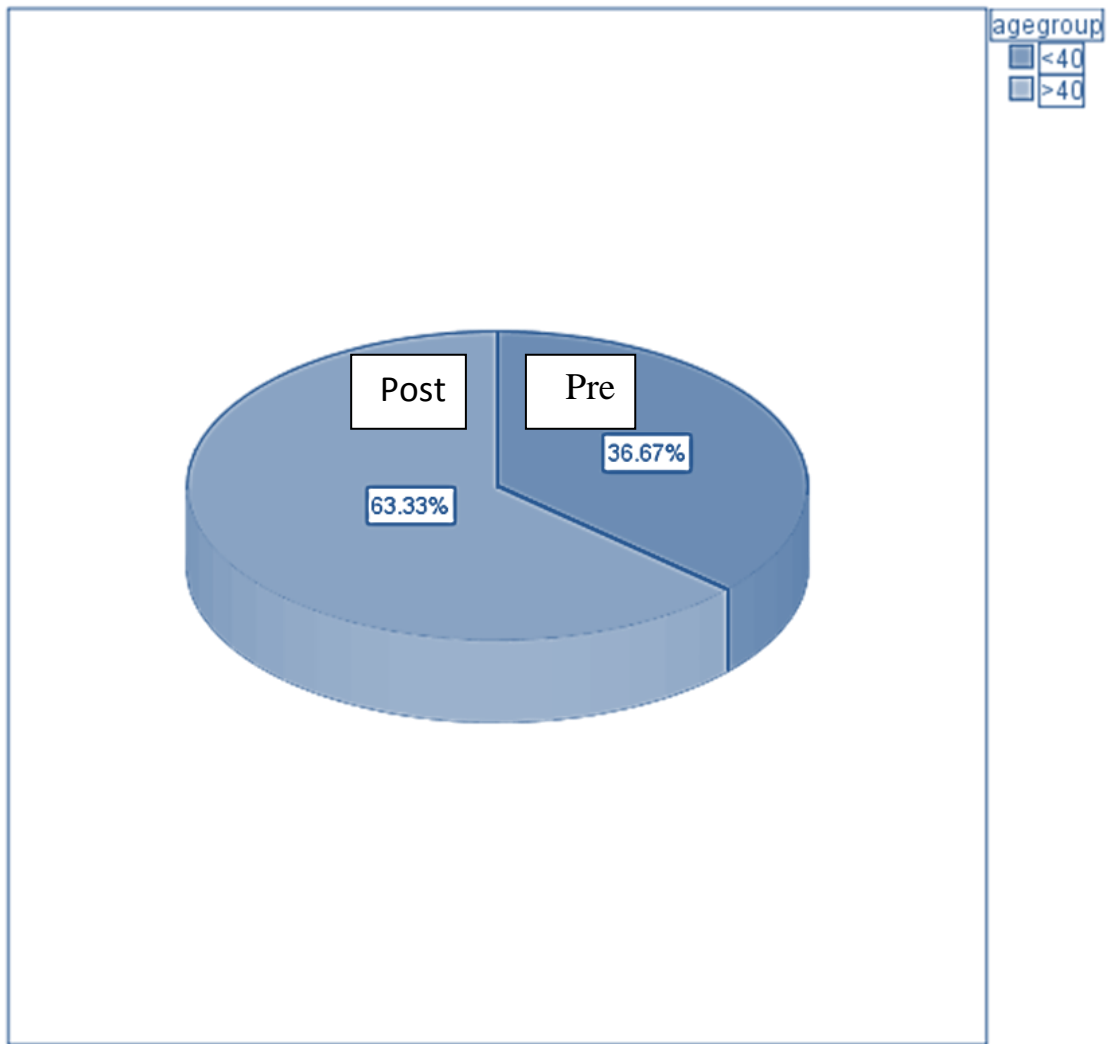
There was moderate positive correlation between glucose level and insulin level ( $r = 0.579$ ,  $p = 0.001$ ) as shown in **figure (3.5)**.

There was strong positive correlation between glucose level and insulin resistance index ( $r = 0.788$ ,  $p = 0.000$ ) as shown in **figure (3.6)**.

There was strong positive correlation between insulin resistance index and insulin level ( $r = 0.755$ ,  $p = 0.000$ ) as shown in **figure (3.7)**.



**Figure (3.1): sex distribution among patients**



**Figure (3.2): Distribution of age among patients**

**Table (3.1): the frequency and percentage of patients BMI**

| <b>BMI</b>           | <b>Frequency</b> | <b>Percentage (%)</b> |
|----------------------|------------------|-----------------------|
| <b>Normal Weight</b> | 11               | 36.7 %                |
| <b>Overweight</b>    | 9                | 30.0 %                |
| <b>Obese</b>         | 10               | 33.3 %                |
| <b>Total</b>         | 30               | 100.0 %               |

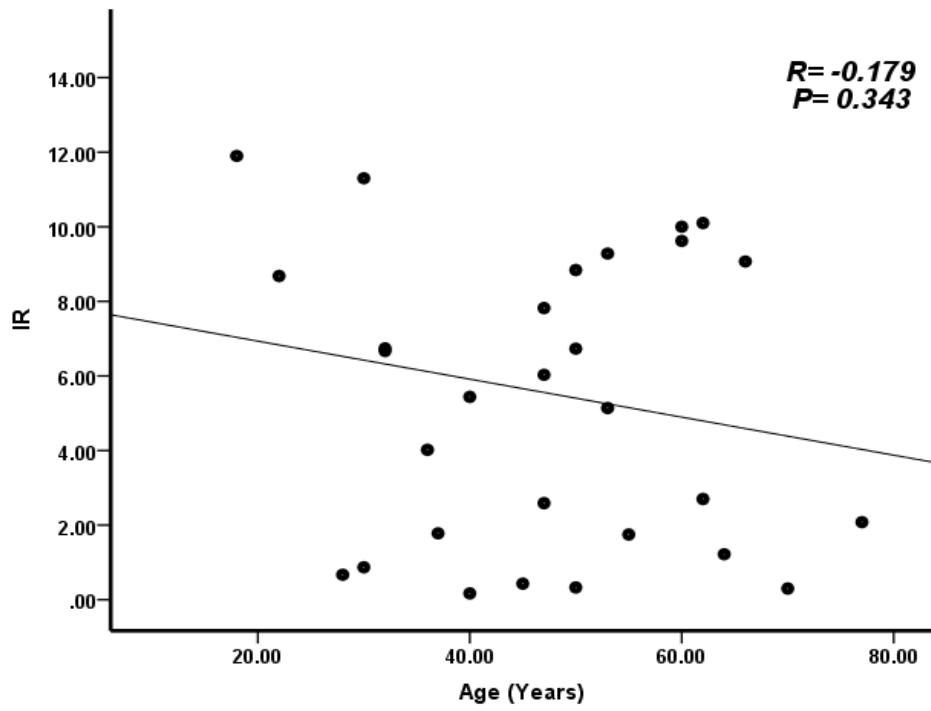
**Table (3.2): Mean concentration of glucose, insulin, IR index and BMI in case VS control groups**

| <b>Parameters</b>      | <b>Case<br/>(Mean±SD)</b> | <b>Control<br/>(Mean±SD)</b> | <b><i>P</i>-value</b> |
|------------------------|---------------------------|------------------------------|-----------------------|
| <b>Glucose (mg/dL)</b> | 161 ±34.8                 | 106±17.7                     | 0.000                 |
| <b>Insulin (µu/mL)</b> | 14.1±8.94                 | 10.2±5.16                    | 0.043                 |
| <b>IR</b>              | 5.57±4.19                 | 2.81±1.61                    | 0.001                 |
| <b>BMI</b>             | 28.2±6.98                 | 25.6±3.84                    |                       |

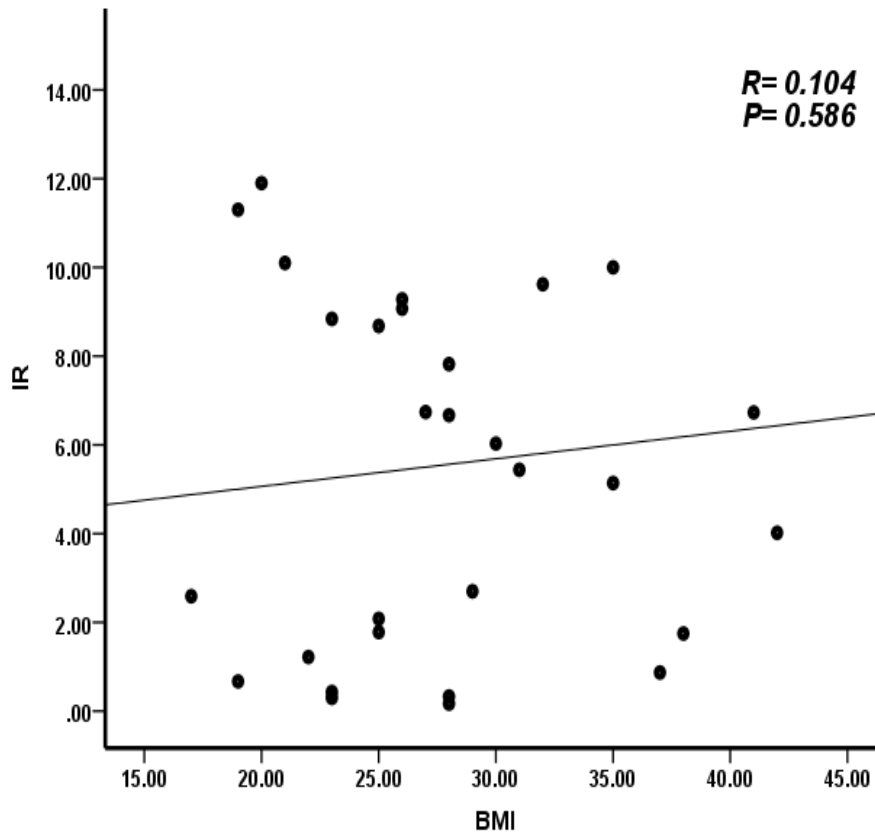


**Table (3.3): mean concentration of glucose, insulin and IR index in males VS females**

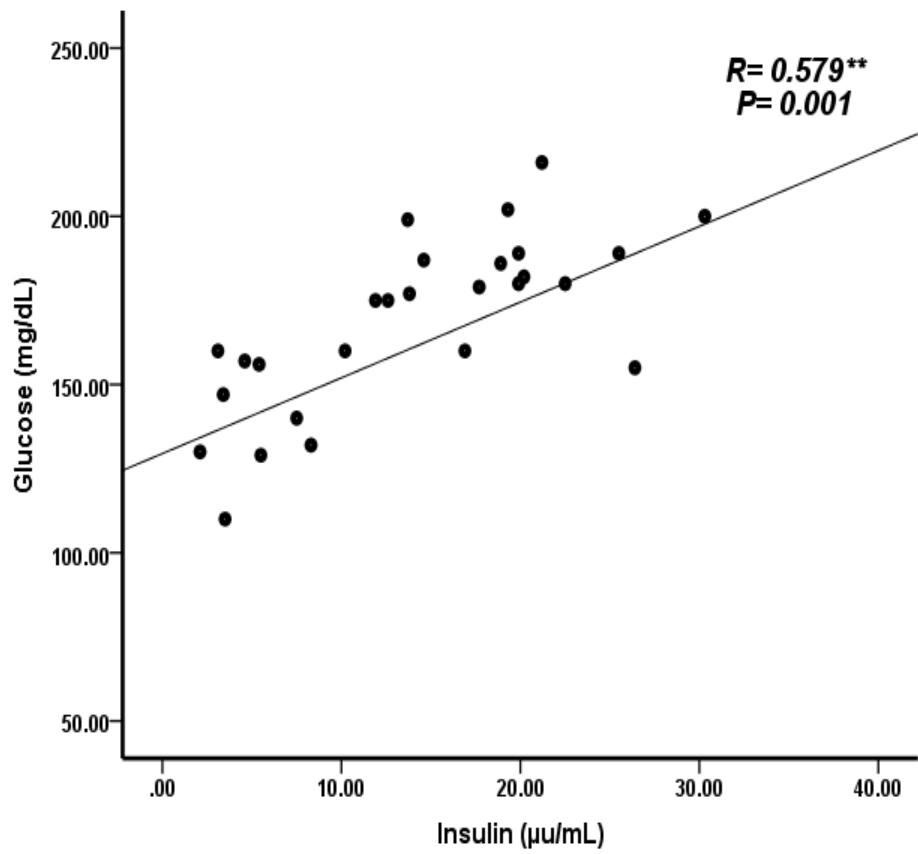
| <b>Parameters</b>          | <b>Male<br/>(Mean±SD)</b> | <b>Female<br/>(Mean±SD)</b> | <b><i>P-value</i></b> |
|----------------------------|---------------------------|-----------------------------|-----------------------|
| <b>Glucose<br/>(mg/dL)</b> | 166±33.3                  | 159±35.8                    | 0.611                 |
| <b>Insulin (µu/mL)</b>     | 18.4±8.14                 | 12.5±7.71                   | 0.045                 |
| <b>IR</b>                  | 6.21±4.94                 | 5.34±3.99                   | 0.623                 |



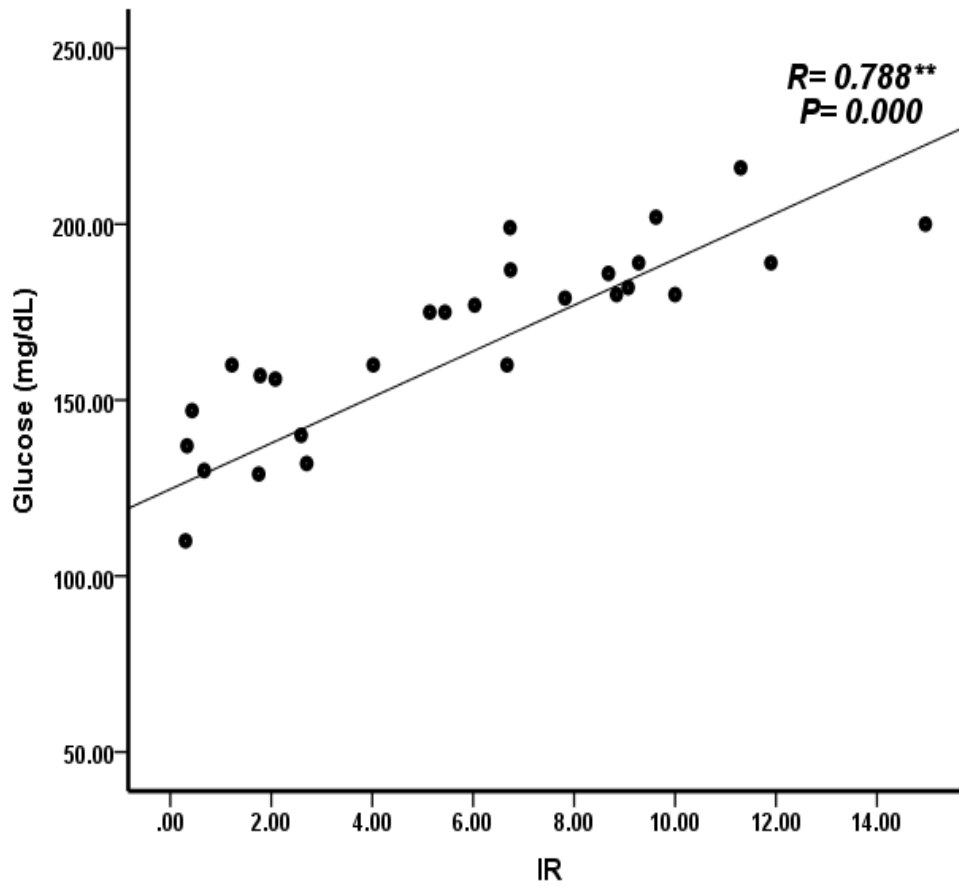
**Figure (3.3): correlation between age and insulin resistance ( $r = 0.179$ ,  $p = 0.343$ )**



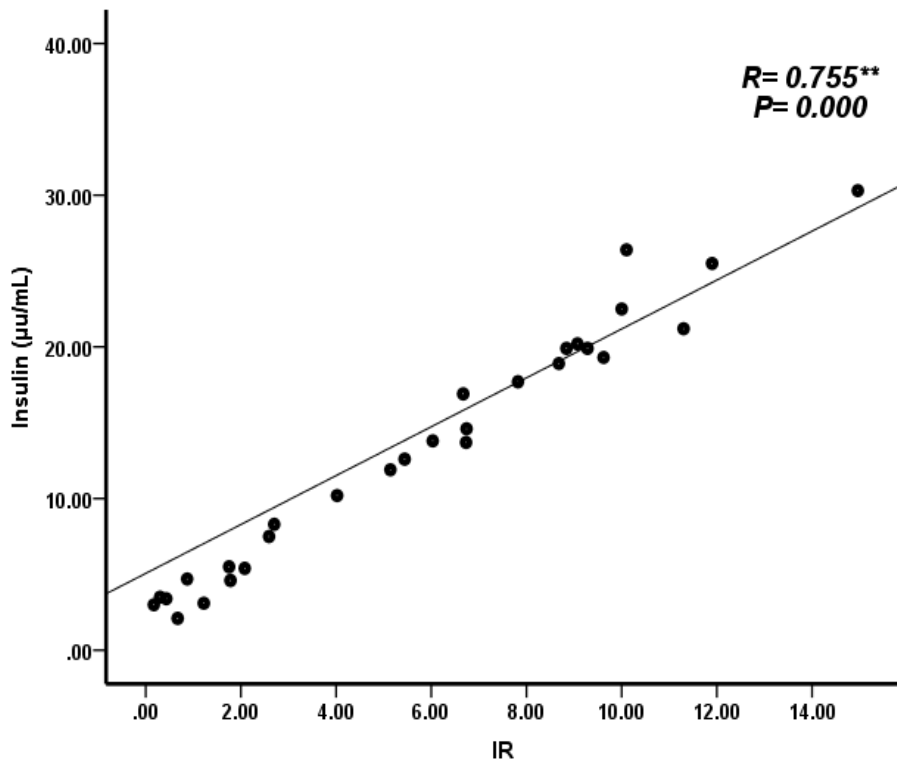
**Figure (3.4): correlation between BMI and IR**  
**( $r = 0.104$ ,  $p = 0.586$ )**



**Figure (3.5): correlation between insulin level and glucose level (r = 0.579, p=0.001)**



**Figure (3.6): correlation between insulin resistance index and glucose level ( $r = 0.788$ ,  $p = 0.000$ )**



**Figure (3.7): correlation between insulin resistance index and insulin level**  
**(r = 0.755, P =0.000)**

# ***CHAPTER FOUR***

## ***DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS***

## 4.1 Discussion

A gallstones disease is a worldwide disease and it is known to be one of the most common health problems leading to surgical intervention. Gall stones formation is a multifactorial process and many studies have shown that insulin resistance is one of the associated factors. Therefore we conducted this descriptive cross-sectional study to assess the insulin resistance index (HOMA-IR) and its correlation with the study variables among gall stones Sudanese patients in Khartoum state.

Analyses of frequency showed that gallstone disease is more common in females than in males with percent (73% females and 27% males) with fold 2.7: 1. This finding in agreement with across sectional study which carried out in Iran on a total of 1522 males and females, they found that females were 2.73 times more likely to have disease compared to males (Alireza-Ansari, 2015). This could be possibly due to sex hormone estrogen differences (Murshid, 1998). Estrogen known as the primary sex hormone in females, this hormone may increases the cholesterol saturation in bile and it could probably lead to cholesterol gallstone formation (Sharma et al., 2013).

The present study showed that the frequencies of premenopausal and postmenopausal females among gallstone patients are (63.3%) and (36.7%) respectively with fold 1.7:1 .This is similar to results of a study in Taiwan which confirmed that increasing age had a direct relationship with the development of gallstones (ChenCY *et al.*, 1998), this may be due to the long-term exposure to other risk factors irrespective of locality or standard of living (Shlomo *et al.*, 2011).



Also the present study showed that the majority of patients have an abnormal weight with percent 63.3% (30% of them were overweight while 33.3% were obese), and 36.7% have a normal weight. This means that the risk of disease is 1.7 times higher with abnormal weight. This finding is in agreement with a prospective case-control study performed on 118 Korean subjects and found that there is significant association between abnormal weight and gall stones disease (Kim *et al.*, 2007).

Results of the present study revealed significant increase in the mean concentration of glucose, insulin and insulin resistance index (HOMA-IR) in gall stones patients when compared with control group ( $p$ -value =0.000, 0.043 and 0.001) respectively. Similar results from previous case-control study found that Insulin resistance is associated with gall stones disease (Cynthia *et al.*, 2008).

Also the study showed that there were insignificant differences in mean concentrations of glucose and insulin resistance index of males in comparison with females ( $p$ -value =0.611 and 0.623) respectively. Sarah *et al.* conducted community- based longitudinal cohort of 292 children studied annually from 9 to 16 years to associate between glucose, insulin resistance index and gender. They concluded that at 16 years the gender difference was not significant (Sarah *et al.*, 2017).

This study revealed significant increase in the mean concentration of insulin of males in comparison to females ( $p$ -value =0.045). Contrary to what was reported by other researchers (Luz-Marina *et al.*, 2007), who found no differences in insulin between girls and boys. This attributed to differences in study subjects; males in this study were elder.

In this study we found that there were no correlation between age and insulin resistance index ( $r = 0.179$ ,  $p = 0.343$ ). This finding is similar to a Non-matched case-controlled study in a university hospital in Mexico

City, in which two hundred and eighty-seven subjects were included (Nahum *et al.*, 2005).

Researches and data indicate a correspondence between greater obesity and higher insulin resistance. A cross-sectional study was performed to examine the relationship between BMI and insulin resistance in US adults, Data were obtained from the National Health and Nutrition and Examination Surveys (1999–2006), concluded that BMI is strongly correlated with insulin resistance (Keilah *et al.*, 2017). This finding is not in a line with our results which show that there was no correlation between BMI and insulin resistance (**r =0.104, p =0.586**). This mismatching is probably due to difference in subjects, in this study the correlation was done for gall stones patients, whom have a high BMI and high insulin resistance index.

More ever, personal correlation results show strong positive correlation of insulin resistance index with fasting glucose and fasting insulin level (**r = 0.788, p = 0.000**) and (**r = 0.755, P =0.000**) respectively. This finding confirmed by a study in Venezuela in which Fasting insulin and glucose were measured in 418 children and adolescents (191 boys and 227 girls) then HOMA- IR was calculated. A significant correlation was found between insulin and glucose with HOMA- IR concentrations, which was expected, because HOMA-IR values are derived from insulin and glucose concentrations (Luz-Marina *et al.*, .2007).

## **4.2 Conclusions**

The data suggests that, gallstones disease is common in Sudanese post-menopausal females than males and obese subjects being more susceptible to gall stones disease. HOMA-IR is higher in gallstones patients. Moreover, insulin resistance index not correlate with age and BMI while the insulin resistance index is positively correlate with glucose and insulin. Thus HOMA-IR should be monitored for gall stones patients to predict and diagnose related complications.

## **4.3 Recommendation**

Estimation of lipid profile is recommended as further study and also to study the nutritional status and lifestyle as risk factors for gall stones formation.

## References

- Ahima, R.S. and Lazar, M.A.**, 2008. Adipokines and the peripheral and neural control of energy balance. *Molecular Endocrinology*, 22(5), pp. 1023-1031.
- Ansari-Moghaddam, A., Khorram, A., Miri-Bonjar, M., Mohammadi, M. and Ansari,H.**, 2016. The prevalence and risk factors of gallstone among adults in South-East of Iran: A population-based study. *Global journal of health science*, 8(4), p.60.
- Barrett, K E. and Keely, S.J.**, 2006. Integrative physiology and pathophysiology of intestinal electrolyte transport. *Physiology of the gastrointestinal tract*, 4, pp.1931-1951.
- Beale, E. G. 2013.** Insulin signaling and insulin resistance. *Journal of Investigative Medicine*, 61(1), pp.11-14.
- Bonner-Weir ,S.**, 2000. Perspective: postnatal pancreatic  $\beta$  cell growth. *Endocrinology*, 141(6), pp.1926-1929.
- Das, U.N.**, 1999. GLUT-4, tumor necrosis factor, essential fatty acids and daf-genes and their role in insulin resistance and non-insulin dependent diabetes mellitus. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)*, 60(1), pp.13-20.
- Després, J.P.**, 2007. The endocannabinoid system: a new target for the regulation of energy balance and metabolism. *Critical pathways in cardiology*, 6(2), pp.46-50.
- Dulloo, A.G.**, 2005. A role for suppressed skeletal muscle thermogenesis in pathways from weight fluctuations to the insulin resistance syndrome. *Acta Physiologica*, 184(4), pp.295-307.
- Ferrannini, E. and Mari, A.**, 1998. How to measure insulin sensitivity. *Journal of hypertension*, 16(7), pp.895-906.

- Fox, S.I.**, 2009. Fundamentals of human physiology. McGraw-Hill.
- Geor, R.J.**, 2013. Nutritional management of insulin resistance. *Journal of Equine Veterinary Science*, 33(10), pp.852-854.
- Goldstein, B.J. and Müller-Wieland, D. eds.**, 2016. Type 2 diabetes: principles and practice. CRC Press.
- Griffin, M.J. and Sul, H.S.**, 2004. Insulin Regulation of Fatty Acid Synthase Gene Transcription: Roles of USF and SREBP-1c. *IUBMB life*, 56(10), pp.595-600.
- Hale, W.G., Margham, J.P. and Saunders, V.A.**, 1995. Collins dictionary of biology. Harpercollins Pub Ltd.
- Hansotia, T. and Drucker, D.J.**, 2005. **GIP and GLP-1** as incretin hormones: lessons from single and double incretin receptor knockout mice. *Regulatory peptides*, 128(2), pp.125-134.
- Havel, P.J.**, 2001. Peripheral signals conveying metabolic information to the brain: short-term and long-term regulation of food intake and energy homeostasis. *Experimental Biology and Medicine*, 226(11), pp.963-977.
- Hirosumi, J., Tuncman, G., Chang, L. and Gorgun, C.Z.**, 2002. A central role for JNK in obesity and insulin resistance. *Nature*, 420(6913), p.333.
- Ishiki, M. and Klip, A.**, 2005. Minireview: recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners. *Endocrinology*, 146(12), pp.5071-5078.
- Jeffery, S.C., Hosking, J., Jeffery, A.N., Murphy, M.J., Voss, L.D., Wilkin, T.J. and Pinkney, J.**, 2017. Insulin resistance is higher in prepubertal girls but switches to become higher in boys at age 16: A Cohort Study (EarlyBird 57). *Pediatric Diabetes*.
- Kim, S. S., Lee, J. G., Kim, D. W., Kim, B. H., Jeon, Y. K., Kim, M. R., Kim, I. J.** (2011). Insulin Resistance as a Risk Factor for Gallbladder Stone

Formation in Korean Postmenopausal Women. *The Korean Journal of Internal Medicine*, 26(3), 285–293.

**Leibowitz, M.D., Biswas, C., Brady, E.J., Conti, M., Cullinan, C.A., Hayes, N.S., Manganiello, V.C., Saperstein, R., Wang, L.H., Zafian, P.T. and Berger, J.**, 1995. A novel insulin secretagogue is a phosphodiesterase inhibitor. *Diabetes*, 44(1), pp.67-74.

**Lammert, F. and Sauerbruch, T.**, 2005. Mechanisms of disease: the genetic epidemiology of gallbladder stones. *Nature Reviews. Gastroenterology & Hepatology*, 2(9), p.423.

**Liu, L.F., Purushotham, A., Wendel, A.A. and Belury, M.A.**, 2007. Combined effects of rosiglitazone and conjugated linoleic acid on adiposity, insulin sensitivity, and hepatic steatosis in high-fat-fed mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 292(6), pp.G1671-G1682.

**Martinez, K.E., Tucker, L.A., Bailey, B.W. and LeCheminant, J.D.**, 2017. Expanded Normal Weight Obesity and Insulin Resistance in US Adults of the National Health and Nutrition Examination Survey. *Journal of diabetes research*, 2017.

**Mather, K.J., Lteif, A., Steinberg, H.O. and Baron, A.D.**, 2004. Interactions between endothelin and nitric oxide in the regulation of vascular tone in obesity and diabetes. *Diabetes*, 53(8), pp.2060-2066.

**Méndez-Sánchez, N., Bermejo-Martínez, L.B., Viñals, Y., Chavez-Tapia, N.C., Vander Graff, I., Ponciano-Rodríguez, G., Ramos, M.H. and Uribe, M.**, 2005. Serum leptin levels and insulin resistance are associated with gallstone disease in overweight subjects. *World journal of gastroenterology: WJG*, 11(39), p.6182.

**Meshkani, R. and Adeli, K.,** 2009. Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. *Clinical biochemistry*, 42(13), pp.1331-1346.

**Morales, L.M., Raleigh, X., Fernandez, V. and Molero-Conejo, E.,** 2007. Distribution of fasting glucose, insulin, homeostasis model assessment (HOMA) insulin resistance (IR) and HOMA beta cell in children and adolescents from Maracaibo, Venezuela. *Revista medica de Chile*, 135(2), pp.205-211.

**Mues, C., Zhou, J., Manolopoulos, K.N., Korsten, P., Schmall, D., Klotz, L.O., Bornstein, S.R., Klein, H.H. and Barthel, A.,** 2009. Regulation of glucose-6-phosphatase gene expression by insulin and metformin. *Hormone and metabolic research*, 41(10), pp.730-735.

**Murshid, K.R.,** 1998. Symptomatic gallstones: a disease of young Saudi women. *Saudi journal of gastroenterology*, 4(3), p.159.

**Nolan, C.J., Damm, P. and Prentki, M.,** 2011. Type 2 diabetes across generations: from pathophysiology to prevention and management. *The Lancet*, 378(9786), pp.169-181.

**Park, S., Hong, S.M. and Ahn, I.S.,** 2010. Exendin-4 and exercise improve hepatic glucose homeostasis by promoting insulin signaling in diabetic rats. *Metabolism*, 59(1), pp.123-133.

**Pedersen, O., Bak, J.F., Andersen, P.H., Lund, S., Moller, D.E., Flier, J.S. and Kahn, B.B.,** 1990. Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. *Diabetes*, 39(7), pp.865-87

**Pratley, R.E.,** 1998. Gene–environment interactions in the pathogenesis of type 2 diabetes mellitus: lessons learned from the Pima Indians. *Proceedings of the Nutrition Society*, 57(2), pp.175-181.

- Rajpathak, S.N., Gunter, M.J., Wylie-Rosett, J., Ho, G.Y., Kaplan, R.C., Muzumdar, R., Rohan, T.E. and Strickler, H.D.,** 2009. The role of insulin-like growth factor-I and its binding proteins in glucose homeostasis and type 2 diabetes. *Diabetes/metabolism research and reviews*, 25(1), pp.3 - 12.
- Romao, I. and Roth, J.,** 2008. Genetic and environmental interactions in obesity and type 2 diabetes. *Journal of the American Dietetic Association*, 108(4), pp.S24-S28.
- Rossi, A.S., Lombardo, Y.B. and Chicco, A.G.,** 2010. Lipogenic enzyme activities and glucose uptake in fat tissue of dyslipemic, insulin-resistant rats: effects of fish oil. *Nutrition*, 26(2), pp.209-217.
- Sanderson, T.H., Kumar, R., Sullivan, J.M. and Krause, G.S.,** 2008. Insulin blocks cytochrome c release in the reperfused brain through PI3-K signaling and by promoting Bax/Bcl-XL binding. *Journal of neurochemistry*, 106(3), pp.1248-1258.
- Seneviratne, H.R., Lankeshwara, D., Wijeratne, S., Somasunderam, N. and Athukorale, D.,** 2009. Serum insulin patterns and the relationship between insulin sensitivity and glycaemic profile in women with polycystic ovary syndrome. *BJOG: An International Journal of Obstetrics & Gynaecology*, 116(13), pp.1722-1728.
- Sharma, R., Sachan, S.G. and Sharma, S.R.,** 2013. Preponderance of gallstone in female. *Korea*, 1(1), pp.12-13.
- Shulman, G.I.,** 2000. Cellular mechanisms of insulin resistance. *Journal of Clinical Investigation*, 106(2), p.171.
- Simonson, G.D. and Kendall, D.M.,** 2005. Diagnosis of insulin resistance and associated syndromes: the spectrum from the metabolic syndrome to type 2 diabetes mellitus. *Coronary artery disease*, 16(8), pp.465-472.



**Taylor, S.I.**, 1992. Lilly Lecture: molecular mechanisms of insulin resistance: lessons from patients with mutations in the insulin-receptor gene. *Diabetes*, 41(11), pp.1473-1490.

**Thulasi, R., Dias, P., Houghton, P.J. and Houghton, J.A.**, 1996. Alpha 2a-interferon-induced differentiation of human alveolar rhabdomyosarcoma cells: correlation with down-regulation of the insulin-like growth factor type I receptor. *Cell growth & differentiation: the molecular biology journal of the American Association for Cancer Research*, 7(4), pp.531-541.

**Torlinska, T., Mackowiak, P., Nogowski, L., Hryniewiecki, T., Witmanowski, H., Perz, M., Madry, E. and Nowak, K.M.**, 2000. Age dependent changes of insulin receptors in rat tissues. *Journal of Physiology and Pharmacology*, 51(4, 2).

**Varea, O., Arevalo, M.A., Garrido, J.J., Garcia-Segura, L.M., Wandosell, F. and Mendez, P.**, 2010. Interaction of estrogen receptors with insulin-like growth factor-I and Wnt signaling in the nervous system. *Steroids*, 75(8), pp.565-569.

**Wallace, T.M. and Matthews, D.R.**, 2002. The assessment of insulin resistance in man. *Diabetic Medicine*, 19(7), pp.527-534.

**Watson, R.T. and Pessin, J.E.**, 2001. Intracellular organization of insulin signaling and GLUT4 translocation. *Recent progress in hormone research*, 56, pp.175-193.

**Wang, Z., Iwasaki, Y., Zhao, L.F., Nishiyama, M., Taguchi, T., Tsugita, M., Kambayashi, M., Hashimoto, K. and Terada, Y.**, 2009. Hormonal regulation of glycolytic enzyme gene and pyruvate dehydrogenase kinase/phosphatase gene transcription. *Endocrine journal*, 56(8), pp.1019-1030.

**Warttig, S., Ward, S. and Rogers, G.,** 2014. Diagnosis and management of gallstone disease: summary of NICE guidance. *BMJ: British Medical Journal* (Online), 349.

**Wilson, J.L.,** 1988. *Biochemistry*; (Stryer, Lubert).

**Woodcroft, K.J., Hafner, M.S. and Novak, R.F.,** 2002. Insulin signaling in the transcriptional and posttranscriptional regulation of CYP2E1 expression. *Hepatology*, 35(2), pp.263-273.

**Zac-Varghese, S., Tan, T. and Bloom, S.R.,** 2010. Hormonal interactions between gut and brain. *Discovery medicine*, 10(55), pp.543-552.

**Zhao, M., Wan, Z.L., Whittaker, L., Xu, B., Phillips, N.B., Katsoyannis, P.G., Ismail-Beigi, F., Whittaker, J. and Weiss, M.A.,** 2009. Design of an Insulin Analog with Enhanced Receptor Binding Selectivity RATIONALE, STRUCTURE, AND THERAPEUTIC IMPLICATIONS. *Journal of Biological Chemistry*, 284(46), pp.32178-32187.

# ***APPENDICES***

بسم الله الرحمن الرحيم

Sudan University for Science and Technology

Collage of Graduated studies

Clinical Chemistry Department

**Assessment of insulin resistance index (HOMA-IR) among  
gall stones patients**

Questionnaire NO: ..... Date.....

Age: ..... Sex: .....

Residence: urban (.....) Rural (.....)

Body weight (kg): ..... Body height (cm): .....

Body mass index: .....

Other disease: .....

Drugs use: .....

Have you ever been used contraceptive? Yes (.....) No (.....)

Type of contraceptive oral (.....) injection (.....)

Age at first menses: ..... Age at last menses: .....

Premenopausal (.....) Postmenopausal (.....)

**Investigations:**

LH level: .....

FSH level: .....

Insulin level.....

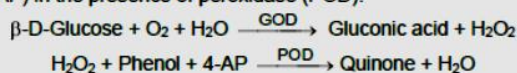
Blood glucose level: .....

**Quantitative determination of glucose IVD**

Store at 2-8°C

**PRINCIPLE OF THE METHOD**

Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The formed hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), is detected by a chromogenic oxygen acceptor, phenol, 4 – aminophenazone (4-AP) in the presence of peroxidase (POD):



The intensity of the color formed is proportional to the glucose concentration in the sample<sup>1,2</sup>.

**CLINICAL SIGNIFICANCE**

Glucose is a major source of energy for most cells of the body; insulin facilitates glucose entry into the cells.

Diabetes is a disease manifested by hyperglycemia; patients with diabetes demonstrate an inability to produce insulin<sup>1,5,6</sup>.

Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

**REAGENTS**

|   |                           |            |
|---|---------------------------|------------|
| R | TRIS pH 7.4               | 92 mmol/L  |
|   | Phenol                    | 0.3 mmol/L |
|   | Glucose oxidase (GOD)     | 15000 U/L  |
|   | Peroxidase (POD)          | 1000 U/L   |
|   | 4 – Aminophenazone (4-AP) | 2.6 mmol/L |

**PREPARATION**

The reagent is ready to use.

**STORAGE AND STABILITY**

All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8°C, protected from light and contaminations prevented during their use. Do not use reagents over the expiration date.

**SIGNS OF REAGENT DETERIORATION:**

- Presence of particles and turbidity.
- Blank absorbance (A) at 505 nm ≥ 0.32.

**ADDITIONAL EQUIPMENT**

- SPIN640 Autoanalyzer.
- General laboratory equipment.

**SAMPLES**

Serum or plasma, free of hemolysis<sup>1</sup>:

Serum should be removed from the clot as quickly as possible.

Stability of the sample: Glucose in serum or plasma is stable at 2-8° for 3 days.

**BARCODED REAGENTS LOAD MUST BE PRECEDED OF A SPINREACT "DATABASE" COPY INTO THE ANALYZER SOFTWARE. IT IS AVAILABLE UNDER REQUEST TO SPINREACT.**

**SPIN640 APPLICATION**

|                            |              |                       |                |
|----------------------------|--------------|-----------------------|----------------|
| <b>TEST INFORMATION</b>    |              | <b>REAGENT VOLUME</b> |                |
| Nº                         | **           | Vol. R1               | 300            |
| Test                       | GLU          | Vol. R2               |                |
| Full Name                  | Glucose      | Vol. R3               |                |
| Standard nº                | 1            | Vol. R4               |                |
| <b>SAMPLE VOLUME</b>       |              | <b>RESULT SETUP</b>   |                |
| Vol. Sample Stand.         | 3            | Decimal               | 1 Slope 1      |
| Vol. Sample Increas.       |              | Unit                  | mg/dL Inter. 0 |
| Vol. Sample Dec            |              |                       |                |
| <b>REACTION PARAMETERS</b> |              |                       |                |
| Reac. Type                 | End Point    | Direction             | Increase       |
| Pri. Wave.                 | 505          | Reagent Blank         | 10-11          |
| Sec. Wave.                 |              | React. Time           | 79-80          |
| <b>JUDGEMENT CRITERIA</b>  |              |                       |                |
| Absorbance                 | -30000/30000 | Lin. Range            | 0-500          |
| Incre.Test                 |              | Lin. Limit            |                |
| Decre.Test                 |              | Subs. Limit           |                |
| Prozone (Rate-Antigen)     |              | Q1                    |                |
| PC                         |              | Q2                    |                |
| ABS                        |              | Q3                    |                |
|                            |              | Q4                    |                |

The Calibration is stable until **36 days**. After this period the Calibration must be performed again in order to obtain good results.

**QUALITY CONTROL**

Control sera and calibrators are recommended to monitor the performance of assay procedures: SPINTRON H Calibrator, SPINTRON H Normal and Pathologic (Ref. 1002011, 1002120 and 1002210).

If control values are found outside the defined range, check the instrument, reagents and technique for problems.

Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

**REFERENCE VALUES<sup>1</sup>**

Serum or plasma:  
 60 – 110 mg/dL ≅ 3.33 – 6.10 mmol/L

These values are for orientation purpose; each laboratory should establish its own reference range.

**NOTES**

1. Calibration with the aqueous standard may cause a systematic error in automatic procedures. In these cases, it is recommended to use a serum Calibrator.
2. Use clean disposable pipette tips for its dispensation.

**BIBLIOGRAPHY**

1. Kaplan L.A. Glucose. Kaplan A et al. Clin Chem The C.V. Mosby Co. St Louis. Toronto, Princeton 1984; 1032-1036.
2. Trinder P. Ann Clin Biochem 1969; 6 24-33.
3. Young DS. Effects of drugs on Clinical Lab. Tests, 4th ed AACC Press, 1995.
4. Young DS. Effects of disease on Clinical Lab. Tests, 4th ed AACC 2001.
5. Burtis A et al. Tietz Textbook of Clinical Chemistry, 3rd ed AACC 1999.
6. Tietz N W et al. Clinical Guide to Laboratory Tests, 3rd ed AACC 1995.

**PACKAGING**

Ref: MD41011

Cont.

R: 6 x 40 mL

### **Important Information for Users**

The University of Minnesota periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

### **NHANES 2007-2008 Public Release Data Set Information**

This document details the Lab Protocol for testing items in the following table:

| <b>Data File Name</b> | <b>Variable Name</b> | <b>SAS Label</b>       |
|-----------------------|----------------------|------------------------|
| GLU_E                 | LBXIN                | Insulin ( $\mu$ U/mL ) |
|                       | LBDINSI              | Insulin (pmol/L)       |

### Insulin in Serum NHANES 2007-2008

#### **1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE**

Insulin is the primary hormone responsible for controlling glucose metabolism, and its secretion is governed by plasma glucose concentration. The insulin molecule is synthesized in the pancreas as pro-insulin and is later cleaved to form C-peptide and insulin. The principal function of insulin is to control the uptake and utilization of glucose in the peripheral tissues. Insulin concentrations are severely reduced in insulin-dependent diabetes mellitus (IDDM) and some other conditions, while concentrations are raised in non-insulin-dependent diabetes mellitus (NIDDM), obesity, and some endocrine dysfunctions.

The Merocodia Insulin ELISA is a two-site enzyme immunoassay utilizing the direct sandwich technique with two monoclonal antibodies directed against separate antigenic determinants of the insulin molecule. Specimen, control, or standard is pipetted into the sample well, then followed by the addition of peroxidase-conjugated anti-insulin antibodies. Insulin present in the sample will bind to anti-insulin antibodies bound to the sample well, while the peroxidase-conjugated anti-insulin antibodies will also bind to the insulin at the same time. After washing to remove unbound enzyme-labelled antibodies, TMB-labelled substrate is added and binds to the conjugated antibodies. Acid is added to the sample well to stop the reaction, and the colorimetric endpoint is read on a microplate spectrophotometer set to the appropriate light wavelength.

## **2. SAFETY PRECAUTIONS:**

Follow all procedures and policies listed the Fairview-University Medical Center Laboratory Safety Manual. Consider all specimens, control materials, and calibrator materials as potentially infectious.

## **3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT:**

### **RESULT ENTRY:**

- A. Open the Study Patients Folder located in the “aren0085” server folder.
- B. Open the *Mercodia Insulin in duplicate* file with the matching date of the results.
- C. Click on the Read Data tab (at the bottom of the page).
- D. Put in your floppy disk with the OD readings on it.
- E. Open the floppy disk under My Computer, 3 ½ Floppy Disk, open the data file that you want.
- F. Highlight the data, right-click, and copy.

G. Open the appropriate results file, click the Read Data tab, right-click in the top left cell, and paste the results.

H. **If samples are run singly**, while the data is highlighted click on the Excel Sort button to order the results so that no spaces remain between lines. Do not do this step if samples are run in duplicate.

I. Highlight and copy the well numbers to the appropriate positions in the Samples to Run page.

Insulin in Serum NHANES 2007-2008

J. Go back to the Read Data tab and highlight and copy the result values.

K. Go to the Samples to Run page and paste the values in the correct positions corresponding to the well numbers.

L. Check your control values to make sure that they are in the specified range.

M. Plot the QC values in the appropriate QC file in the computer.

N. Print the Samples to Run page.

O. Check your results against your spectrophotometer raw data.

P. Open the appropriate results file.

Q. Find the Lab ID numbers that correspond to those you have data for. Place the cursor in the correct position in the list.

R. Go back to the Insulin results file and highlight your results. Right-click, then select Copy.

S. Go back to the appropriate results file, right-click, and paste the results.

T. Double-check your spreadsheet (copy and paste procedure) against your results hard copy.

U. Fill in the other information on the Result spreadsheet: date, volume used, kit lot number, and any other comments about the assay run.

V. Save the information on the spreadsheet.



W. Have a second Technologist review your work, sign off, and file the results.

#### **4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

Serum or EDTA- or heparin-plasma may be used to test for insulin concentration with this kit. Either type of sample should be stored at -20oC or lower if not assayed immediately (within 24 hours). Avoid repeated freeze-thaw cycles of the sample. The CSCL laboratory has determined that insulin is stable up to five freeze-thaw cycles. Twenty-five (25) uL of each sample of serum or plasma is required to test each specimen singly per assay. Grossly lipemic, icteric, or hemolyzed samples do not interfere with this assay. All patient samples should be handled as of capable of transmitting infections.

No dilution is required for most samples. If a dilution is required, use Calibrator 0 as the diluent.

Samples from individuals undergoing insulin therapy may produce incorrect results due to formation of anti-insulin antibodies that are capable of interfering with this assay.

#### **PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

4 Insulin in Serum NHANES 2007-2008

#### **6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

A. Instrumentation

1. Microplate reader capable of measuring absorbance at 450 nm. (Molecular Devices, SpectraMax 250).
2. Beckman Coulter Biomek 2000 Workstation, Beckman Coulter Biomek P250 pipette tips (catalog #373689) as pictured in the “Biomek Workstation” figure below, Biomek 48-tube holders, Biomek quarter reservoirs

## B. Materials and Reagents

### **Reagents:**

All reagents must be brought to room temperature before use. \* **Follow appropriate reagent preparation guidelines according to either a single kit or a ten-pack of kits, depending upon what is used. The instructions below are for a ten-pack of kits.**

#### 1. MilliQ Water:

MilliQ is the trade name of the water system purchased from the Millipore Corporation (Continental Water System). MilliQ water is deionized water treated with activated carbon and deionization cartridges and filtered to remove microorganisms larger than 0.22 micrometers.

2. Merckodia Insulin ELISA Kit. Catalog number 10-1113-10 (10 x 96 determinations) or 10-1113-01 (96 determinations). Merckodia AB, Uppsala, Sweden. See outer label of kit for expiration date. The kit includes the following reagents:

#### a. Microplates:

Ten 96-well microplates coated with a murine monoclonal anti-insulin antibody. Store at 2-8°C. After opening package, any unused microplate wells should be returned to the foil pouch (containing the desiccant pack) and sealed; these may be stored for up to 2 months at 2-8°C. The expiration date is printed on the outer packaging of the plates; unopened plates that are stored at 2-8°C are stable until this date.

b. Enzyme Conjugate 11X:

Peroxidase conjugated mouse monoclonal anti-insulin, ~6 $\mu$ g/mL. Store at 2-8 $^{\circ}$ C.

Dilute as instructed below. Diluted enzyme conjugate should be used within one day. The expiration date is printed on the outer packaging of the enzyme conjugate; enzyme conjugate that is stored at 2-8 $^{\circ}$ C is stable until this date.

c. Enzyme Conjugate Buffer:

One hundred twenty (120) mL of solution, ready for use. Store at 2-8 $^{\circ}$ C. The expiration date is printed on the outer packaging of the enzyme

Insulin in Serum NHANES 2007-2008 conjugate buffer; enzyme conjugate buffer that is stored at 2-8 $^{\circ}$ C is stable until this date.

d. Calibrators:

Recombinant human insulin in concentrations of 2, 3, 10, 30, 100, and 200 mU/L, ready to use, each at a volume of 1mL, except calibrator 2.0 which contains 0.5mL.

e. Calibrator 0:

Five (5) mL of solution, ready to use. This calibrator is used as a plate blank in the calculation of results and for the dilution of any samples with an insulin concentration greater than 200mU/L. Store at 2-8 $^{\circ}$ C.

f. Wash Buffer 21X:

Store at 2-8 $^{\circ}$ C. Dilute as instructed below. Store diluted buffer at 2-8 $^{\circ}$ C for up to 4 weeks. The expiration date is printed on the outer packaging of the wash buffer; wash buffer that is stored at 2-8 $^{\circ}$ C is stable until this date.

g. Substrate TMB:

3, 3',5,5'-tetramethylbenzidine (TMB) colorless solution, ready to use. *Note: light sensitive.* Store at 2-8°C. The expiration date is printed on the outer packaging of the substrate TMB; substrate TMB that is stored at 2-8°C is stable until this date.

h. Stop Solution:

0.5M H<sub>2</sub>SO<sub>4</sub>, ready to use. Store at 2-8°C. The expiration date is printed on the outer packaging of the stop solution; stop solution that is stored at 2-8°C is stable until this date.

*Caution: This is an acid solution. Wear eye, hand, face, and clothing protection when handling this substance.*

#### **Equipment and Supplies Required:**

1. 20 µL, 200 µL, and 1000 µL pipets (for manual method; or See 10.)
2. Pipet tips appropriate for above pipets (1, for manual method; or See 10.)
3. 20-200µL 12-channel pipet
4. 1L graduated cylinder
5. Nunc-Immuno Wash 12 (catalog #470175) microplate washer
6. 0.5 mL and 2.0mL microcentrifuge tubes, graduated free-standing
7. 10mL disposable serological pipettes
8. Plastic reagent reservoirs
9. Test tube tipper
10. Plastic transfer pipettes

Insulin in Serum NHANES 2007-2008

#### **C. Reagent Preparation**

All reagents must be brought to room temperature before use.

1. Wash buffer: If catalog #10-1113-01 (the single is kit) is used: dilute the bottle of 21X wash buffer in 800mL of MilliQ water. If catalog #10-1113-10 (**the 10 pack of kits**) is used, prepare wash buffer according to the table below. Using 40mL of wash buffer and 800mL of water (1 plate instructions) will be enough for two plates. Discard any remaining wash after two plates and make fresh. If only one plate is done in a day, save the wash until the next plate is run.

**Note:** Prepare the conjugate directly in a Biomek quarter reservoir, using a disposable 10mL serological pipet.

#### D. Standards Preparation

1. Calibrators:

Upon receipt of the ten-pack of kits, aliquot each calibrator into 14, 0.5mL microcentrifuge vials, each containing 70uL, calibrator 2 will only have 7 vials due to its smaller volume. Each vial is adequate for 1 plate. Store at 2-8oC. The expiration date is printed on the outer packaging of the calibrators; calibrators that are stored at 2-8oC are stable until this date.

1. Calibrator 0:

Upon receipt of the ten-pack of kits, aliquot calibrator 0 into 14, 0.5mL microcentrifuge vials, each containing 70uL. Each vial is adequate for 1 plate. The remaining solution can be kept in the original vial to use in making

Insulin in Serum NHANES 2007-2008

sample dilutions. Store at 2-8oC. The expiration date is printed on the outer packaging of calibrator 0; calibrator 0 that is stored at 2-8oC is stable until this date.

#### E. Preparation of Quality Control Materials

Control Low (Catalog #10-1134-01)

Control High (Catalog #10-1164-01)

**\*The control materials are manufactured from human blood components, therefore all controls should be handled as if capable of transmitting infections.**

Reconstitute each Mercodia Diabetes-antigen Control by adding 500uL deionized water to each vial. Replace the rubber stopper and allow standing for 5 minutes. Gently swirl until contents are thoroughly mixed. Avoid foaming.

Aliquot each control into 11, 0.5mL microcentrifuge vials, each containing 45uL.

Each vial is adequate for 1 plate. Store at -70°C. Avoid repeated freezing and thawing. Reconstituted Controls are stable for three months at -70°C.

## **7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES**

A daily position calibration is performed on the Biomek, as well as a monthly cleaning procedure, and a calibration of the pipet tools every 6 months. \*See Biomek Maintenance Procedures.

## **8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS**

### **A. PROCEDURE**

#### **Making the Worksheet**

Open the Study Patients Folder located in the “aren0085” server or the “shortcut to study patients” folder on the desktop.

1. Open the appropriate Study Folder (Mesa, NHANES, etc...)

1. Open the *Mercodia Insulin in duplicate template* file.

1. Click on “Samples to Run” tab (at the bottom of page).

1. Place the cursor in the S1 box.

1. Open the file that contains the ID numbers of the appropriate study.
1. Find Lab ID numbers you will assay and “highlight” them by selecting with the cursor.
1. Right-click on the selection, choose Copy, then go back to the *Mercodia Insulin in duplicate template* and Right-click on the S1 box and choose Paste.
2. Click on the “Sample Template” tab on the bottom of the page and check the plate layout and lot numbers of controls and reagents. Update if necessary.

#### Insulin in Serum NHANES 2007-2008

sample dilutions. Store at 2-8oC. The expiration date is printed on the outer packaging of calibrator 0; calibrator 0 that is stored at 2-8oC is stable until this date.

#### E. Preparation of Quality Control Materials

Control Low (Catalog #10-1134-01)

Control High (Catalog #10-1164-01)

**\*The control materials are manufactured from human blood components, therefore all controls should be handled as if capable of transmitting infections.**

Reconstitute each Mercodia Diabetes-antigen Control by adding 500uL deionized water to each vial. Replace the rubber stopper and allow standing for 5 minutes. Gently swirl until contents are thoroughly mixed. Avoid foaming.

Aliquot each control into 11, 0.5mL microcentrifuge vials, each containing 45uL.

Each vial is adequate for 1 plate. Store at -70°C. Avoid repeated freezing and thawing. Reconstituted Controls are stable for three months at -70°C.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A daily position calibration is performed on the Biomek, as well as a monthly cleaning procedure, and a calibration of the pipet tools every 6 months. \*See Biomek Maintenance Procedures.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### A. PROCEDURE

#### Making the Worksheet

Open the Study Patients Folder located in the “aren0085” server or the “shortcut to study patients” folder on the desktop.

2. Open the appropriate Study Folder (Mesa, NHANES, etc...)
2. Open the *Mercodia Insulin in duplicate template* file.
2. Click on “Samples to Run” tab (at the bottom of page).
2. Place the cursor in the S1 box.
2. Open the file that contains the ID numbers of the appropriate study.
2. Find Lab ID numbers you will assay and “highlight” them by selecting with the cursor.
3. Right-click on the selection, choose Copy, then go back to the *Mercodia Insulin in duplicate template* and Right-click on the S1 box and choose Paste.
4. Click on the “Sample Template” tab on the bottom of the page and check the plate layout and lot numbers of controls and reagents. Update if necessary.

#### NHANES 2007-2008

1. Save the worksheet in the folder of the appropriate study, under Insulin Results Save **as** *Mercodia Insulin in duplicate results* “date of run”.



1. Print your Worksheet.

B. Quality Control Materials

1. Calibrators: Arrange the 70 $\mu$ L calibrator aliquots in the Biomek 48-tube holder as shown below. The calibrator aliquots are sufficient for one plate.

1. Controls: Arrange the low and high Mercodia controls and lab (pooled)

Control in the Biomek 48-tube holder as shown below.

| Calibrators & Controls (tube holder #1), for samples in duplicate Tube holder #1 | 1               | 2                    | 3                  | 4        | 5        | 6            | 7         | 8 |
|--|-----------------|----------------------|--------------------|----------|----------|--------------|-----------|---|
| A  | Calibrator<br>0 | Cal 2.0              | Cal<br>3.0         | Cal 10.0 | Cal 30.0 | Cal<br>100.0 | Cal 200.0 |   |
| B  |                 | Mercodia low<br>ctrl | Mercodia high ctrl |          |          | Lab ctrl     |           |   |

The samples in the microplate will then be arranged as shown in the table below.

9 Insulin in Serum NHANES 2007-2008

| Number of strips/plates | Wash buffer 21X | MilliQ water |
|-------------------------|-----------------|--------------|
| 6 strips                | 20mL            | 400mL        |
| <b>1 plate</b>          | <b>40mL</b>     | <b>800mL</b> |
| 2 plates                | 70mL            | 1400mL       |
| 3 plates                | 110mL           | 2200mL       |

2. Enzyme conjugate: Dilute the 11X enzyme conjugate according to the table below.

*if catalog #10-1113-01 (the single is kit) is used:*

| Number of strips | Enzyme conjugate 11X | Enzyme conjugate buffer |
|------------------|----------------------|-------------------------|
| 4                | 400uL                | 4.0mL                   |
| 6                | 600uL                | 6.0mL                   |
| 8                | 700uL                | 7.0mL                   |
| 10               | 900uL                | 9.0mL                   |
| 12               | 1 vial (1.2mL)       | 1 vial (12mL)           |

*if catalog #10-1113-10 (the 10 pack of kits) is used:*

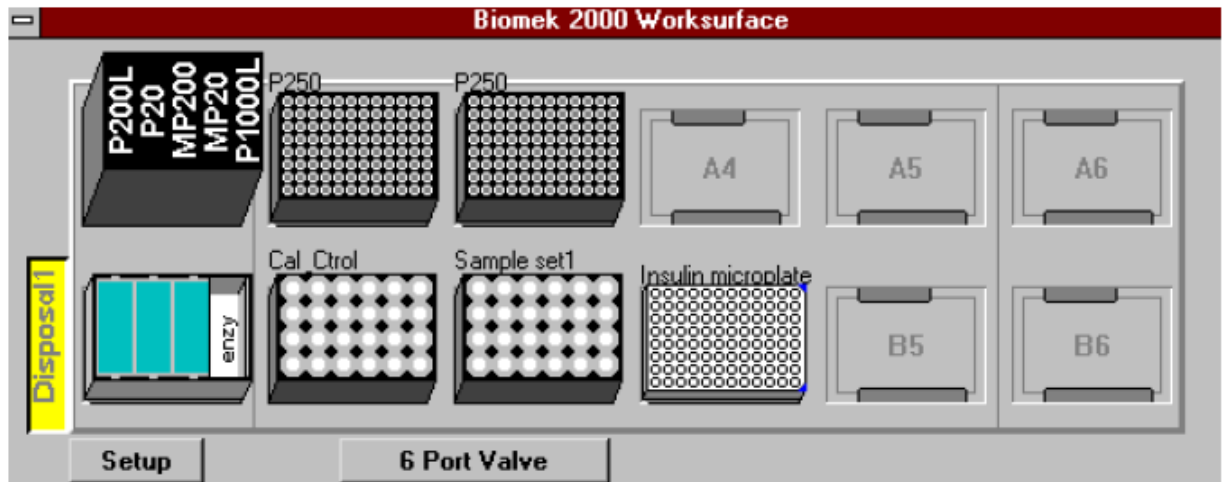
| Number of strips/plates | Enzyme conjugate 11X | Enzyme conjugate buffer |
|-------------------------|----------------------|-------------------------|
| 6 strips                | 0.5mL                | 5mL                     |
| 1 plate                 | 1.0mL                | 10mL                    |
| 2 plates                | 2.0mL                | 20mL                    |
| 3 plates                | 3.0mL                | 30mL                    |

| Sample distribution chart, for samples in duplicate 1 | 2          | 3          | 4           | 5               | 6               | 7                | 8                | 9                | 10               | 11               | 12               |                  |
|---|------------|------------|-------------|-----------------|-----------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|   | A          | Cal<br>0   | Cal<br>0    | Lab<br>Ctrl     | sam<br>ple<br>4 | sam<br>ple<br>8  | sam<br>ple<br>12 | sam<br>ple<br>16 | sam<br>ple<br>20 | sam<br>ple<br>24 | sam<br>ple<br>28 | sam<br>ple<br>32 |
| B   | Cal<br>2.0 | Cal<br>2.0 | Lab<br>Ctrl | sam<br>ple<br>4 | sam<br>ple<br>8 | sam<br>ple<br>12 | sam<br>ple<br>16 | sam<br>ple<br>20 | sam<br>ple<br>24 | sam<br>ple<br>28 | sam<br>ple<br>32 | sam<br>ple<br>36 |
| C   | Cal<br>3.0 | Cal<br>3.0 | sam<br>ple  | sam<br>ple      | sam<br>ple      | sam<br>ple       | sam<br>ple       | sam<br>ple       | sam<br>ple       | sam<br>ple       | sam<br>ple       | sam<br>ple       |

|   |                  |                  |                 |                 |                  |                  |                  |                  |                  |                  |                  |                  |
|---|------------------|------------------|-----------------|-----------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|   |                  |                  | 1               | 5               | 9                | 13               | 17               | 21               | 25               | 29               | 33               | 37               |
| D | Cal<br>10.0      | Cal<br>10.0      | sam<br>ple<br>1 | sam<br>ple<br>5 | sam<br>ple<br>9  | sam<br>ple<br>13 | sam<br>ple<br>17 | sam<br>ple<br>21 | sam<br>ple<br>25 | sam<br>ple<br>29 | sam<br>ple<br>33 | sam<br>ple<br>37 |
| E | Cal<br>30.0      | Cal<br>30.0      | sam<br>ple<br>2 | sam<br>ple<br>6 | sam<br>ple<br>10 | sam<br>ple<br>14 | sam<br>ple<br>18 | sam<br>ple<br>22 | sam<br>ple<br>26 | sam<br>ple<br>30 | sam<br>ple<br>34 | sam<br>ple<br>38 |
| F | Cal<br>100.<br>0 | Cal<br>100.<br>0 | sam<br>ple<br>2 | sam<br>ple<br>6 | sam<br>ple<br>10 | sam<br>ple<br>14 | sam<br>ple<br>18 | sam<br>ple<br>22 | sam<br>ple<br>26 | sam<br>ple<br>30 | sam<br>ple<br>34 | sam<br>ple<br>38 |
| G | Cal<br>200.<br>0 | Cal<br>200.<br>0 | sam<br>ple<br>3 | sam<br>ple<br>7 | sam<br>ple<br>11 | sam<br>ple<br>15 | sam<br>ple<br>19 | sam<br>ple<br>23 | sam<br>ple<br>27 | sam<br>ple<br>31 | sam<br>ple<br>35 | sam<br>ple<br>39 |
| H | Lo<br>w<br>Ctrl  | Hig<br>h<br>ctrl | sam<br>ple<br>3 | sam<br>ple<br>7 | sam<br>ple<br>11 | sam<br>ple<br>15 | sam<br>ple<br>19 | sam<br>ple<br>23 | sam<br>ple<br>27 | sam<br>ple<br>31 | sam<br>ple<br>35 | sam<br>ple<br>39 |

**\*Note: If calibrators, control types, sample types, tube type, dilution factor, position or number of samples are changed, make sure the Biomek method is changed accordingly.**

## BIOMEK WORK SURFACE



PROTOCOL: Method name = **Insulin in dup (Other programs depending on Study)**

Lab book = ELISA

DECK CONFIGURATION: set up the Biomek work surface as follows:

**B1 – prepared Enzyme conjugate**

**B2 – Cal and control (tube holder #1)**

**B3 – Samples (tube holder #2)**

**B4 – Insulin microplate**

**A2 – P250**

**A3 – P250**

Insulin in Serum NHANES 2007-2008 SEQUENCE:

**Time**

Add 25 $\mu$ L calibrators, controls, and samples(in duplicate) 20 min

- Add 100 $\mu$ L enzyme conjugate 10 min

**After the set up of the ELISA plate is complete, continue the assay manually, as described below:**

1. Cover the plate with an adhesive plate cover. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker set at 450 rpm (setting 45).

2. Aspirate each well and wash with a microplate washer, repeating the process 5 times for a total of 6 washes. Invert the plate and blot against clean paper towels between each wash. **Complete removal of the liquid at each step is essential to good performance.** After the last wash, remove remaining wash buffer, then invert the plate and blot it against clean paper towels, making sure all the liquid is removed.

**\*\*Avoid prolonged exposure of the wells to vacuum aspiration apparatus. Excessive drying of the wells can lead to poor assay performance. Subsequent reagents should be added immediately after washing the plate.** Do not let the plate dry out before adding the Substrate.

3. Add 200 $\mu$ L of Substrate TMB to each well using a 12-channel pipet. Cover plate with a new adhesive cover. Incubate for 15 minutes at room temperature. (Requires 20 mL per plate)

1. Add 50 $\mu$ L of Stop solution to each well using a 12-channel pipet. Pipet up and down a few times to mix solutions thoroughly; take care to avoid foaming or cross-contamination. (Need 6mL per plate)

1. Read plate on a microplate reader set to 450 nm immediately, if for some reason the plate can not be read immediately the assay plate is stable for 30 minutes after adding Stop solution.

#### D. Reading Plate and Calculation of Results:

Computerized data reduction of absorbance for the Calibrators (2-200 mU/l) versus the concentration using log/log regression should be performed to obtain the concentration of insulin.

1. To read plate, set the parameters for the plate reader for this assay:

Endpoint reading

L1=450nm

L2=650nm

Plate blanking ON

Standard curve calculation: log/log curve fit

2. Turn on the plate reader (5 to 10 minutes before use) by pressing the switch at the rear of the instrument.

3. Double-click on the SoftMax Pro 2.6 icon, located on the desktop, and open the folder containing the *Mercodia Insulin in duplicate template* for the

11 Insulin in Serum NHANES 2007-2008

Soft Max Pro software. If any changes need to be made to the template, follow the directions below; otherwise, proceed to step 4.

a. To change the number of wells to be read: Click the “Setup” box. Click the “Strips” box. Highlight the number of columns of wells to be read (whole columns only). Click “OK.”

b. To change information about the calibrators or their placement: Click the “Template” box. Choose the appropriate units for measurement. Highlight each standard box and name the standards and set the concentration of each by typing the value in the space at the top of the dialog box. Press return to enter each value. Select the boxes (representing each well) individually to set the unknown wells. Each unknown may be named, if desired. Select the boxes (representing each well) individually to set the control wells. Each control may be named, if desired. Click “OK.”

c. To change the dilution factor used in the assay (if necessary): Click the triangle next to “Unknowns” to open the results list. Click on the column labeled “FinalResult.” Click on the “f(x)” button for the Unknowns list. Change the value listed (i.e. \*100) to the correct dilution factor used in the assay. Click “OK.” **Note: this will change the dilution for all specimens read.**

d. If necessary, save these changes to the template file.

4. Open the door of the plate reader by pressing the “Drawer” button the top of the machine. Insert the plate in the correct orientation. Close the door by pressing the “Drawer” button again.

5. Click the “Read” button at the top of the open template on the computer desktop.

6. Examine the optical density (OD) values generated to ensure they look appropriate. Also examine the standard line generated (by clicking on the

triangle next to “Standards”). Finally, examine the calculated values for the unknowns.

7. Print the results to the Tsai Research printer.

8. Save the results on the computer in the appropriate folder as *Insulin <assay date>*. 9. Insert a 3 ½ floppy disk.

10. Highlight your results.

11. Under Edit, choose copy.

12. Open Excel, and open a blank worksheet.

13. Under Edit, choose paste.

14. Save the results to the 3½ floppy disk: File, Save As, My computer, 3½ floppy, **save as** *Insulin <assay date>*.

15. Close the SoftMax Pro program on the computer.

16. Remove the microplate from the plate reader, close the drawer, then switch the machine off.

12 Insulin in Serum NHANES 2007-2008



## **REPORTABLE RANGE OF RESULTS**

The CSCL laboratory has determined that the Mercodia Insulin Kit is linear up to 135mU/L. Specimens above 135mU/L should be diluted with the 0 calibrator and reassayed. Values less than 2mU/L are repeated. If the duplicate agrees, the value is reported as <2mU/L.

The detection limit is <1mU/L calculated as two standard deviations above the Calibrator 0.

## **10. QUALITY CONTROL (QC) PROCEDURES**

Two commercial Mercodia controls, Low and High, as well as a pooled in-house control are run in each plate. The values of these controls need to be evaluated after each plate is run. The controls are plotted daily with each run, and if the controls fall out of the established range, the run needs to be repeated.

Mercodia Low Control (Lot #13734) Range: 4.2-9.1mU/L

Mercodia High Control (Lot #13736) Range: 52-71mU/L

In-house Control: Range established within laboratory

## **11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS**

### **FAIL TO MEET ACCEPTABLE CRITERIA**

The values of these controls need to be evaluated after each plate is run. The controls are plotted daily with each run, and if the controls fall out of the established range, the run needs to be repeated.

## **12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

A definitive clinical diagnosis should not be based on the results of a single test, but should be made after all clinical findings have been evaluated. Sample results from individuals already undergoing insulin therapy may be complicated by formation of anti-insulin antibodies that

are capable of interfering in the assay. Grossly lipemic, icteric, or hemolyzed samples do not interfere in the assay.

The following cross-reactions have been found:

C-peptide <0.01% (by weight)

Proinsulin <0.01% (by weight)

Proinsulin des (31-32) <0.5%

Proinsulin split (32-33) <0.5%

Proinsulin des (64-65) 98%

Proinsulin split (65-66) 56%

Insulin lispro (Humalog®,Eli Lilly) <0.006%

Insulin aspart <0.006%

Rat Insulin 0.7%

### **13. REFERENCE RANGES (NORMAL VALUES)**

13 Insulin in Serum NHANES 2007-2008

Fasting levels for 137 tested, apparently healthy individuals, yielded a mean of 9.2mU/L, a median of 6.9mU/L and a range of 2-25mU/L.

#### **14. CRITICAL CALL RESULTS (“PANIC VALUES”)**

There are no panic values for insulin.

#### **15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens are stored at -70°C until analyzed. On the day of testing, the specimens are thawed and kept in the refrigerator when not on the instrument. The specimens are refrozen within 1-2 days.

#### **16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

If testing cannot be performed, the specimens are stored at -70°C.

#### **17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

NHANES insulin results are entered onto a spreadsheet provided electronically by WESTAT, Inc for NHANES. The spreadsheet is found on the Q drive in the NHANES folder. Select the insulin (023) folder and choose the file named with the corresponding box number.

Enter the analysis date, run number, technologist’s initials, insulin value, and result comment code.

The spreadsheet will be sent electronically by the contact person.

#### **18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

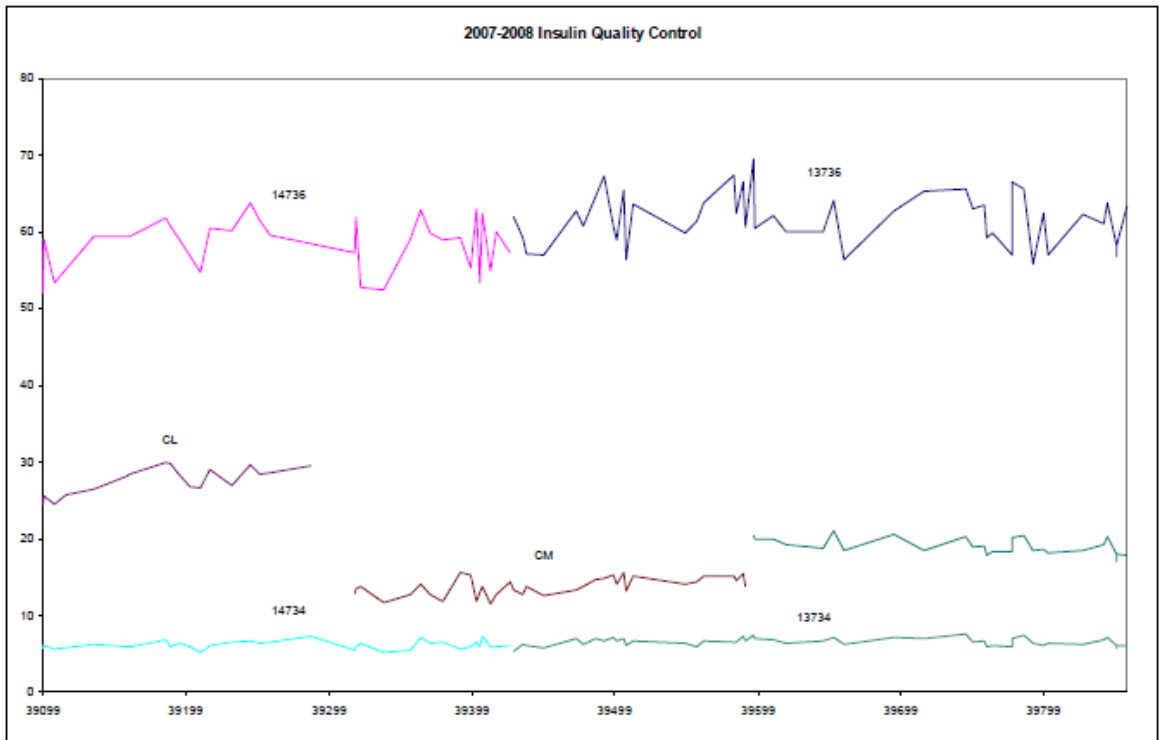
All shipments are recorded on the NHANES Shipping Log upon receipt.

Actions taken during the course of analysis, result reporting, and specimen retention are also recorded on the log.

14 Insulin in Serum NHANES 2007-2008 15

## 19 SUMMARY STATISTICS AND QC GRAPHS

| <b>Summary Statistics for Insulin by Lot Lot</b> | <b>N</b> | <b>Start Date</b> | <b>End Date</b> | <b>Mean</b> | <b>Standard Deviation</b> | <b>Coefficient of Variation</b> |
|--|----------|-------------------|-----------------|-------------|---------------------------|---------------------------------|
| 14734  | 32       | 1/17/2007         | 12/10/2007      | 6.125       | 0.539                     | 8.8                             |
| CL   | 18       | 1/17/2007         | 7/23/2007       | 27.604      | 1.778                     | 6.4                             |
| 14736  | 31       | 1/17/2007         | 12/10/2007      | 58.501      | 3.379                     | 5.8                             |
| 13734  | 48       | 3/16/2007         | 2/13/2009       | 6.518       | 0.503                     | 7.7                             |
| 13736  | 48       | 3/16/2007         | 2/13/2009       | 61.651      | 3.381                     | 5.5                             |
| CM   | 36       | 8/23/2007         | 5/22/2008       | 13.767      | 1.177                     | 8.5                             |
| CN   | 27       | 5/28/2008         | 2/13/2009       | 19.020      | 1.052                     | 5.5                             |



## REFERENCES

Mercodia AB. Mercodia Insulin ELISA kits insert. Mercodia AB, Sylveniusgatan 8A, SE-754 50 Uppsala, Sweden.