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"Expression of adipokine genes associated with gestational diabetes mellitus"

Thesis

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List Of Abbreviation

GDM	Gestational diabetes mellitus
RETN	Resistin
ADIPOQ	Adiponectin
ADIPOR1	Adiponectin Receptor 1
ADIPOR2	Adiponectin Receptor 2
LEP	Leptin
DM	Diabetes mellitus
DMT1	Diabetes Mellitus Type 1
DMT2	Diabetes Mellitus Type 2
IDDM	Insulin-dependent diabetes mellitus
NIDDM	Noninsulin-dependent diabetes mellitus
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
WHO	World Health Organization
ADA	American Diabetes Association
IDF	International Diabetes Federation
EASD	The European Association for the Study of Diabetes
IR	Insulin Resistance
OGTT	Oral Glucose Tolerance Test
WAT	White Adipose Tissue
BAT	Brown Adipose Tissue
ADSF	Adipose Secretory Factor
LMW	Low-molecular Weight
MMW	Middle-molecular Weight
AMPK	Activated Protein Kinase
HMW	High-molecular Weight
ER	Endoplasmic Reticulum
Mrna	messenger Ribonucleic Acid
cDNA	complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid

RNA	Ribonucleic Acid
ARC	Arcuate Nucleus
VMH	Ventromedial Nucleus
DMH	Dorsomedial Nucleus
PMH	Posteromedial Hypothalamus
3V	Third Ventricle
Ob	Obese
PE	Preeclampsia
PCR	Polymerase Chain Reaction
RT PCR	Reverse Transcription Polymerase Chain Reaction
rt-PCR	Real Time Polymerase Chain Reaction
q PCR	Quantitative Polymerase Chain Reaction
PCA	Polymerase Cycling Assembly
dPCR	Digital Polymerase Chain Reaction
ISSR	Intersequence-specific Polymerase Chain Reaction
MSP MLPA	Methylation-specific Polymerase Chain Reaction
SOEing	Overlap-extension Polymerase Chain Reaction
TAIL PCR	Thermal asymmetric interlaced Polymerase Chain Reaction
EDTA	Ethylenediamine Tetra-acetic Acid
UV	Ultra-Violet
DMRT	Duncan's multiple range test

Abstract

Abstract

Background: Gestational diabetes mellitus (GDM), defined as a carbohydrate intolerance of varying severity, is the most frequent metabolic disorder of pregnancy, affecting 1-10% of all pregnancies. Although most of the women with GDM return to normal glucose tolerance after delivery, they have an increased risk of developing diabetes, mainly type 2 diabetes mellitus (DM2), later on, with an incidence ranging from 6-62 %, depending on the population examined and the length of follow up considered. Adipokines comprise a variety of proteins with signaling properties produced in body fat. The adipokines or adipocytokines (Greek adipo-, fat; cyto-, cell; and -kinos, movement) are cytokines (cell-to-cell signaling proteins) secreted by adipose tissue

Aim of the work: The purpose of the study was to assess the expression profile of genes with potential role in the development of insulin resistance (adipokines). RT-PCR was used for expression analysis of the studied genes which are leptin, Adiponectin, Adiponectin receptor1, Adiponectin receptor2 and Resistin.

Results: Leptin gene expression is higher in patients with GDM than in normal pregnant women. Expression of adiponectin genes in pregnant women with GDM decreases in comparison with their matched healthy pregnant group. Adiponectin Receptor 1 is the same range in both groups with slight high in GDM compared with controls. While Adiponectin Receptor 2 decreases in patients with GDM than normal ones. The last expression for Resistin gene is significantly elevated in GDM women compared to their matched controls.

Conclusion: From the present study, it could be concluded that all studied adipokines showed remarkable variations in their levels between GDM and control subjects.Leptin and Resistin increases in GDM and Adiponectin R1&2 are slightly in the same range and Adiponectin gene decreases in GDM.

Keywords: GDM, Adipokines, Leptin, Resistin, Adiponectin, AdipoR1, AdipoR2 and RT-PCR.

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Review

Introduction

Diabetes mellitus

Introduction to DM

Diabesity–the biggest epidemic in human history **(*Zimmet, 2007*)**. Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic diseases in which there are high sugar levels over a prolonged period. Symptoms of high blood sugar include frequent urination, increased thirst, and increased hunger. If left untreated, diabetes can cause many complications. Acute complications include diabetic ketoacidosis and nonketotic hyperosmolar coma **(*Kitabchi; et al. ,2009*)** Serious long-term complications include cardiovascular disease, stroke, chronic kidney failure, foot ulcers, and damage to the eyes.

Diabetes is due to either the pancreas not producing enough insulin or the cells of the body not responding properly to the insulin produced **(*Shoback and Dolores 2011*).**

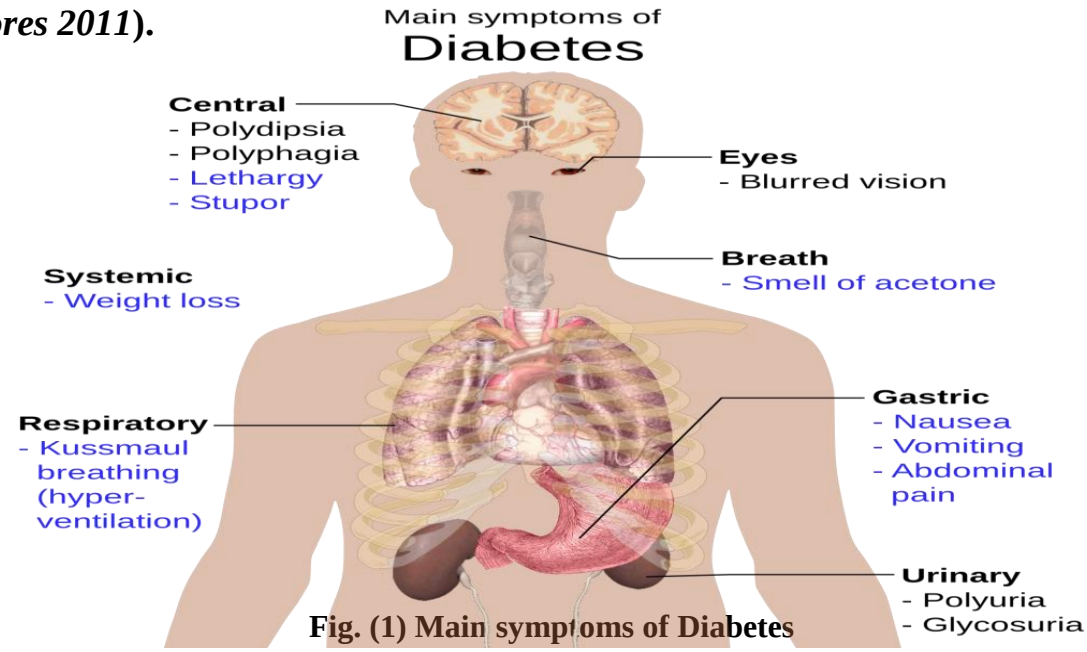


Fig. (1) Main symptoms of Diabetes

There are three main types of diabetes mellitus:

A. Diabetes Mellitus Type 1 (DMT1) :

Identification:

"Insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes". The cause is unknown.

Type one diabetes (T1D) is a chronic autoimmune disease culminating in the destruction of insulin secreting beta (β) cells in the pancreas **(*Bluestone, et al, 2010; Todd, 2010*)**.

In T1D the absence of insulin results in higher than optimum amounts of glucose in blood plasma known as hyperglycemia **(*Giugliano, et al., 2008*).**

Signs and symptoms*:*

Overview of the most significant symptoms of diabetes:

The classical symptoms of type 1 diabetes include: polyuria (excessive urination), polydipsia (increased thirst), xerostomia (dry mouth), polyphagia (increased hunger), fatigue, and weight loss **(*Hotu et al., 2004*)**.

Cause:

The cause of type 1 diabetes is unknown. A number of explanatory theories have been put forward, and the cause may be one or more of the following: genetic susceptibility, a diabetogenic trigger, and/or exposure to an antigen **(*Williams 2010*)**

Laboratory diagnosis:

Diabetes mellitus is characterized by recurrent or persistent hyperglycemia, and is diagnosed by demonstrating any one of the followings ***(Vijan, S, 2010).***

Fasting plasma glucose level at or above 7.0 mmol/L (126 mg/dL). Plasma glucoseAt or above 11.1 mmol/L (200 mg/dL) two hours after a 75 g oral glucose load as in a glucose tolerance test. Symptoms of hyperglycemia and casual plasma glucose at or above 11.1 mmol/L (200 mg/dL). Glycated hemoglobin (hemoglobin A1C) at or above 48 mmol/mol (≥ 6.5 DCCT %). (This criterion was recommended by international Expert Committee Diabetes Care 2009 and the American Diabetes Association in 2010, although it has yet to be adopted by the WHO).

About a quarter of people with new type 1 diabetes have developed some degree of diabetic ketoacidosis (a type of metabolic acidosis which is caused by high concentrations of ketone bodies, formed by the breakdown of fatty acids and the deamination of amino acids) by the time the diabetes is recognized.

Prevention:

Type 1 diabetes is not currently preventable. Some researchers believe it might be prevented at the latent autoimmune stage, before it starts destroying beta cells **(*Bluestone JA, et al. 2010*).**

There is increasing prevalence of 'maternal obesity' ('obesity' in pregnancy) GDM **(*Lawrence, Sela et al., 2011*)** and T2DM in pregnancy **(*Feig & Palda, Murphy, 2010*).**

B. Diabetes mellitus type 2 (DMT2):

Introduction:

Diabetes mellitus type 2 (formerly noninsulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes) is a metabolic disorder that is characterized by hyperglycemia (high blood sugar) in the context of insulin resistance and relative lack of insulin **(*Touma and Pannain 2011*),** This is in contrast to diabetes mellitus type 1, in which there is an absolute lack of insulin due to breakdown of islet cells in thepancreas **(*Christian & Stewart, 2010*).**

Type 2 diabetes makes up about 90% of cases of diabetes, with the other 10% due primarily to diabetes mellitus type 1 and gestational diabetes. Obesity is thought to be the primary cause of type 2 diabetes in people who are genetically predisposed to the disease.

Type 2 diabetes is initially managed by increasing exercise and dietary changes.

Rates of type 2 diabetes have increased markedly since 1960 in parallel with obesity. As of 2010 there were approximately 285 million people diagnosed with the disease compared to around 30 million in 1985 **(*Smyth and Heron, 2006*)** Type 2 diabetes is typically a chronic disease associated with a ten-year-shorter life expectancy **(*Williams 2010*)**.

Long-term complications from high blood sugar can include heart disease, strokes, diabetic retinopathy where eyesight is affected, kidney failure which may require dialysis, and poor blood flow in the limbs leading

to amputations. The acute complication of ketoacidosis, a feature of type 1 diabetes, is uncommon, **(*Fasanmade; et al., 2008*)** .

Symptoms

The classic symptoms of diabetes are polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger), and weight loss **(*Ijan, 2010*)**. Other symptoms that are commonly present at diagnosis include a history of blurred vision, itchiness, peripheral neuropathy, recurrent vaginal infections, and fatigue. Many people, however, have no symptoms during the first few years and are diagnosed on routine testing **(*Shoback et al., 2011*).** People with type 2 diabetes mellitus may rarely present with hyperosmolar hyperglycemic state (a condition of very high blood sugar associated with a decreased level of consciousness and low blood pressure) **(*Shoback et al., 2011*)**.

Cause:

The development of type 2 diabetes is caused by a combination of lifestyle and genetic factors **(*Ripsin et al., 2009; Risérus et al., 2009*)**. While some of these factors are under personal control, such as diet and obesity, other factors are not, such as increasing age, female gender, and genetics **(*Williams textbook of endocrinology, 2009*)**.

A lack of sleep has been linked to type 2 diabetes **(*Touma et al., 2011*)**. This is believed to act through its effect on metabolism. The nutritionalstatus of a mother during fetal development may also play a role, with one proposed mechanism being that of altered DNA methylation **(*Christian et al., 2010*)**.

Diagnosis:

Table (1): (*Vijan*, 2010).

Condition	2 hour glucose	Fasting glucose	HbA_{1c}	
Unit	mmol/l(mg/dl)	mmol/l(mg/dl)	mmol/mol	DCCT %
Normal	<7.8 (<140)	<6.1 (<110)	<42	<6.0
Impaired fasting glycaemia	<7.8 (<140)	≥6.1(≥110) & <7.0(<126)	42-46	6.0–6.4
Impaired glucose tolerance	≥7.8 (≥140)	<7.0 (<126)	42-46	6.0–6.4
Diabetes mellitus	≥11.1 (≥200)	≥7.0 (≥126)	≥48	≥6.5

The World Health Organization definition of diabetes (both type 1 and type 2) is for a single raised glucose reading with symptoms, otherwise raised values on two occasions, of either: *(*World Health Organization, 2007*)* fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) Or with a glucose tolerance test, two hours after the oral dose a plasma glucose ≥ 11.1 mmol/l (200 mg/dl)

A random blood sugar of greater than 11.1 mmol/l (200 mg/dL) in association with typical symptoms **(*Ijan, 2010*)** or a glycated hemoglobin (HbA_{1c}) of ≥ 48 mmol/mol (≥ 6.5 DCCT %) is another method of diagnosing diabetes. ***Williams textbook of endocrinology In 2009*** an International Expert Committee that included representatives of the American Diabetes Association (ADA), the International Diabetes Federation (IDF), and the European Association for the Study of Diabetes (EASD) recommended that a threshold of ≥ 48 mmol/mol (≥ 6.5 DCCT %) should be used to diagnose diabetes **(*International Expert, Committee, 2009*)**.

Prevention:

Onset of type 2 diabetes can be delayed or prevented through proper nutrition and regular exercise **(*Raina and Kenealy, 2008; Orozco* et al., *2008*)**. According to WHO T2DM is, 'largely the result of excess body weight and physical inactivity' (2011b, unpaginated) (***Diabetes UK, 2005*).** T2DM is perceived as a 'lifestyle disease' **(*Al-Maskari, 2010; Fullagar, 2009*)** carrying attendant negative moral valence **(*Gollust & Lantz, 2009*)**. It is epidemiologically associated with socioeconomic disadvantage **(*Gollust & Lantz, 2009; ImKampe & Gulliford, 2010*)**.

Medication to reduce blood glucose levels such as hypo-glycaemic agents may also be required **(*NHS Choices, 2010a; NICE, 2008a*).**

C. Type 3 Gestational Diabetes Mellitus:

Introduction

It is the third main form and occurs when pregnant women without a previous history of diabetes develop a high blood-sugar level.

Gestational diabetes mellitus (GDM) "is carbohydrate intolerance that begins or is first recognized during pregnancy" (*American Diabetes Association, 2006*). Gestational diabetes mellitus (GDM) is the most frequent metabolic disorder in pregnancy, affecting 1–10% of all pregnancies **(*Beischer et al., 1996 and Metzger et al., 2010*).** Although most of the women with GDM return to normal glucose tolerance after delivery, they still have significantly increased risk of developing diabetes later in life **(*Kim et al., 2002*).**

Obesity, high weight gain during pregnancy, increased parity and advanced maternal age are recognized risk factors for developing GDM **(*Anna et al., 2008; Alshami et al., 2010*)**.

Pregnancy and insulin resistance:

Pregnancy is normally linked with distinct changes in metabolism of glucose and insulin resistance (IR) to aid stipulation of fuel substrate for the growing fetus.

During late gestation, the insulin decreases lipolysis, which leads to larger postprandial increases in free fatty acids, amplified gluconeogenesis and insulin resistance (IR) **(*Homko et al., 1999*).** Pregnancy is physiologically associated with insulin resistance and hyperinsulinaemia, which predispose vulnerable women to develop diabetes in pregnancy. This insulin resistance is predominantly prominent in the second half of gestation, with increased insulin resistance subsequently contributing to the development of gestational diabetes (GDM).

Diagnosis:

Table (2): Tests for gestational diabetes

Non-challenge blood glucose test Fasting glucose test 2-hour postprandial (after a meal) glucose test Random glucose test
Screening glucose challenge test
Oral glucose tolerance test (OGTT)

A number of screening and diagnostic tests have been used to look for high levels of glucose in plasma or in serum defined circumstances. One method is a stepwise approach where a suspicious result on a screening test is followed by diagnostic test.

Prevention*:*

A 2015 review found that when done during pregnancy moderate physical exercise is effective for the prevention of gestational diabetes (***Sanabria-Martínez et al., 2015*****)**. A 2014 review however did not find a significant effect **(*Yin et al., 2014*)**.

Theoretically, smoking cessation may decrease the risk of gestational diabetes among smokers.

According to the American Diabetes Association **(*American Diabetes Association*, *1999*)**, you're considered at high risk for this condition (and should be screened early) if:

- You're obese (your body mass index is over 30).
- You've had gestational diabetes in a previous pregnancy.
- You have sugar in your urine.
- You have a strong family history of diabetes.

In addition, a study published in the March 2010 issue of Obstetrics & Gynecology found an association between excessive weight gain during pregnancy – particularly in the first trimester – and the risk of gestational diabetes.

Researchers found the risk highest in women who were overweight to begin with and in nonwhite women. Keep in mind that many women who develop gestational diabetes don't have any risk factors. That's why most practitioners will order the screening at 24 to 28 weeks for all their pregnant patients as a matter of course.

Adipokines

Introduction

A large variety of proteins identified as adipokines are secreted in adipose tissue which controls diverse metabolic functions through various pathways like auto, para, or endocrine. Out of more than 50 adipokines identified, leptin and adiponectin have been extensively researched **(*Gavrilova et al., 2000*).**

The adipokines or adipocytokines (Greek adipo-, fat; cyto-, cell; and -kinos, movement) are cytokines (cell-to-cell signaling proteins) secreted by adipose tissue. The first adipokine to be discovered was leptin in 1994 **(*Conde et al., 2011*).** Since that time hundreds of adipokines have been discovered **(*Lehr et al., 2012*)**.

Adipokines are protein molecules released by the adipocytes and are molecules that are biologically active and that have been shown to mediate various stages of metabolism. Adipokines help to mediate the regulation of food consumption, the metabolism of glucose, protein and lipids, blood pressure regulation, modulation of inflammation and immunity, and they work together to regulate inflammation, insulin action, and glucose metabolism locally and systemically **(*Striensa, 2006*).** In an obese person, the adipokine/cytokine network is altered leading to a state that encourages inflammation and the impairment of adipocyte metabolism **(*Treyhurn and Wood, 2010*).**

Adipokines have now totaled over 50 different molecular proteins. Recent statistics show that about 520 million people worldwide are obese (WHO 2014 review) and this statistic is significant in relation to recent research which

demonstrates a link between obesity, cardiovascular diseases and type 2 diabetes mellitus (***Bravo et al., 2006, Barnes 2011***).

Types of Adipose tissue

Adipose tissue can be divided into 2 major types: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT represents the vast majority of adipose tissue in humans and is the physiological site of triglyceride storage during energy consumption and NEFA release when energy expenditure exceeds energy intake **(*Vázquez-Vela et al., 2008*).**

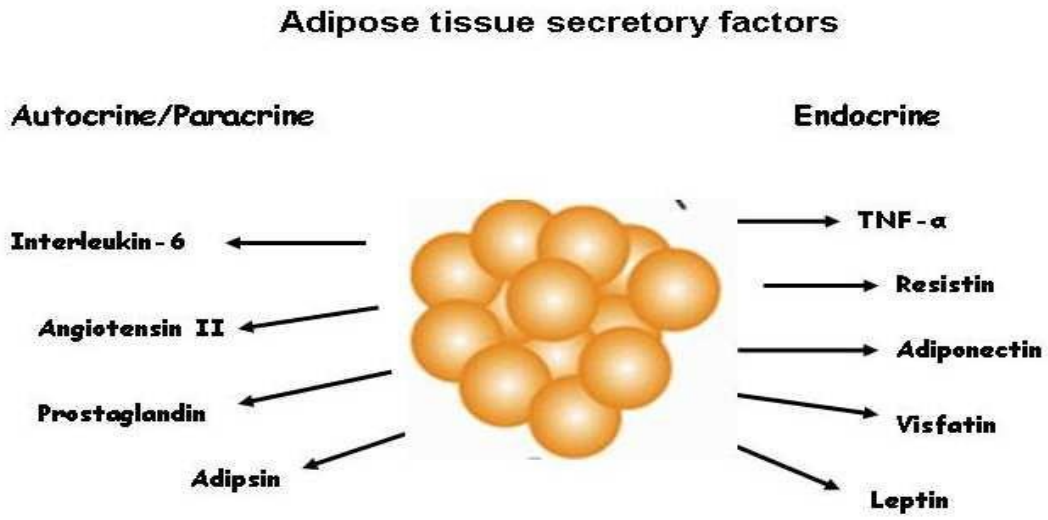


Figure (2): Biological functions of adipocytes**.** Previously, adipocytes were considered to be inert and passive reservoir for energy storage releasing fuel as fatty acids and glycerol in time of fasting or starvation. More recently it has become clear that adipocytes are highly active metabolic and endocrine organ that secrete important hormones, cytokines, vasoactive substances, and other peptides.

Adipokines and T2D:

Obesity is associated with a low-grade inflammation of WAT which can subsequently lead to insulin resistance and impaired glucose tolerance. In obesity, WAT is characterized by abnormal adipokine production and secretion of a wide range of inflammatory molecules which result in activation of some pro-inflammatory signaling pathways and induction of several biological markers of inflammation **(*Hotamisligil et al., 1993, Fried et al., 1998*).** The altered adipokine production in obesity has been shown to have local effects on WAT physiology but also systemic effects on other organs including the pancreatic beta-cells **(*Bastard et al., 2002; Sartipy et al., 2003*)**.

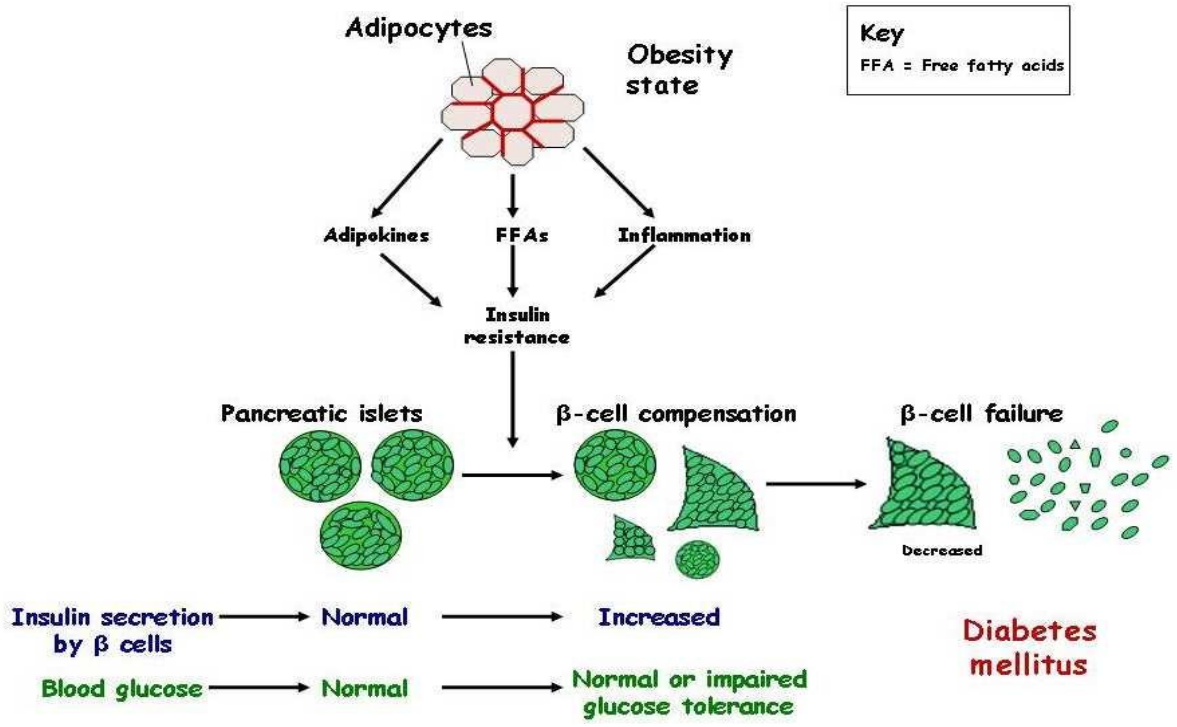


Figure (3): Adipose tissue secreted factors and β-cell function / failure. Pathways leading to insulin resistance associated with obesity which are induced by adipokines, free fatty acids, and chronic inflammation in adipose tissue. Figure adapted from **(Kasuga, 2006)**.

Resistin

Introduction

Resistin was discovered by three different independent groups using modern genomic approaches **(*Steppan et al., 2001; Kim et al., 2001*)**. Kim and co-workers used microarray analyses and were able to identify resistin as adipose secretory factor (ADSF) while Holcomb's group initially found resistin, which they termed 'FIZZ3', as an expressed sequence tag related to a protein they found to be induced during lung inflammation.

Resistin is a recently identified adipocyte-derived hormone that has been shown to play a substantial role in the development of insulin resistance **(*Steppan et al., 2001a*)**. Resistin was originally identified by three independent groups using a variety of techniques **(*Kim et al., 2001; Steppan et al., 2001a*)**.

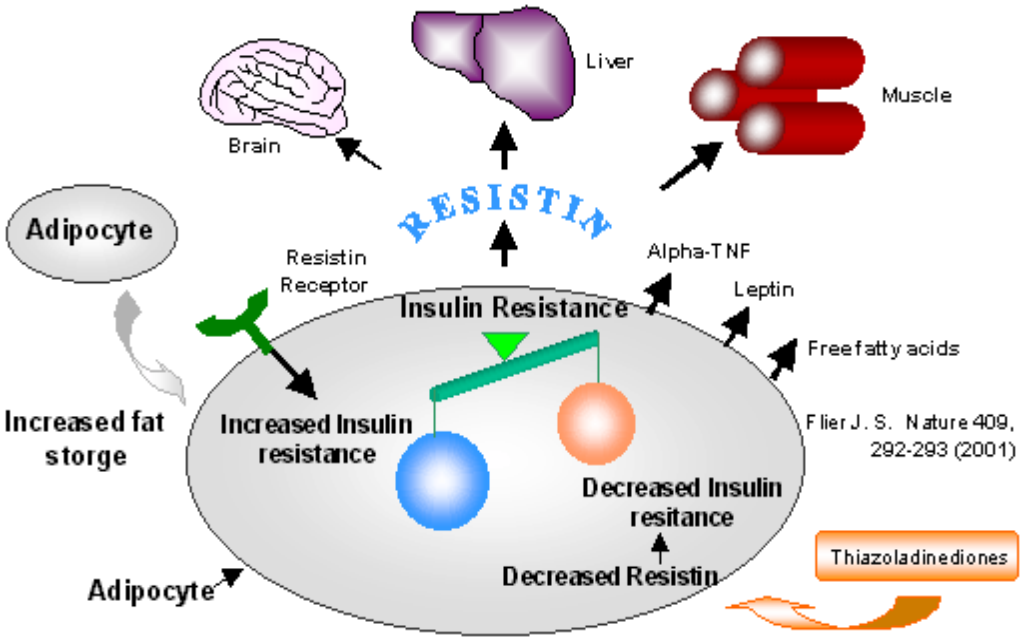


Figure (4): Resistin to be identified as an adipocyte-derived mediator of insulin resistance **.**

Resistin is a 12.5 kDa peptide which was first described in 2001 **(*Steppan et al., 2001*).** This protein is a pro-inflammatory mediator which has been shown to increase inflammatory cytokine production and release from several cells, including adipocytes, leucocytes and endothelial cells **(*Stofkova, 2010*).**

Initially, resistin was thought to be secreted by adipocytes, but it has been shown that the presence of resistin in adipose tissue is largely due to preadipocytes **(*Janke et al., 2002*),** leucocytes **(*Kaser et al., 2003*),** and other non-adipose tissue cells **(*Fain et al., 2003*)** rather than mature adipocytes. Serum resistin concentration may be elevated in obese insulin resistant humans compared to obese insulin sensitive humans **(*Baranova et al., 2006*),** but this finding followed a study showing that serum resistin concentrations are only related to insulin sensitivity in lean humans **(*Heilbronn et al., 2004*)**.

Other studies found no difference in serum resistin concentrations **(*Kielstein et al., 2003; Chen et al., 2006*)** or adipose tissue resistin mRNA levels **(*Janke et al., 2002*)** between healthy, insulin resistant and diabetic patients. Furthermore, the relationship between resistin and obesity remains unclear.

Resistin: obesity and T2D

Serum resistin is elevated in diabetes and obesity **(*Kim et al., 2001; Azuma et al., 2003*)** hence it has been suggested that resistin may have a causative role in the insulin resistance and T2D **(*Steppan et al., 2001*).** Functionally, administration of resistin resulted in impaired glucose tolerance and insulin sensitivity in wild-type mice, while immuno-neutralization of circulating resistin in diet-induced obese mice resulted in reduced blood glucose levels and significantly improved hyperglycemia and insulin sensitivity **(*Steppan et al., 2001*).** However, the physiological role of

resistin in humans and the mechanism by which it neutralizes insulin action in rodents are still unclear.

Serum resistin levels and expression in WAT have previously been reported to be reduced in fasted mice and increased on re-feeding **(*Kim et al., 2001; Steppan et al., 2001*)** suggesting diet may also play a significant role.

GDM is characterized by an amplification of low-grade inflammation already existing in normal pregnancy and increased circulating concentrations of inflammatory cytokines including TNFα and IL-6. TNFα and IL-6 are putative negative regulatory factors of the adiponectin gene.

Adiponectin

Introduction:

Adiponectin is a 30 kDa protein first discovered in the mid-1990s to be expressed exclusively in adipocytes **(*Scherer et al. 1995*).** Adiponectin was originally discovered by four research groups **(*Maeda et al., 1996*, *Nakano et al., 1996*)**. In 1995, Scherer and colleagues identified a protein by cDNA cloning from the mouse adipocyte cell line 3T3-L1 and named it adipose complement related protein (Acrp30) **(*Scherer et al., 1995*)**. Hu and colleagues (1996) using the same cell line isolated the same protein and called it AdipoQ **(*Hu et al., 1996*)**. The name adiponectin was proposed by Arita and colleagues, in 1998 but all four names are used **(*Kihara et al., 1998*).** In this thesis the most common name of the protein, adiponectin will be used.

The biological effects of adiponectin are via binding onto the adiponectin receptors, two of which have been identified: AdipoR1 and AdipoR2. AdipoR1 and AdipoR2 are found predominantly in skeletal muscle and liver, respectively, but are also ubiquitously expressed in peripheral tissues **(*Yamauchi et al. 2003; Kadowaki & Yamauchi 2005*).**

AdipoR1 and AdipoR2, although exert similar physiological effects, have distinct coupling pathways. AdipoR1 activation tends to cause phosphorylation and activation of AMPK, whereas AdipoR2 is more prominent in activation of peroxisome proliferator-activated receptor **(*Yamauchi et al. 2007*).**

In general, patients with obesity, insulin resistance, metabolic syndrome and hypertension have lower concentrations of adiponectin in their blood **(*Yamauchi et al. 2001; Chow et al. 2007*).**

Adiponectin is also important in blood pressure regulation. Adiponectin produced vasodilation in porcine retina arterioles and some of its effect are endothelium-dependent **(*Omae et al. 2013*).**

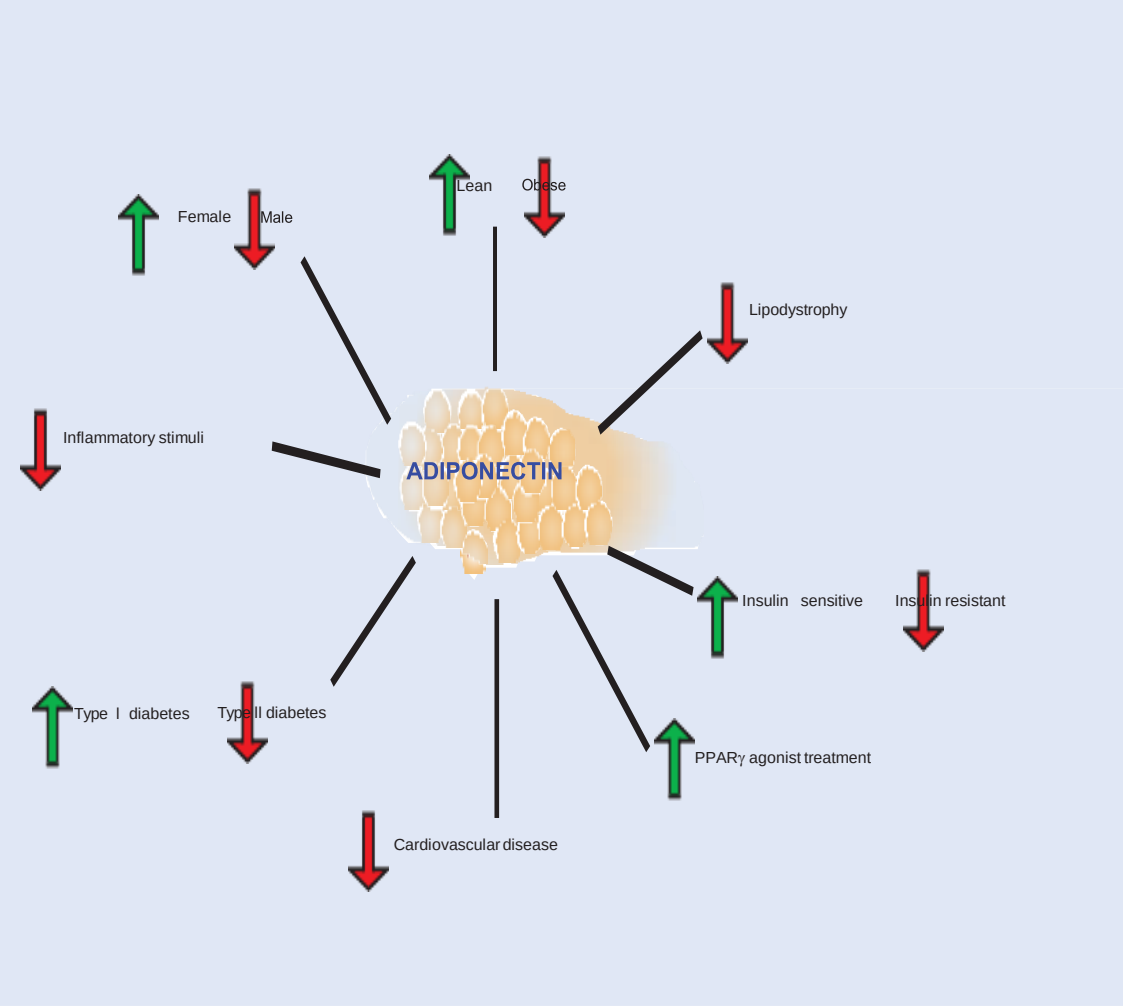


Figure (4): Summary of the most important factors and disease states that lead to an up-regulation (indicated by green arrow) or down-regulation (indicated by a red arrow) of adiponectin in adipose tissue.

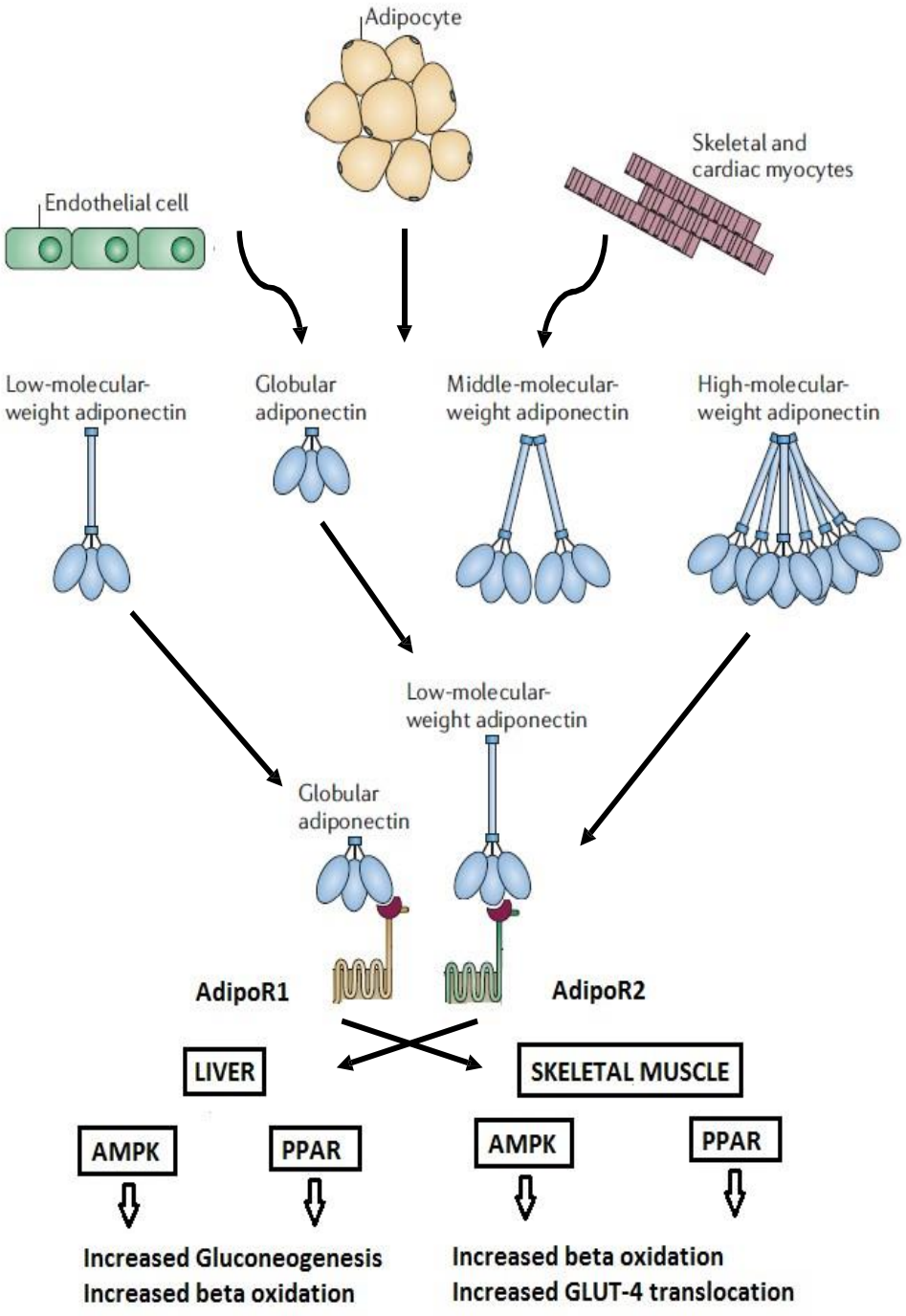


Figure (5): Structure and hypothesized signalling pathways of adiponectin.

Figure 5 Adiponectin is produced predominantly by adipocytes but also by other cell types including skeletal muscle. The full-length molecule undergoes post-translational modifications forming trimers, hexamers and 12-18-mers called low (LMW), middle (MMW) and high-molecular weight (HMW) respectively. These are thought to be essential to its final function and to the insulin sensitizing features of the molecule. Adiponectin interacts with its two membrane receptors to initiate changes in glucose and insulin signaling via AMPK and PPARα found that HMW adiponectin binds the most avidly to its adiponectin receptors, thus stimulating AMP activated protein kinase (AMPK) . Modified from **(*Tilg et al. 2006*)**.

Synthesis of adiponectin

The molecular details of adiponectin synthesis pathway remain poorly defined. Recent studies with murine derived fAd have shown that chaperone proteins such as BiP, ERp44, Ero1-Lα and Dslb aid the assembly of adiponectin multimers and secretion in vitro **(*Liu et al., 2008*, *Simpson and Whitehead, 2010*).**

Furthermore, high levels of expression of these chaperones result in efficient adiponectin secretion and are associated with high circulating adiponectin levels. The secretion of adiponectin is regulated by the binding of adiponectin in the endoplasmic reticulum (ER) to the protein ERp44 through a thiol bond with cysteine 39, therefore adiponectin is retained in the ER **(*Wang and Scherer, 2008*)**.

Adiponectin receptors

There are two distinct adiponectin receptors identified, called AdipoR1 and AdipoR2, which are thought to facilitate the biological actions of adiponectin **(*Yamauchi et al., 2003*).** Northern blot analysis of human and murine tissue found mRNA from AdipoR1 was abundantly expressed in skeletal muscle and to a lesser extent in the heart and spleen. In contrast, AdipoR2 expression was mostly restricted to the liver **(*Yamauchi et al., 2003*).**

The expression of these adiponectin receptors has been identified in monocytes (mRNA and protein) and are thought to play an important role in the immune system **(*Weigert et al., 2008*).** The existence of these adiponectin receptors in platelets is not clear. Kato and colleagues have found AdipoR1 and AdipoR2 (mRNA) expressed in murine platelets **(*Kato et al., 2006*).** Furthermore, AdipoR1 (mRNA) was detected in human platelets, whereas both AdipoR1 and AdipoR2 were found in megakaryocyte cell lines **(*Kato et al., 2006*).**

AdipoR1 and AdipoR2 are composed of seven transmembrane domains similar to that of G-protein coupled receptors. However, AdipoR1 and AdipoR2 do not transduce G-protein coupled signaling (***Yamauchi et al., 2003*).**

In 2004, a third receptor for adiponectin was proposed, T-cadherin, a member of the cadherin superfamily which are involved in cell adhesion and signaling. T-cadherin is thought to bind to hexamers and HMW adiponectin oligomers **(*Hug et al., 2004*).** The existence of these adiponectin receptors can be explained by different isoforms of adiponectin which require different receptor conformations to ensure a high binding affinity, and by the wide biological actions of adiponectin in different tissues.

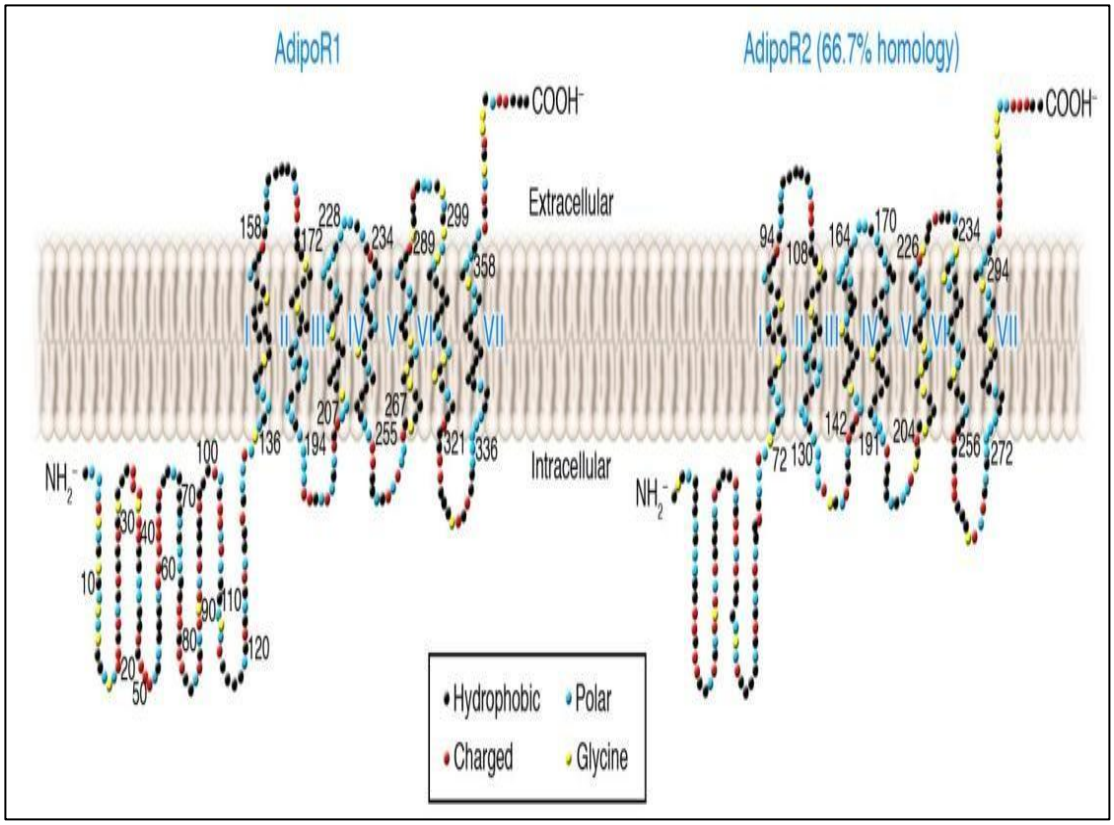


Figure (6): Adiponectin receptors**.** Adiponectin signals through two G- protein like receptors, AdipoR1 and AdipoR2 which consist of seven transmembrane domains **(*Kadowaki and Yamauchi, 2005*)**.

Leptin

Leptin (from Greek λεπτός leptos, "thin"), the "satiety hormone, is a hormone made by adipose cells that helps to regulate energy balance by inhibiting hunger. Leptin is opposed by the actions of the hormone ghrelin, the "hunger hormone". Both hormones act on receptors in the arcuate nucleus of the hypothalamus to regulate appetite to achieve energy homeostasis (***Brennan AM, Mantzoros CS , 2006***). In obesity, a decreased sensitivity to leptin occurs, resulting in an inability to detect satiety despite high energy stores **(** ***Zhang F , Basinski MB ,etal 1997*** **)** .

Leptin is primarily produced by white adipose tissue, nevertheless, it is also secreted by a range of other tissues including the heart **(*Purdham et al. 2004*).** Adipocytes secrete leptin in a slow, continuous fashion, however, if stimulated (by insulin and glycolytic substrates, for example) secretion is markedly increased **(*Cammisotto et al. 2005*).** Leptin secretion is reduced as a response to starvation or cold with starvation leading to increased sympathetic nervous activity, and, as a consequence leptin production is reduced **(*Rayner and Trayhurn, 2001*)**.

Leptin is the primary adipose hormone that relays information about energy stores to the brain through interact ion with the leptin receptor . The leptin receptor is expressed in various regions of the brain and within the hypothalamus leptin can act on two sub-populations of Ob-Rb expressing neurons within the ARC of the hypothalamus to mediate its anorexigenic effect **(*Morris & Rui, 2009*)**.

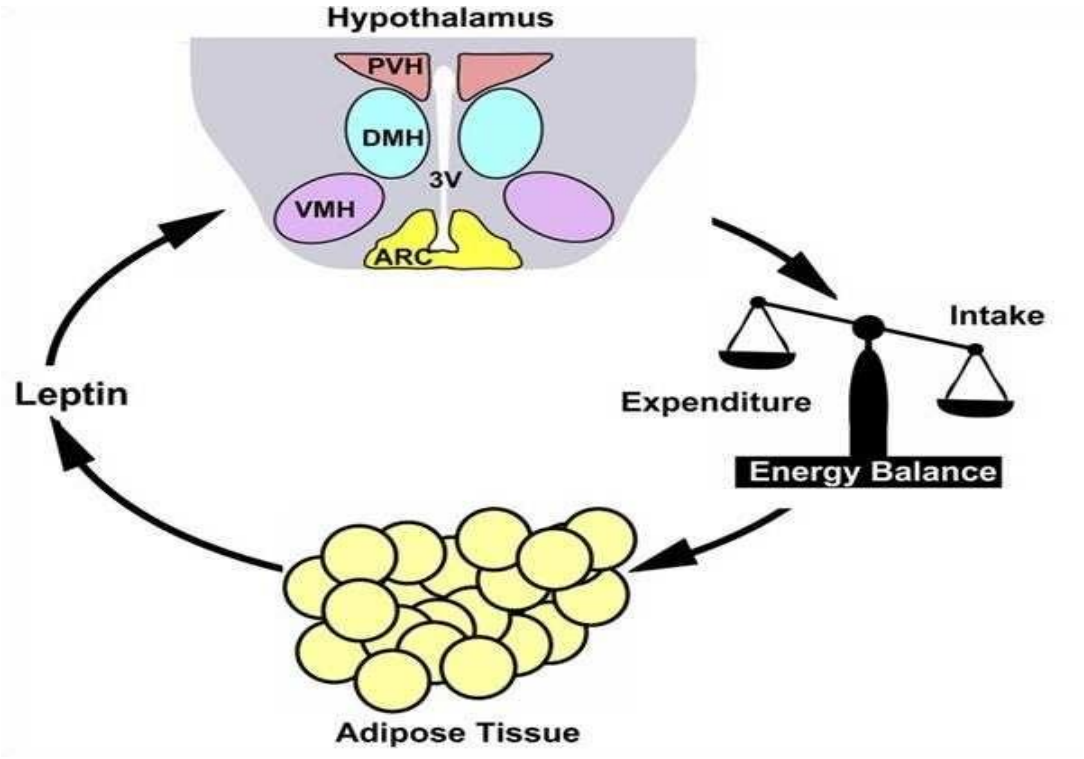


Figure (7): Leptin as a satiety factor. Leptin's primary role is as a satiety factor in energy homeostasis. Leptin is produced in and secreted from the white adipose t issue into the blood. Leptin is transported into the CNS and acts on leptin receptors in the hypothalamus to induce leptin signaling and mediate anorexigenic effects. Arcuate nucleus (ARC) , ventromedial nucleus (VMH), dorsomedial nucleus (DMH), posteromedial hypothalamus (PMH) , third ventricle (3V) (***Morris and Rui, 2009)*.**

The Ob(Lep) gene (Ob for obese, Lep for leptin) is located on chromosome 7 in humans **(*Kolata GB , 2007*)** . Human leptin is a 16-kDa protein of 167 amino acids.

Leptin mRNA is primarily expressed in adipocytes and to a lesser extent in other tissues like the ovaries, placenta, skeletal muscle and stomach.

White adipose tissue is the predominant site of leptin synthesis although there is some evidence to suggest that brown adipose t issue is also a site for the product ion of leptin. Studies into levels of leptin mRNA in different sites of adipose tissue have shown that there is site specific variation in both humans and rodent s. For example, in humans, leptin mRNA expression is greater in subcutaneous fat depot s than in omental adipose t issue **(*Marget ic et al., 2002*)**.

Table (3): Sites of leptin m RNA expression. Leptin m RNA is expressed in various tissues in both human and mouse. Corresponding sites of leptin receptor m RNA expression are also indicated **(*Margetic et al., 2002*).**

	Lepti	**Leptin**
White adipose tissue	√	
Brown adipose tissue	√	
Placenta	√	√
Ovary	√	√
Stomach	√	
Skeletal muscle	√	
Liver	√ (Protein)	√
Heart	√ (Fetal)	√

Leptin is translated as a 167 amino acid protein the sequence of which is highly conserved between many species including human, mouse and cow indicating that leptin and its role are important. Leptin is composed of four very similar g- helices and post-translational modifications cleave the protein into a functional molecule that consists of two peptides **(*Zhang et al., 1997*)**.

Leptin resistance is associated with impaired leptin signaling, particularly in the hypothalamus, and leads to increased appetite and decreased energy expenditure **(*Enriori et al. 2006*).** Leptin resistance is thought to be an important step in the development of diet-induced obesity and its subsequent problems **(*Tschop et al. 2007*).** Two mechanisms have been put forward to explain leptin resistance. First, it has been suggested that it may occur as a result of decreased leptin transport to the brain **(*Banks et al. 2004*)** .

The importance of leptin, with regard to energy balance and metabolic status, is clearly demonstrated in the presence of interrupted leptin signaling **(*Chua, Jr. et al. 1996; Chen & Wang, 2005*)**.

Leptin circulates in blood in free form and bound to proteins. Leptin is involved in reproduction by stimulating gonadotropin-releasing hormone from the hypothalamus **(*Comninos et al., 2014*)**.

From the earliest stages of pregnancy, maternal leptin concentrations increase implying that the increases are not only originating from maternal weight gain **(*Henson and Castracane, 2006*).**

Leptin in gestational diabetes mellitus. Most studies have found increased **(*Ategbo et al., 2006; Chen et al., 2010*)**. leptin concentrations in GDM. Moreover, hyperleptinemia in early pregnancy appears to be predictive of an increased risk to develop GDM later in pregnancy independent of maternal adiposity. Thus, ***(Qiu et al. 2004*)** have studied 823 pregnant women from 13 weeks of gestation to delivery.

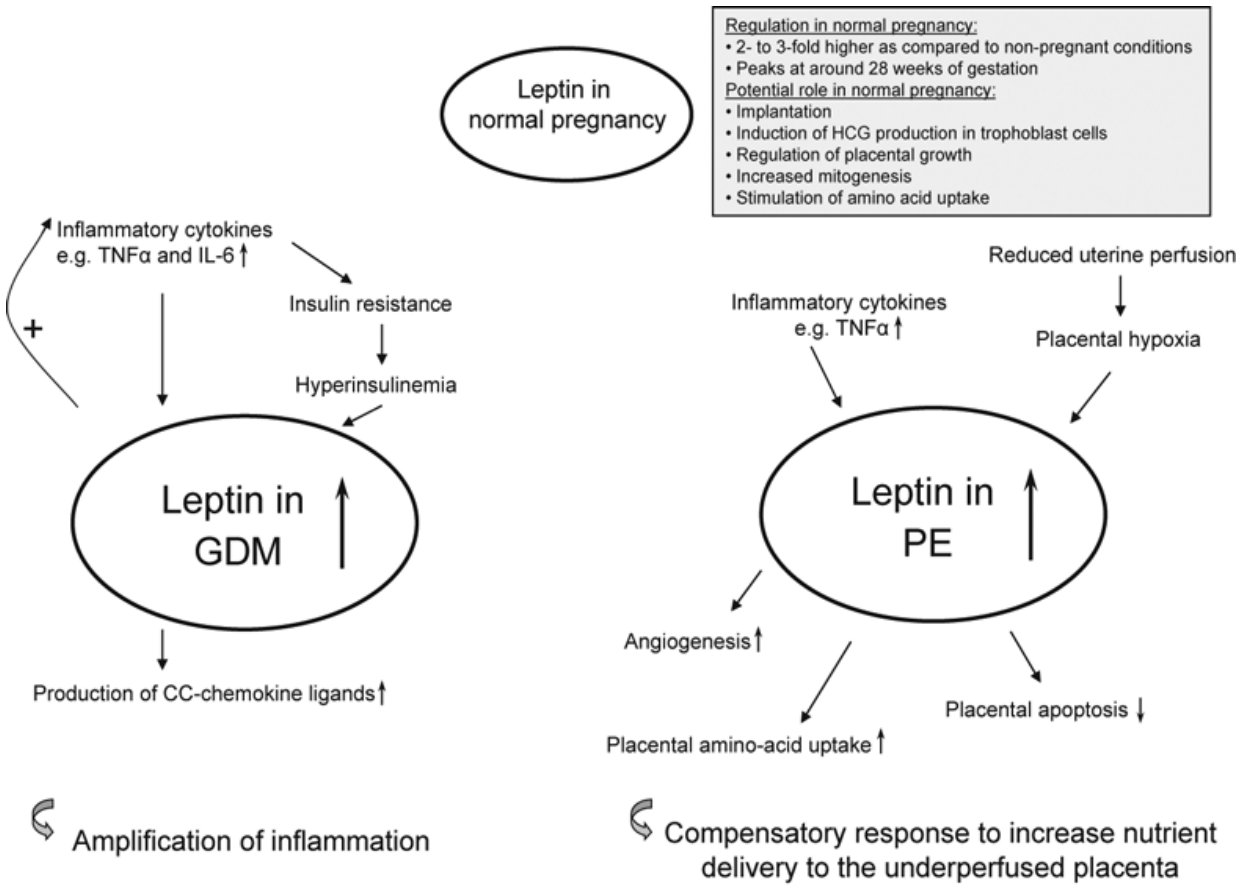


Figure (8) Leptin in normal pregnancy, PE, and GDM .

Immunoreactive leptin has been found in human breast milk; and leptin from mother's milk has been found in the blood of suckling infant animals **(*Casabiell et al., 1997*)**.

Leptin's ability to regulate bone mass was first recognized in 2000 **(*Ducy et al., 2000*)**. Leptin can affect bone metabolism via direct signaling from the brain.

There is a potential for treatment of diseases of bone formation - such as impaired fracture healing - with leptin **(*Rőszer et al., 2014*)**. In humans, low circulating plasma leptin has been associated with cognitive changes associated with anorexia **(*Casanueva et al., 1997*)**, depression, and HIV **(*Lieb et al., 2009*)**.

In humans, many instances are seen where leptin dissociates from the strict role of communicating nutritional status between body and brain and no longer correlates with body fat levels:

- Leptin level is decreased after short-term fasting (24–72 hours), even when changes in fat mass are not observed **(*Henson et al., 2006; Ladyman and Grattan, 2005*)**.
- Leptin plays a critical role in the adaptive response to starvation **(*Schubring et al., 1998; Catalano et al., 2006*)**.
- In obese patients with obstructive sleep apnea, leptin level is increased, but decreased after the administration of continuous positive airway pressure **(*Yura et al. 2003; Ategbo et al., 2006*)** in non-obese individuals, however, restful sleep (i.e., 8–12 hours of unbroken sleep) can increase leptin to normal levels.
- Serum level of leptin is reduced by sleep deprivation **(*Chen et al., 2010; Kautzky-Willer et al., 2010*)**.
- Leptin level is increased by perceived emotional stress **(*Vitoratos et al., 2001*)**.
- Leptin level is decreased by increases in testosterone levels and increased by increases in estrogen levels **(*Festa et al., 1999*)**.
- Leptin level is chronically reduced by physical exercise training **(*McLachlan et al., 2006; Adali et al., 2009*)**.
- Leptin release is increased by dexamethasone **(*Haugen et al., 2006*)**.
- Leptin level is increased by insulin **(*Hendler et al., 2005*)**.
- Leptin levels are paradoxically increased in obesity **(*Masuyama et al., 2010*)**.

It has been proposed to have a role in such processes as inflammation, angiogenesis, immunity, hematopoiesis and reproduction **(*Marget ic et al, 2002*).**

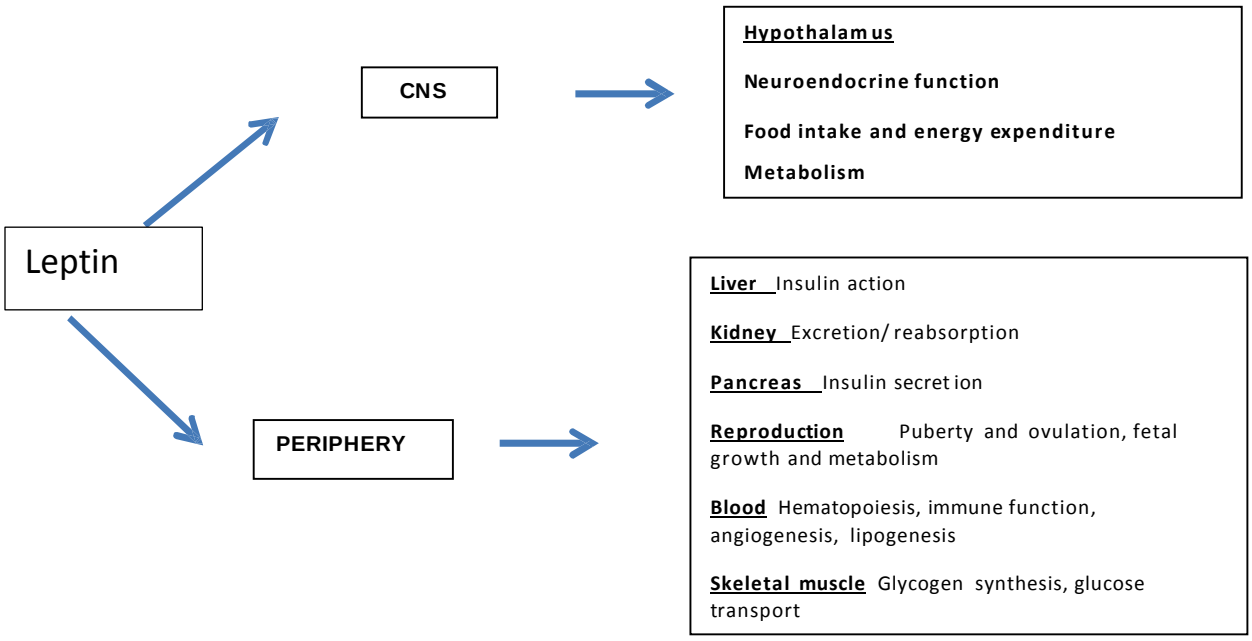


Figure (9) : *The central and peripheral actions of leptin*.

In its primary role as a modulator of energy homeostasis, leptin exhibits circadian oscillations and a strong relationship has been suggested between feeding and the diurnal rhythm of leptin **(*Bodosi et al., 2004*)**.

Although variations in leptin protein levels may be normal in the regulation of energy homeostasis, there have been many reports of abnormal leptin expression levels in relation to disease and dysfunction. During gestation, plasma leptin levels in the mother increase. This coincides with an increase in leptin receptor binding protein levels in maternal circulation and could lead to prolonged leptin circulation or to increased synthesis by either maternal fat depots and or the placenta **(*Marget ic et al., 2002*).**

Leptin was approved in the United States in 2014 for use in congenital leptin deficiency and generalized lipodystrophy **(*Sinha, 2014*)**.

Polymerase chain reaction (PCR)

Introduction

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Polymerase Chain Reaction was developed in 1984 by the American biochemist, Kary Mullis. Mullis received the Nobel Prize and the Japan Prize for developing PCR in 1993 **(*Bartlett and Stirling, 2003*).**

However, the basic principle of replicating a piece of DNA using two primers had already been described by Gobind Khorana in 1971.

Progress was limited by primer synthesis and polymerase purification issues **(*Kleppe et al., 1971*).**

PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications **(*Saiki et al., 1985*)**.

The polymerase chain reaction is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple. The technique amplifies specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality **(*Erlich, 1989*).**

PCR; the quick, easy method for generating unlimited copies of any fragment of DNA, is one of those scientific developments that actually deserve timeworn superlatives like "revolutionary" and "breakthrough.

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase, which the method is named after, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations (Joshi M, Despande J.2010, Lauerman LH. 2004).

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq.polymerase, an enzyme originally isolated from the thermophilic bacterium *Thermus aquaticus*. If heat-susceptible DNA polymerase is used, it will denature every cycle at the denaturation step. Before the use of Taq polymerase, DNA polymerase had to be manually added every cycle, which was a tedious and costly process (Angela R. Porta, & Edward Enners. (2012).

Real time PCR

Real time PCR or quantitative PCR (qPCR) is other adaptation of the PCR method to quantify the number of copies of nucleic acids during PCR. Thus, qPCR is used to quantify DNA o cDNA, determining gene or transcript numbers present within different samples **(*Marty et al., 2004; Lobert et al., 2010*)**. qPCR offers advantages such as speed in the result, the

reduced risk of contamination and the ease in handling technology **(*Mackay et al., 2002; Maibach et al., 2003*)**.

This PCR uses fluorescence detection systems which are generally of two types: intercalating agents and labeled probes with fluorophores. Intercalating agents such as SYBR Green are fluorochromes that dramatically increase the fluorescence by binding to a double-stranded DNA **(*Ke et al., 2000; Vlková et al., 2010*)**.

Thus, the increase of DNA in each cycle reflects a proportional increase in the emitted fluorescence. However, it is considered that intercalating agents offer a low specificity because they can be bind to nonspecific products or primer dimers. Several studies have shown that careful selection of primers and using of optimal PCR conditions may minimize this no specificity **(*Mackay et al., 2002*)**.

The use of a high temperature to start the synthesis reaction (hot-start PCR) decreases the risk of nonspecific amplification. Another detection system used in real time PCR are specific hybridization probes labeled with two types of fluorochromes, a donor and an acceptor. The most commonly used probes are hydrolysis or TaqMan probes, molecular beacons probes, and FRET (fluorescent resonance energy transfer) **(*Moretti et al., 1998; Demeke and Jenkins, 2010*)**.

The increase of DNA in each cycle is proportional to hybridization of probes, which in turn is proportional to the increase in the emitted fluorescence. The use of probes allows identifying polymorphisms and mutations; however, these are more complex and expensive than

intercalating agents **(*Chagovetz and Blair, 2009; Smith and Osborn, 2009; Giasuddin et al., 2008*)**.

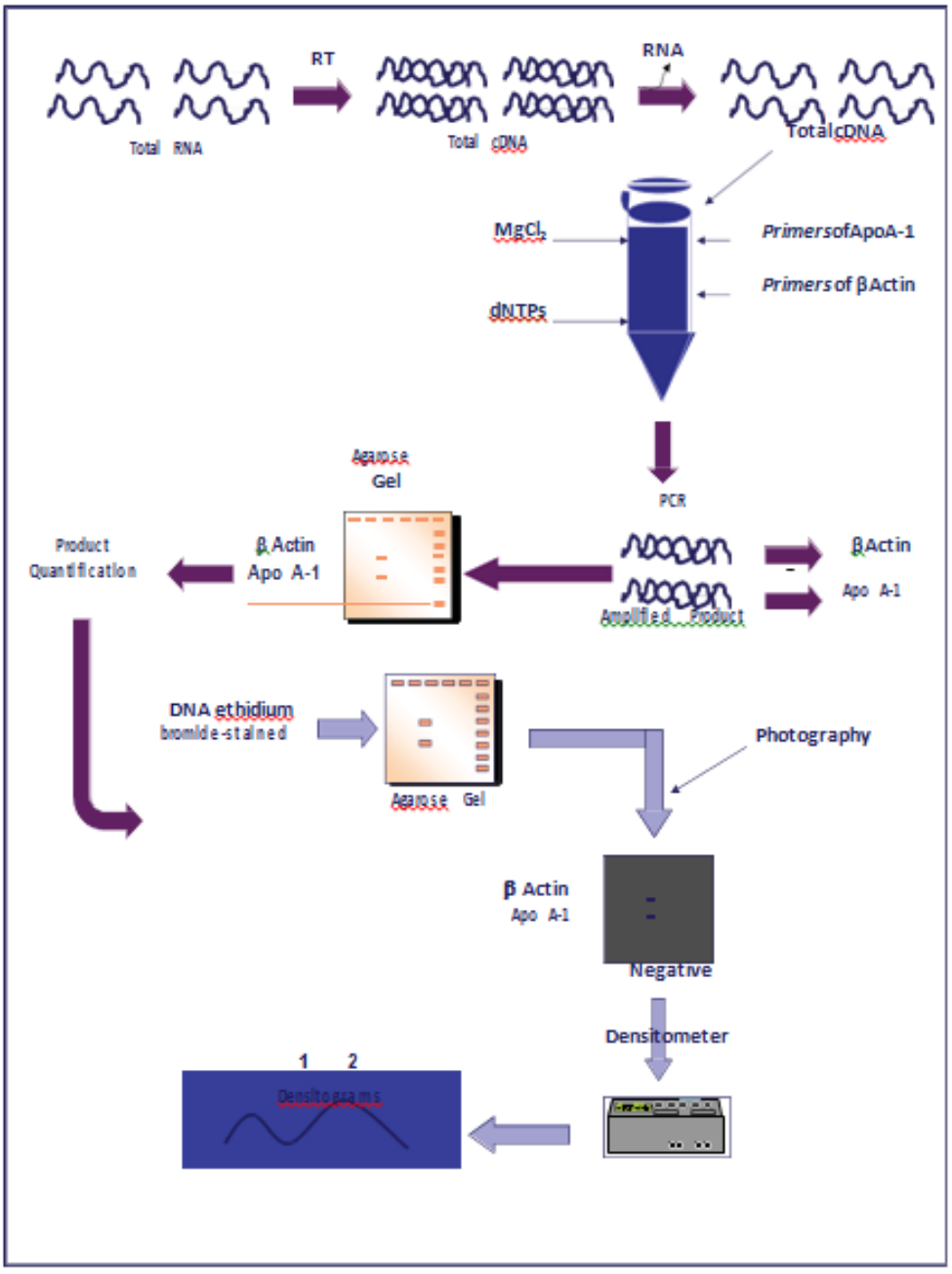


Figure (11). Semiquantitative PCR procedure. This technique is useful for identifying small amounts of nucleic acids.

Stages

The PCR process can be divided into three stages:

Exponential amplification: At every cycle, the amount of product is doubled (assuming 100% reaction efficiency). The reaction is very sensitive: only minute quantities of DNA must be present **(*Sarkar et al., 1990*)**.

Leveling off stage: The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting.

Plateau: No more product accumulates due to exhaustion of reagents and enzyme.

Optimization

In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants **(*Joseph* et *al., 2001*)**.

This usually involves spatial separation of PCR-setup areas from areas for analysis or purification of PCR products, use of disposable plasticware, and thoroughly cleaning the work surface between reaction setups. Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products, and the usage of alternate buffer components or

polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA. Addition of reagents, such as formamide, in buffer systems may increase the specificity and yield of PCR **(*Sarkar et al., 1990*)**. Computer simulations of theoretical PCR results (Electronic PCR) may be performed to assist in primer design.

Amplification and quantification of DNA

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsarand the body of English king Richard III **(*Chemical, 2007*)**.

Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample—a technique often applied to quantitatively determine levels of gene expression. Quantitative PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000 fold higher than that of other methods. PCR allows

for rapid and highly specific diagnosis of infectious diseases, including those caused by bacteria or viruses **(*Cai et al., 2014*)**.

PCR also permits identification of non-cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes **(*Cai et al., 2014*; *Salis, 2009*)**.

Viral DNA can likewise be detected by PCR. The primers used must be specific to the targeted sequences in the DNA of a virus, and PCR can be used for diagnostic analyses or DNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease **(*Cai* et al., 2014)**. Such early detection may give physicians a significant lead time in treatment. The amount of virus ("viral load") in a patient can also be quantified by PCR-based DNA quantitation techniques.

Limitations

DNA polymerase is prone to error, which in turn causes mutations in the PCR fragments that are made. Additionally, the specificity of the PCR fragments can mutate to the template DNA, due to nonspecific binding of primers. Furthermore, prior information on the sequence is necessary in order to generate the primers **(*Garibyan and Avashia, 2013*)**.

Applications of PCR and impact on science

During the past 30 years molecular techniques have been under development, however these have had a rapid and tremendous progress in recent year **(*Fluit et al., 2001*)**. Among molecular techniques, PCR and its different variations are highlighted as the most commonly used in laboratories and research institutes **(*Hernández-Rodríguez et al., 2011; Gomez et al., 2008*)**.

Molecular biology techniques, particularly PCR, have had a major impact on medicine. Clinical microbiology has been transformed with the use of molecular technology because it has generated a benefit to the patient affected by infectious diseases. These applications are transforming and complementing the work of biochemists, immunologists, microbiologists and other health professionals who see in the molecular tools new alternatives for a rapid diagnosis of microorganisms as well as for the determination of multiple factors associated with antibiotic resistance thus expanding the knowledge of microbial epidemiology and surveillance at the genetic level **(*Weile and Knabbe, 2009; Dreier et al., 2007; Hutchins and Grabsch, 2009*)**.

The usefulness of PCR in identification of microorganisms has led to the selection and quality assurance of blood that blood banks are using for patients with different pathologies **(*Dreier et al., 2007*)**. The incorporation of molecular techniques has been of great importance in the identification and characterization of many viruses, including influenza, which through a rapid, sensitive, and effective molecular diagnosis has allowed inclusion

of early treatment to benefit patients and control of a high impact infection **(*Dale, 2010; Wyczałkowska-Tomasik and Zegarska, 2009*)**.

Variations

1.Allele-specific PCR.

2.Assembly PCR or Polymerase Cycling Assembly (PCA).

3.Asymmetric PCR.

4. Reverse Transcription PCR(RT-PCR).

5.Digital PCR (dPCR).

6.Helicase-dependent amplification.

7. Hot start PCR.

8. Solid Phase PCR.

9.Intersequence-specific PCR (ISSR).

10. Ligation-mediated PCR.

11. Methylation-specific PCR (MSP).

12. Miniprimer PCR.

13. Multiplex ligation-dependent probe amplification (MLPA).

14. Multiplex-PCR.

15. NanoparticleAssisted PCR (nanoPCR).

16. Dial-out PCR.

17. PAN-AC.

18. Nested PCR.

19. Reverse Transcription PCR(RT-PCR).

20. Overlap-extension PCR or Splicing by overlap extension (SOEing).

21. Suicide PCR.

22. Silico PCR (digital PCR, virtual PCR, electronic PCR, e-PCR).

23. TouchdownPCR (Step-down PCR).

24. Thermal asymmetric interlaced PCR (TAIL-PCR).

25. Inverse PCR.

26. QuantitativePCR **(qPCR).**

Subjects and methods

Subjects and methods

Study subjects:

This study was done on 50 pregnant women around 2013-2014 selected from Qasr Al Ainy Hospital from the gynecology and obstetrics department clinics and from armed forces hospital in Kafr AlSheikh. The practical part was done at the faculty of Veterinary Medicine at Kafr AlSheikh University.

High risk women, including maternal age26±5 years, relevant past obstetric and family history and impaired glucose metabolism, had been previously screened and diagnosed for gestational diabetes mellitus (GDM). This was obtained by measuring fasting plasma glucose concentration or performing an oral glucose tolerance test (OGTT) at 24 to 28 weeks of gestation. The study groups were age-matched.

Samples are classified into two groups, the first group are pregnant women having gestational diabetes mellitus (GDM) [35 patients] , and the second group the control group are [15 healthy] pregnant women.

Inclusion criteria:

1. Women between 21 to 31 years old.
2. Have normal blood picture.
3. With normal liver and renal functions.
4. No history of other diseases.
5. Has got diabetes after pregnancy.

Exclusion criteria:

1. Patients suffering from diabetes before pregnancy.

2. Hyper or hypotension women.

03. Anemic or have any abnormality in their blood picture.

4. Have age over 31 years or less than 21 years.

5. Pregnancies before week 24 of pregnancy or after 28 week of gestation.

6. Smokers.

An OGTT involves fasting blood sample, then three other samples were taken again through 3 hours sequentially after a 75 g oral glucose. The results were interpreted according to the National Diabetes Data Group (NDDG) criteria and the Third International Workshop-Conference on Gestational Diabetes Mellitus and also American Diabetes Association (Table 1).

Table 1. The National Diabetes Data Group (NDDG) criteria.

For the three-hour test:

Interval	Abnormal reading
Fasting	95 mg/dl or higher
1 hour	180 mg/dl or higher
2 hours	155 mg/dl or higher
3 hours	140 mg/dl or higher

If one of the results is higher than normal, patient will likely need to test again in four weeks. If two or more of the results are higher than normal, you'll be diagnosed with gestational diabetes.

35 pregnant females out of the 50 were diagnosed with gestational diabetes mellitus (GDM).

Blood sampling:

All blood samples were collected Maternal venous blood samples (5 ml) were collected FROM 24 to 28 weeks of pregnancy immediately transferred into two tubes, one for OGTT Test and the other for the gene expression.

For OGTT, the blood was collected on sodium florid tubes then centrifuged at 4000 for 3 minutes then the plasma transferred into sterile blood collection tubes and stored at 4ºC.

All women had normal fasting glucose levels at the beginning of the pregnancy. While the blood samples for gene expression were collected on EDTA tubes and stored at -80ºC until performing biochemical analysis for them.

Molecular investigation:

RNA extraction

Materials

RiboPure™-Blood Kit (Thermo Scientific, Life technologies, #AM1928) which includes; Lysis solution, Acid-Phenol:Chloroform, Wash solution 1, Wash solution 2, Filter Cartridge, and Elution solution.

Principle

The standard protocol is designed for purification of RNA from 0.5 mL of whole blood. In brief, blood samples were lysed in Lysis Solution. The aqueous phase containing the RNA was separated from organic impurities (mainly protein) by Acid-Phenol:Chloroform. This aqueous phase was then mixed with ethanol and

loaded on a Filter Cartridge. The chaotropic salt and ethanol caused RNA to bind to the silica membrane while the lysate is spun through the column. Subsequently, impurities were effectively removed from the membrane by washing the column with wash solution. Pure RNA was then eluted under low ionic strength conditions with elution solution.

Procedure

1) A volume of 0.5 ml blood collected in tubes containing sodium EDTA anticoagulant was added to 2 ml micro-centrifuge tube containing 0.8 ml lysis solution and 50 µl sodium acetate.

2) Vigorous vortexing was done to lyse blood cells.

3) A volume of 500 µL of Acid-Phenol:Chloroform was added to the cell lysate, and the tube was vortexed for 30 sec, stored at room temp for 5 min, then centrifuged at room temp for 1 min to separate the aqueous and organic phases.

4) The aqueous phase containing RNA was recovered in a fresh 2 mL tube**.**

5) A volume of 600 µl of ethanol (100%) was added and mixed by pipetting.

6) A volume of 700 µl of lysate was transferred to Filter Cartridge inserted in a collection tube. Centrifugation was done to the column for 1 min at 10000 rpm, then the flow through was discarded and the Filter Cartridge was placed back into the collection tube. This step was repeated until all of the lysate has been transferred into the column and centrifuged.

7) The collection tube containing the flow-through solution was discarded and the Filter Cartridge was placed into a new 2 ml collection tube.

8) A volume of 700 µl of Wash Solution 1 was added to the Filter Cartridge and centrifuged for 1 min at 10000 rpm, then the flow through was discarded and the Filter Cartridge was placed back into the collection tube.

9) A volume of 700 µl of Wash Solution 2 was added to the Filter Cartridge and centrifuged for 1 min at 10000 rpm, then the flow through was discarded and the Filter Cartridge was placed back into the collection tube.

10) A volume of 700 µl of Wash Solution 2 was added to the Filter Cartridge and centrifuged for 2 min at 10000 rpm.

11) A volume of 100 µl of Elution Solution was added to the center of the Filter Cartridge and centrifugation was done for 1 min at 10000 rpm to elute RNA.

12) The Filter Cartridge was discarded and the purified RNA was used for downstream applications or store RNA at -80°c until use.

cDNA synthesis

Materials

Reverse transcription kits (Thermo Scientific, Fermentas, #EP0451) which include the following:

- Revert Aid H minus Reverse Transcriptase enzyme and 5X Reaction Buffer
- Oligo dT, RiboLock RNase Inhibitor, and dNTP Mix

Principle

This technique was done using Revert Aid H minus Reverse Transcriptase which is a genetically modified M-MuLV RT, to convert RNA into complementary DNA (cDNA).

Procedure

1) A weight of 5 µg of template RNA were added into sterile, nuclease-free tube on ice, 0.5 µg Oligo dT was added then the volume was completed to 12.5 µl using DEPC-treated water.
2) A volume of 4 µl of 5x Reaction Buffer, 0.5 µg of RiboLock RNase Inhibitor, 2 µl of dNTP Mix and 1 µl RevertAid™ H Minus Reverse Transcriptase were added on the nuclease-free tube containing the component of step 1.
3) After gentle mixing, the mixture was incubated for 60 minutes at 42°C.
4) The reaction was terminated by heating at 70°C for 10 minutes.

Quantification of RNA using Nanodrop

To quantify the concentration of RNA and cDNA to be sure that the concentrations are pure enough to conduct real time PCR. For very pure samples (without significant contamination from protein, phenol, free nucleic acids, organic solvents, carbohydrates, etc.), the absorption of Ultra-Violet (UV) light by the ring structure of purines and pyrimidines can be used to measure the amount of nucleic acids. The Q5000 **(Uv-Vis spectrophotometer Q5000/USA)** automatically performs all necessary measurements and calculations.

Procedure

1) The upper arm of instrument was left and 1.5 µl of blank buffer was first used to the lower surface to get the blank reading by closing the upper arm and clicking the "Measure" button to complete all measurements and calculations and then the upper arm was lift and the blank was removed from both upper and lower surfaces with soft dry paper.
2) 1.5 µl of sample was measured by the same method.

3) Data was exported to an Excel worksheet by clicking the "Report" button.
4) The absorbance of the sample was read at 260 and 280 nm wavelength.
5) The concentration based on an optic density (OD) of 1 corresponding to approximately 50µg/ml for RNA was then automatically calculated.

The OD260/OD280 ratio for pure RNA is ≥ 2. Contamination by protein (which has an absorbance maximum of 280 nm) or phenol will cause the ratio to be significantly lower than these values.

Real time PCR

Real-time PCR with SYBR Green was used to measure expression of mRNAs of target genes in blood, with *GAPDH* as an internal reference. The isolated cDNA were amplified using 2X Maxima SYBR Green/ROX qPCR Master Mix following the manufacturer protocol (Thermo scientific, USA, # K0221) and gene specific primers. The primers used in the amplification are shown in Table 1. The web based tool,Primer3(http://www.genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) was used to design these primers based on published human sequences. To ensure primer sequence is unique for the template sequence; we checked similarity to other known sequences with BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Primers preparation

1- Lyophilized primer at -20ºC was equilibrated at room temperature.
2- Equilibrated primer was spin down for 3 sec. using spin-centifuge-vortex.
3- Lyophilized primer was diluted (both forward and reverse) with RNase free water (the volume was added to get 100µM stock) and then the tube was gently invert for 2 min. at room temperature.

4- Stock primer was diluted with RNase free water buffer (pH 8.0) to get 5µM and kept at -20ºC until used.

Table 2: Forward and reverse primers sequence for the candidate genes.

Target Gene	Sense primer sequence (5'-3')	Anti-sense primer sequence (5'-3')
LEP	CTATGTCCAAGCTGTGCCCATCC	CTGCCAGTGTCTGGTCCATCTTG
RETN	ATGAGCCACAGCCAGAGCCACAG	TCCTGCCCCCTGCGCTCTC
ADIPOQ	GTGATGGCAGAGATGGCACCC	AGGCACCTTCTCCAGGTTCTCC
ADIPOR1	TTGTGTACAAGGTCTGGGAGGGA	CAGCACG AAACCAAGCAGATGG
ADIPOR2	CTGATGGCCAGCCTCTACATCAC	CCCGCCGATCATGAAACGAAAC

Relative expression

The polymerase chain reaction mixture was carried out in a 25 µl which contains the following:

cDNA template (10-20 ng/ µl)	3 µl
2X Maxima SYBR Green/ROX qPCR Master Mix	12.5 µl
Primer forward (10 µM)	1 µl
Primer reverse (0.1- 0.5 µM)	1 µl
Water, nuclease free	7.5 µl
Total	25 µl

The final reaction mixture was placed in a StepOnePlus real time thermal cycler (Applied Biosystems, Life technology, USA) and the PCR program was carried out with the PCR conditions as detailed in table 2.

Table (3): The thermal cycler condition used during real time PCR.

Initial denaturation	Denaturation	Annealing/ Extension	Cycles
94˚C/ 10 min	94˚C/ 15 sec	60˚C/ 30 sec	40

At the end of the last cycle temperature was increased from 60 to 95 °C to produce a melt curve. The house keeping gene (*GAPDH*) represented as normalize that used to calculate the relative gene expression or fold change in target gene. Therefore, the quantities critical threshold (Ct) of target gene were normalized with quantities (Ct) of house keeping gene (*GAPDH*) by used the $2^{-\Delta\Delta Ct}$ method **(Livak and Schmittgen, 2001)** as follow:

- The control group was used as calibrator, while other groups represented as test groups in both target and reference gene.
- The threshold cycle numbers (Ct) of target gene were normalized to that of reference (ref.) gene, in both the test groups and the control group by using the following equations:

  ΔCt (test) = Ct (target in test groups) – Ct (ref. in test groups)

  ΔCt (calibator) = Ct (target in control) – Ct (ref. in control)

- The ΔCt of the test genes were normalized to the ΔCt of the calibrator:

$$\Delta\Delta Ct = \Delta Ct \text{ (test)} - \Delta Ct \text{ (calibrator)}$$

- Fold change of relative gene expression was calculated as follow: **Fold change = ($2^{-\Delta\Delta Ct}$)**

Statistical analysis

For consistency and easier reading, continuous parametric data presented in this study were expressed as mean average ± standard error of the mean (SEM) whereas for non-parametric data percentages or ratios were used.

The statistical significance was evaluated by using SPSS, 18.0 software, 2011.

Values were considered statistically significant when $p<0.05$.

Some statistical graphs produced using Microsoft Office Excel 2010 software (Microsoft Corporation, Berkshire, UK). Further details of any statistical test used are provided in the relevant results Chapters.

Results

Results

Before detecting the change in gene expression of some markers associated with gestation diabetes (GDM), we conducted a screening for glucose levels in different pregnant subjects. In this regard, we carried out fasting blood sugar test (FBS) and oral glucose tolerance test (OGGT). The results obtained from these two tests were presented in (Table 1 and Figs.1 and 2). These results exhibited a significant increase in FBS levels in GDM as compared to control (healthy) group (Table 1 and Fig.1). The level of OGGT was also significantly elevated in GDM, except at the first hour which showed insignificant increase, as compared to control group. Comparing the results obtained from the two tests within each subject (either control or GDM), we found a significant elevation in blood sugar level after oral administration of sugar in both control and GDM as compared to during fasting (Fig. 2). However, this increase was either inversely related to time (first hour reading is the highest and the third hour reading was the lowest) as in the control group or proportionally related to time (first hour reading was the lowest and the third hour reading was the highest) as in the GDM group.

Raw data for OGGT (table A)

	FBS- control	FBS- GDM	1st hour-control	1st hour-GDM	2nd hour-control	2nd hour-GDM	3rd hour-control	3rd hour-GDM
	94.2	119	165.9	154.8	145.9	170.1	129.9	196.5
	86	99.5	147.2	159.7	119	173.9	99.2	201.3
	85.4	142.3	139.4	145.3	117.8	175.3	104.5	207
	87.1	121.6	133	153.3	121.6	174	100.6	209.8
	89.7	133.9	141.7	162	129.8	168.4	117.4	194
	93.2	98.9	159.5	148.2	148.3	172.6	137	188.5
	88.5	101.6	145.7	143	133.9	170.9	121.6	207.9
	83.4	124.3	139.3	151.9	113.3	168.8	94	199.8
	91.5	142	150.5	152.2	138.7	171.8	123.4	211.7
	87	153.8	142.2	143	122.8	169.2	103.3	227.9
	80.8	98.7	119.7	149.8	108.3	174.2	93.9	190.3
	91.9	138.2	138.4	150.6	126.5	168.7	118.9	212.3
	79.5	104.7	110.4	146.6	100.7	165.2	89.8	191.2
	94.3	112.4	174.9	160.3	142.3	170.7	135	188
	92	98.9	144.5	158.3	130	174.3	121.2	201.9
		137.6	144.5	162		183.8		208.2
		151.6		149		170		236.4

		124		151.6		168.9		209.5
		102.8		148.7		171.5		188.9
		113.5		161.4		182		202.1
		135		144.2		165.4		213.8
		99.7		163.2		172.3		180
		116.9		146.1		174.4		203.4
		99		156.5		170.3		213.1
		103.8		156.6		173.5		199.7
		96.3		159		175		200.9
		102.6		148.3		170.1		195.8
		118		161.9		188.8		192.5
		131.3		147.4		172.5		223.2
		114.5		155.6		174.8		205.6
		101.1		158.5		169.5		207.4
		122.4		149		172		189.2
		134.7		153.5		167.5		216.3
		103.5		150.2		171.3		198.9
		110.8		152.4		174.3		202.2
Mean	89.16	116.69	147.49	152.33	130.90	172.17	115.74	200.71
SD	3.44	17.42	11.51	7.17	12.50	2.46	14.83	8.02
SE	1.98	10.06	6.65	4.14	7.22	1.42	8.56	4.63

Raw data for CT Values (Table B):

No.	Leptin	AdipoQ	AdipoR1	AdipoR2	Resistin
1	26.69	30.14	29.57	25.61	25.64
2	29.30	27.17	30.89	27.11	27.20
3	31.91	26.22	30.53	26.20	26.60
4	34.37	28.45	29.44	25.53	25.05
5	25.63	27.12	30.74	25.74	26.82
6	27.45	28.49	29.57	27.21	26.13
7	26.30	28.74	28.70	26.41	25.33
8	28.32	29.65	30.41	27.03	27.07
9	28.50	28.88	30.82	26.54	26.62
10	29.87	27.48	29.39	25.98	25.86
11	30.72	30.32	30.15	27.18	26.70
12	29.89	28.23	30.68	26.80	26.61
13	27.42	30.76	29.49	27.29	25.39
14	32.19	28.84	30.14	26.56	26.10
15	25.37	30.50	29.86	26.48	27.87
16	25.70	27.91	28.62	25.78	23.98
17	29.37	30.29	27.83	24.70	25.05
18	30.31	31.20	28.44	25.98	23.63
19	23.91	27.89	28.81	24.44	24.40
20	30.07	32.71	27.98	25.59	26.80
21	24.82	28.99	28.74	25.01	28.05

22	27.69	30.19	27.50	26.18	24.29
23	26.33	30.33	28.49	25.45	25.57
24	31.80	27.75	28.35	26.40	23.84
25	29.19	32.96	27.45	25.57	26.31
26	23.00	30.81	27.77	25.83	24.19
27	27.84	28.80	28.44	24.48	27.32
28	28.14	32.90	28.76	26.23	27.07
29	24.76	27.17	27.95	25.64	23.91
30	27.42	31.10	27.50	26.12	25.80
31	29.49	30.01	28.56	24.69	25.44
32	23.67	32.93	27.61	26.53	23.59
33	29.78	28.20	28.00	25.81	26.19
34	31.14	31.13	27.90	26.08	27.88
35	24.03	26.86	28.17	24.99	24.65
36	31.12	32.11	28.34	25.76	26.14
37	26.33	30.46	28.28	25.94	23.81
38	29.19	28.64	27.53	26.54	24.31
39	33.95	31.08	28.72	26.33	27.67
40	28.48	27.13	27.90	25.17	23.70
41	33.20	30.77	28.64	26.51	25.19
42	28.39	28.37	27.59	24.73	27.44
43	23.48	32.72	28.83	26.50	24.00
44	30.13	26.95	28.08	25.32	27.13
45	26.48	31.43	27.77	26.45	25.82
46	24.29	30.55	28.84	26.49	24.34
47	33.47	28.42	28.50	25.76	27.71
48	22.95	29.91	28.17	24.92	23.76
49	28.54	27.79	27.67	25.24	25.33
50	26.51	27.02	28.68	24.36	24.10

N= Number of pregnant women , GDM = Gestational Diabetes Mellitus

Lep. = Leptin ,Adipo.Q = Adiponectin , Adipo.R1 = Adiponectin Receptor 1, Adipo.R2 = Adiponectin Receptor 2 , Retn. = Resistin .

Table (1): Results of OGGT for control (normal) and GDM pregnant women between 24-28 weeks in pregnancy.

	Control group	**GDM group**
FBS	89.16 ± 1.98	116.69 ± 10.06 **
1st hr.	147.49 ± 6.65	152.33 ± 4.14
2nd hr.	130.90 ± 7.22	172.17 ± 1.42 ***
3rd hr.	115.74 ± 8.56	200.71 ± 4.63 ****

Data expressed as mean ± standard error of mean (SEM). Means within the same row carrying different superscript symbols are significantly different at ** P≤ 0.01, *** P≤ 0.001, **** P≤ 0.0001 as revealed by student t test.

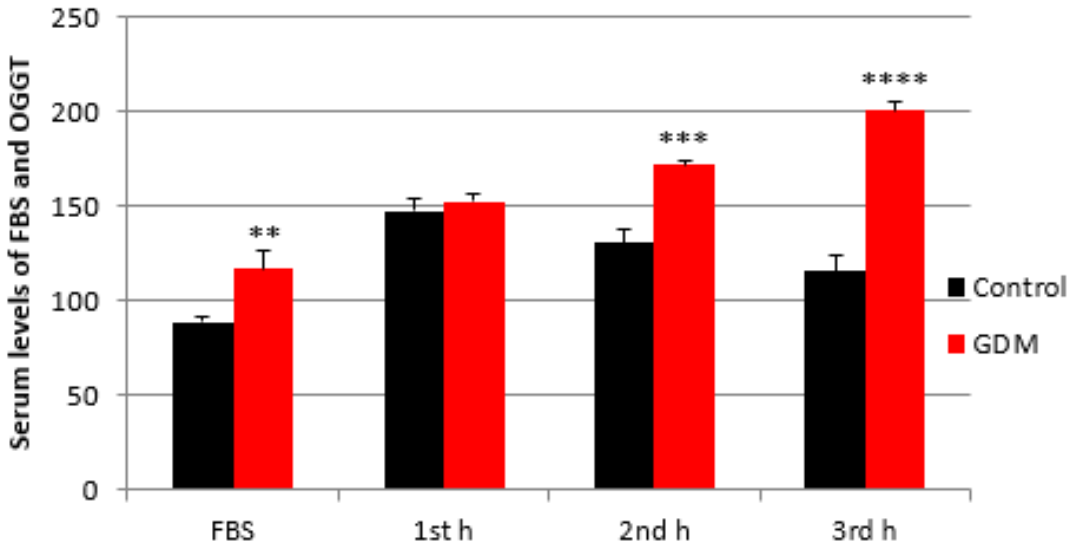


Fig.1. Graphical presentation of FBS and OGGT levels in control (normal) and GDM pregnant women between 24-28 weeks in pregnancy.

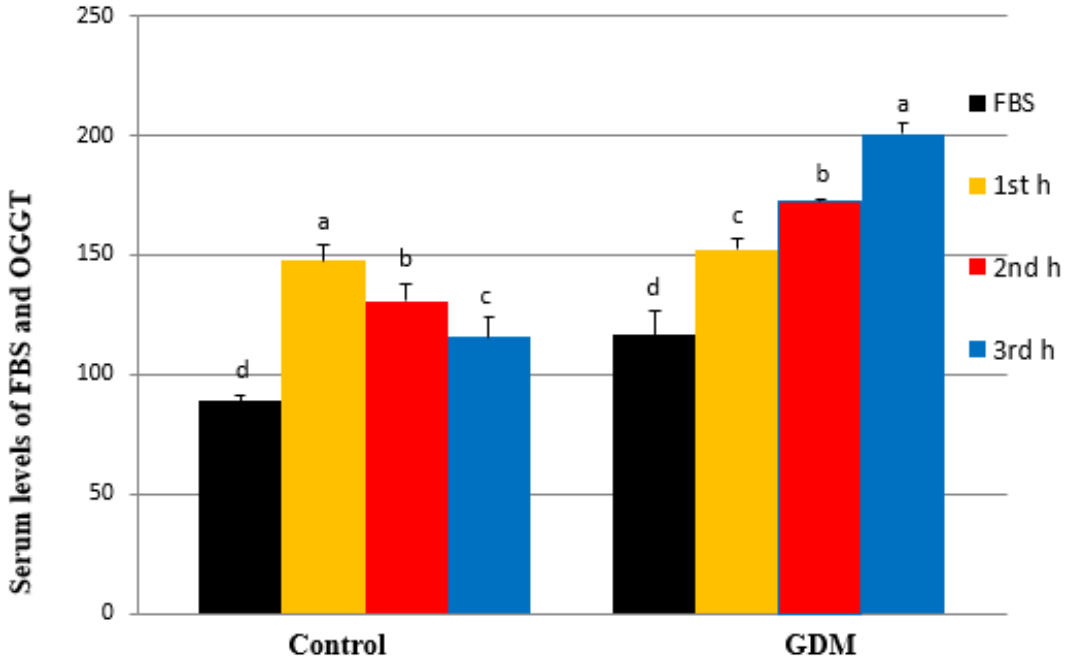


Fig.2. Graphical presentation of FBS and OGGT levels in control (normal) and GDM pregnant women between 24-28 weeks in pregnancy showed comparison between the results of the two tests in each group. Means (columns) within the same groups carrying different superscript letters are significantly different ($P \leq 0.05$) based on results of one way ANOVA and Duncan's multiple range test (DMRT).

Molecular analysis

Real time PCR (qPCR) was used to detect the relative expression of Leptin (*Lep*), Adiponectin (*Adipo*), Adiponectin receptor 1 (*AdipoR1*), Adiponectin receptor 2 (*AdipoR2*), and Resistin (*Retn*) genes in the blood that reflects the changes in transcription levels of these genes in pregnant women with GDM as compared to control (normal).

To conduct real time PCR, we first isolated total RNA from blood of all subjects.

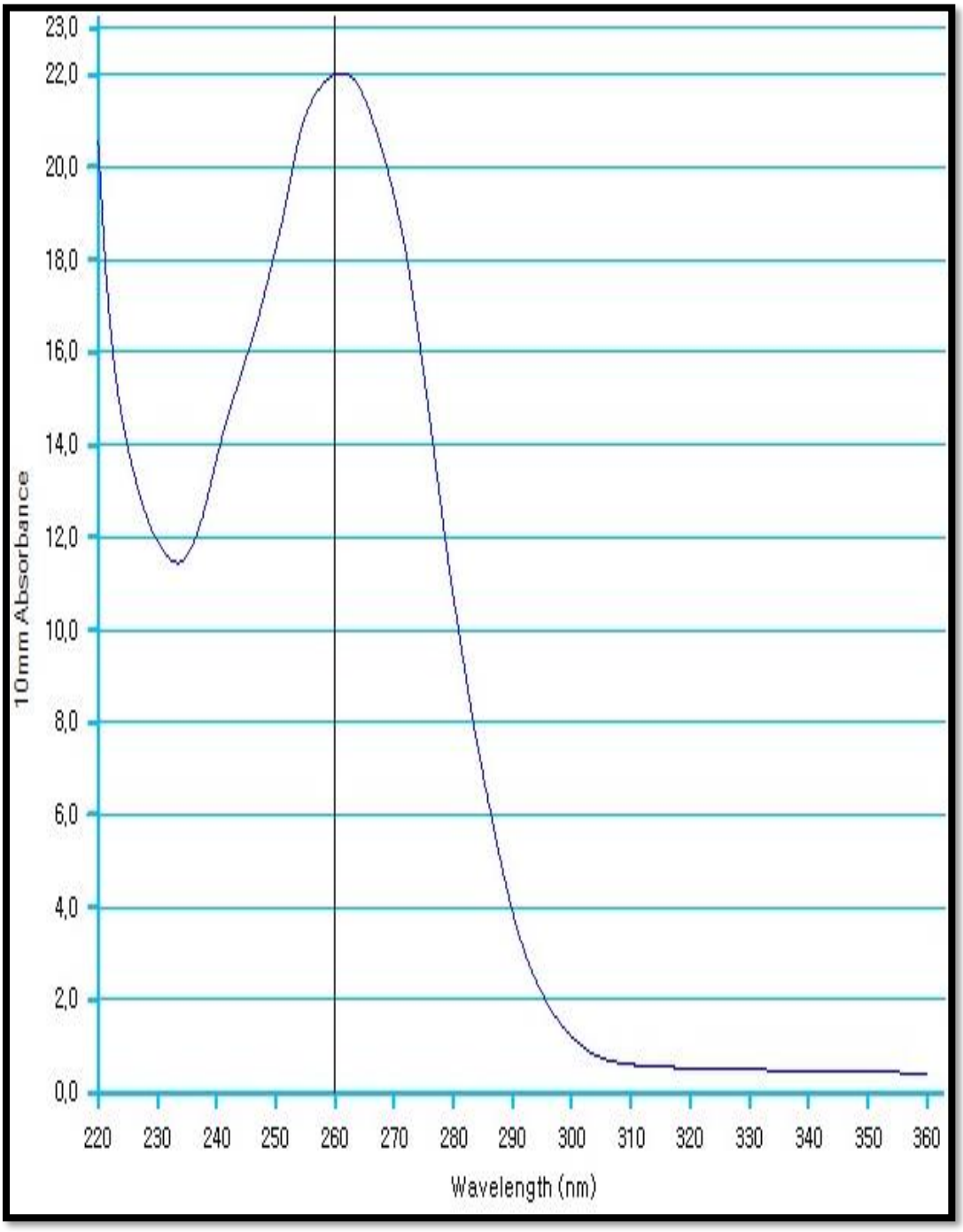


Fig.3: Nanodrop curve showing concentration and purity of extracted RNA from a representative sample which is 1810 ng/µl. In this curve the upper top presents at 260 and the bottom at 230 which indicates the presence of pure RNA.

Effect of GDM on the relative expression of *Lep* gene

Table 3. Changes in relative expression of *Lep* gene in blood of control and GDM groups.

Group	Fold change mean ± SEM
Control (normal)	1.00 ± 0.08
GDM	3.41± 0.13 ****

Data expressed as mean ± standard error of mean (SEM). Means within the same column carrying different superscript symbols are significantly different at **** P≤ 0.0001 as revealed by student *t* test.

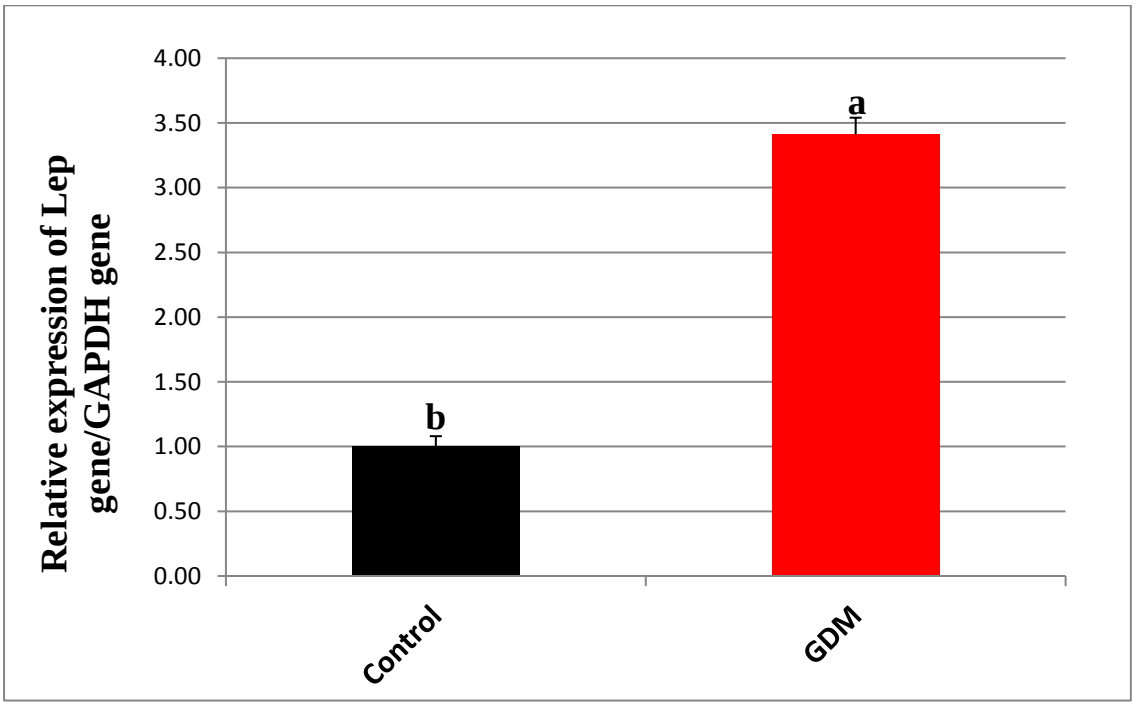


Fig.4. Graphical presentation of real-time quantitative PCR analysis of the expression of *Lep* gene in blood of the GDM group as compared to the control group.

Effect of GDM on the relative expression of *Adipo* gene

The obtained qPCR results revealed significant (P≤0.05) downregulation (0.5 fold decrease) of *Adipo* gene expression level in blood of GDM group as compared to control group (Table 4 and Fig. 5).

Table 4. shows Changes in relative expression of *Adipo* gene in blood of control and GDM groups.

Group	Fold change mean ± SEM
Control (normal)	1.00 ± 0.08
GDM	0.50± 0.04 ****

Data expressed as mean ± standard error of mean (SEM). Means within the same column carrying different superscript symbols are significantly different.

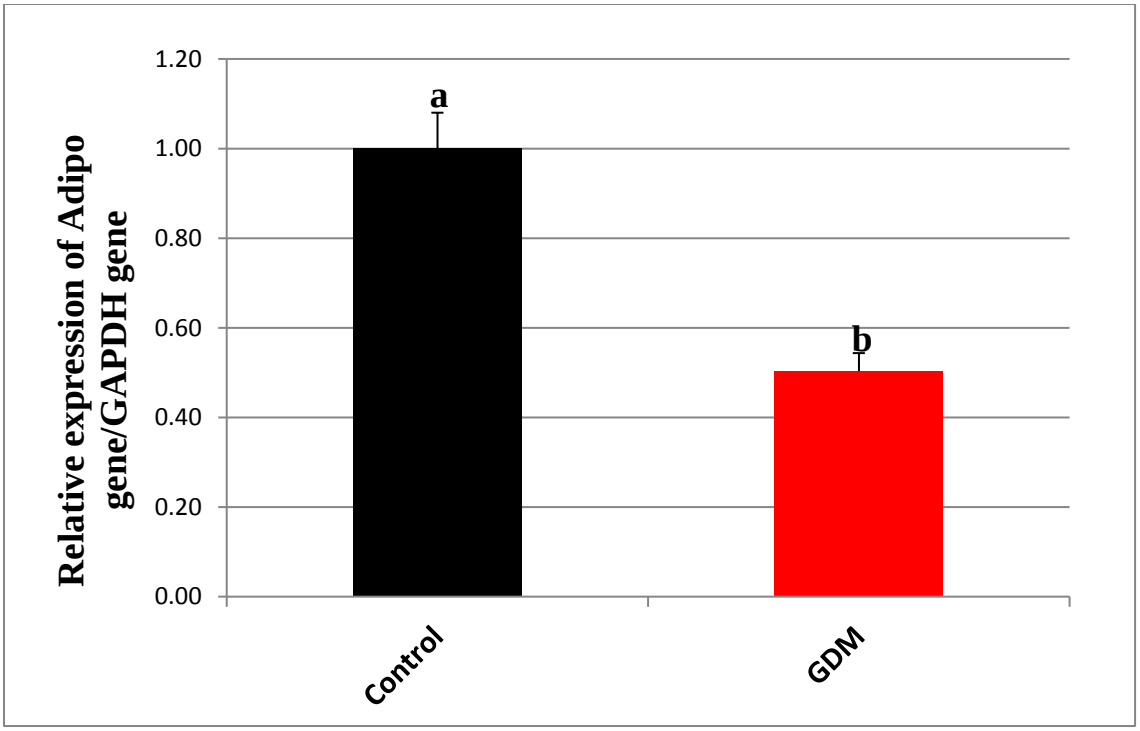


Fig.5. Graphical presentation of real-time quantitative PCR analysis of the expression of *Adipo* gene in blood of the GDM group as compared to the control group.

Effect of GDM on the relative expression of *AdipoR1* gene

The obtained qPCR results revealed insignificant (P≤0.05) upregulation of *AdipoR1* gene expression level in blood of GDM group as compared to control group (Table 5 and Fig. 6).

Table 5. Changes in relative expression of *AdipoR1* gene in blood of control and GDM groups.

Group	Fold change mean ± SEM
Control (normal)	1.00 ± 0.08
GDM	1.09± 0.12

Data expressed as mean ± standard error of mean (SEM) as revealed by student *t* test.

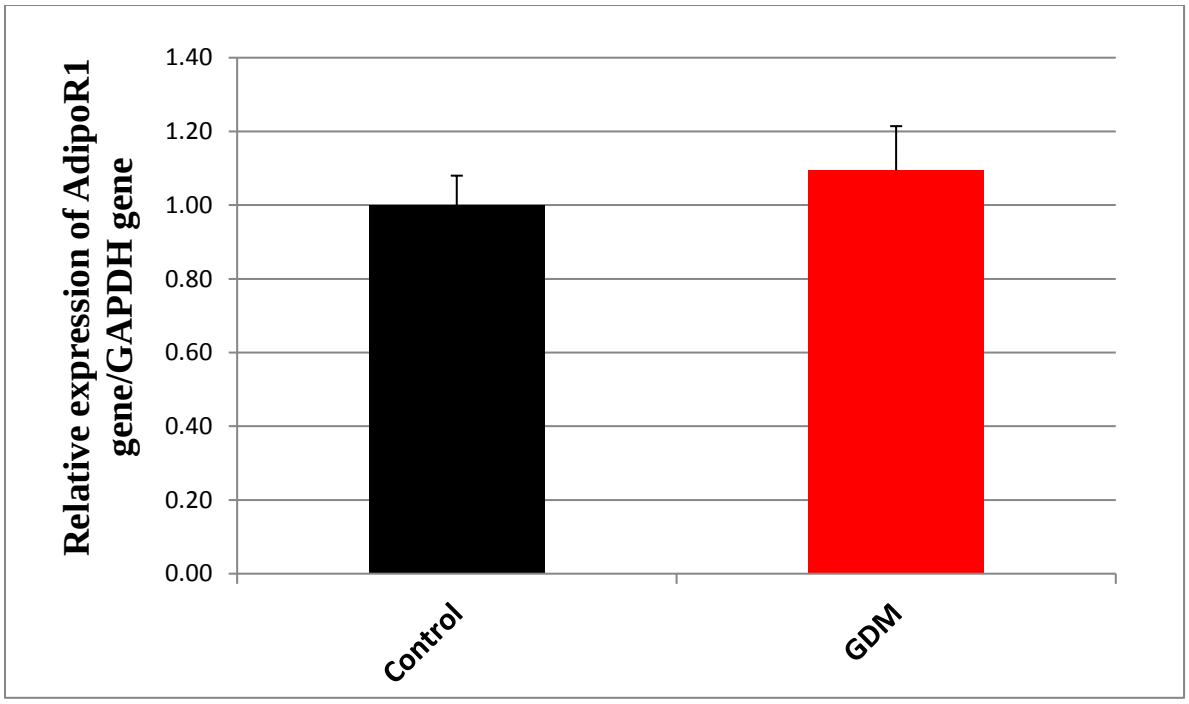


Fig.6. Graphical presentation of real-time quantitative PCR analysis of the expression of *AdipoR1* gene in blood of the GDM group as compared to the control group.

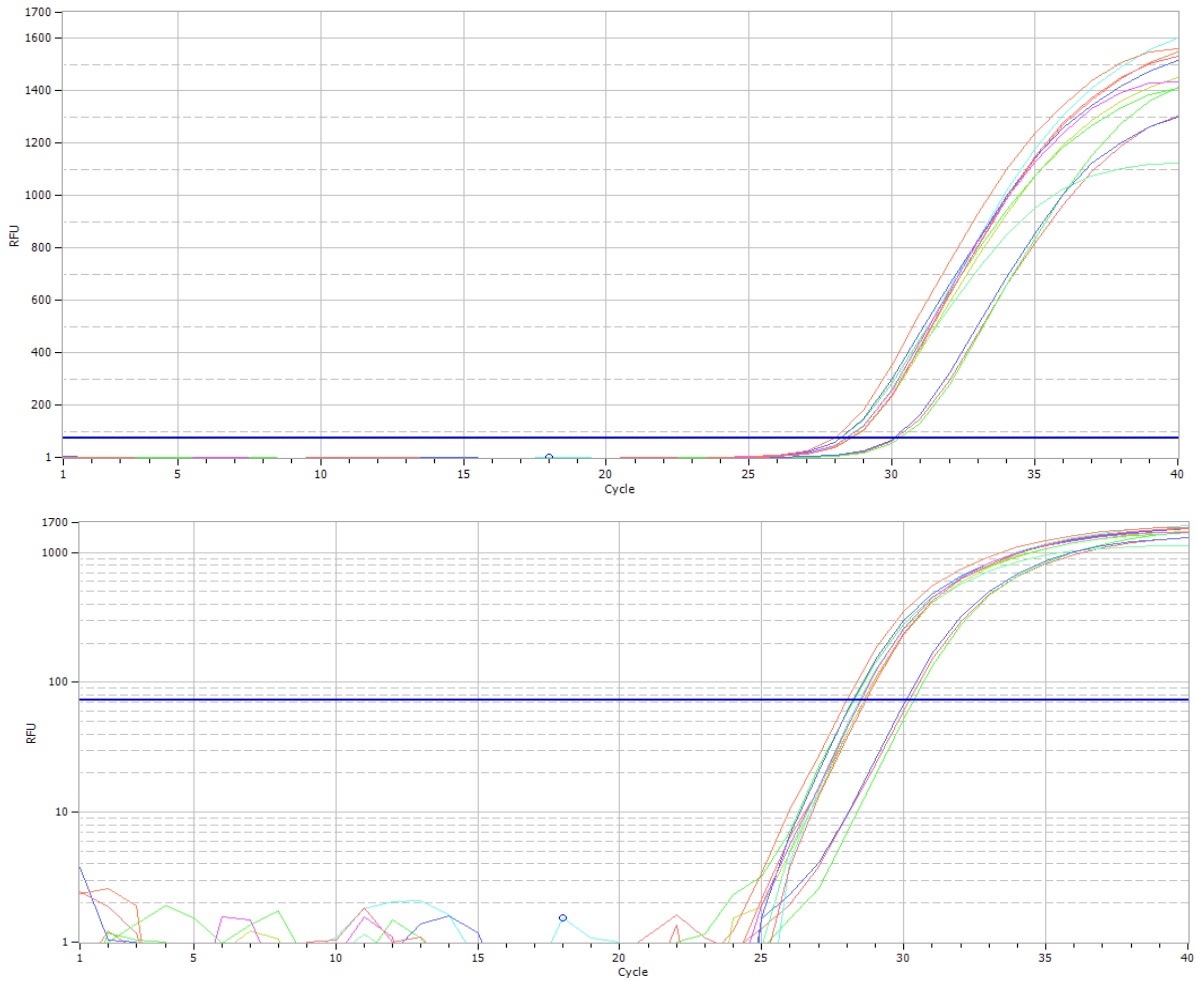


Fig.7.The linear (upper) and log (lower) amplification curves representing the Ct values of *Lep*, *Adipo*, and *AdipoR1* genes in different groups as following in table 6:

Gene	**Group**	**CT value**
Lep	Control	29.87
	GDM	28.48
Adipo	Control	28.49
	GDM	29.91
AdipoR1	Control	30.14
	GDM	28.17

Effect of GDM on the relative expression of *AdipoR2* gene

The obtained qPCR results revealed insignificant (P≤0.005) downregulation (0.87 fold decrease) of *AdipoR2* gene expression level in blood of GDM group as compared to control group (Table 7 and Fig. 8).

Table 7. Changes in relative expression of *AdipoR2* gene in blood of control and GDM groups.

Group	Fold change mean ± SEM
Control (normal)	1.00 ± 0.07
GDM	0.87± 0.13

Data expressed as mean ± standard error of mean (SEM) as revealed by student *t* test.

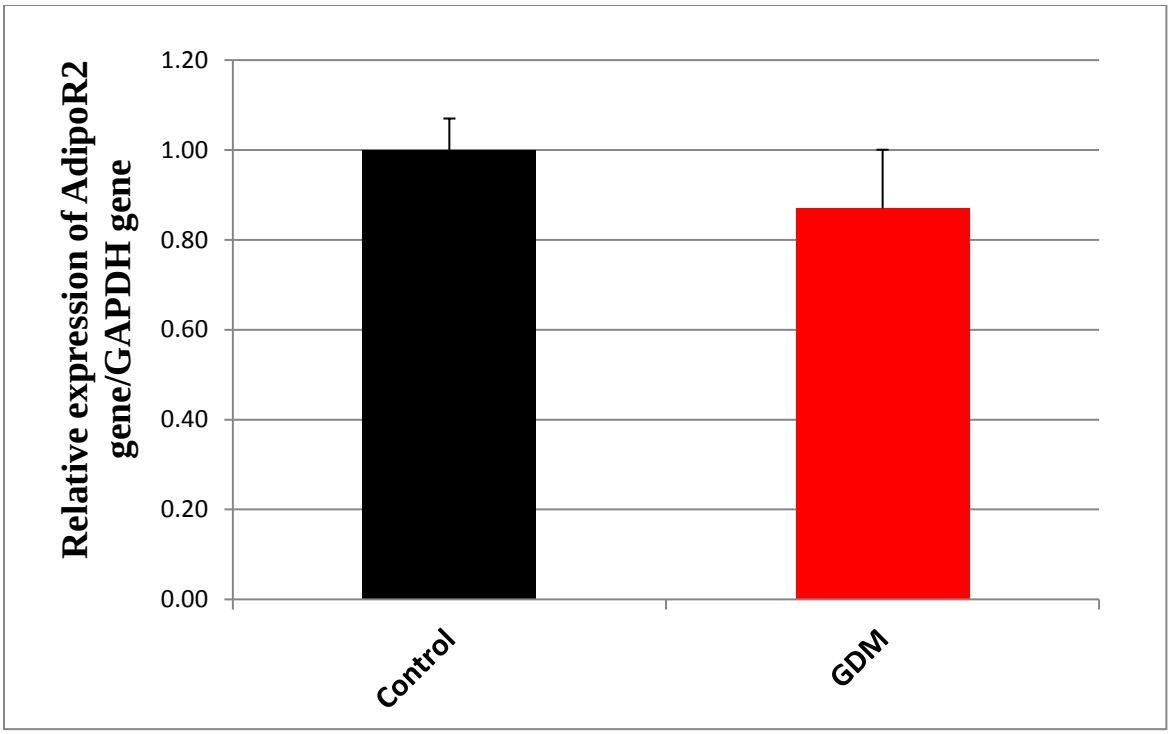


Fig.8. Graphical presentation of real-time quantitative PCR analysis of the expression of *AdipoR2* gene in blood of the GDM group as compared to the control group.

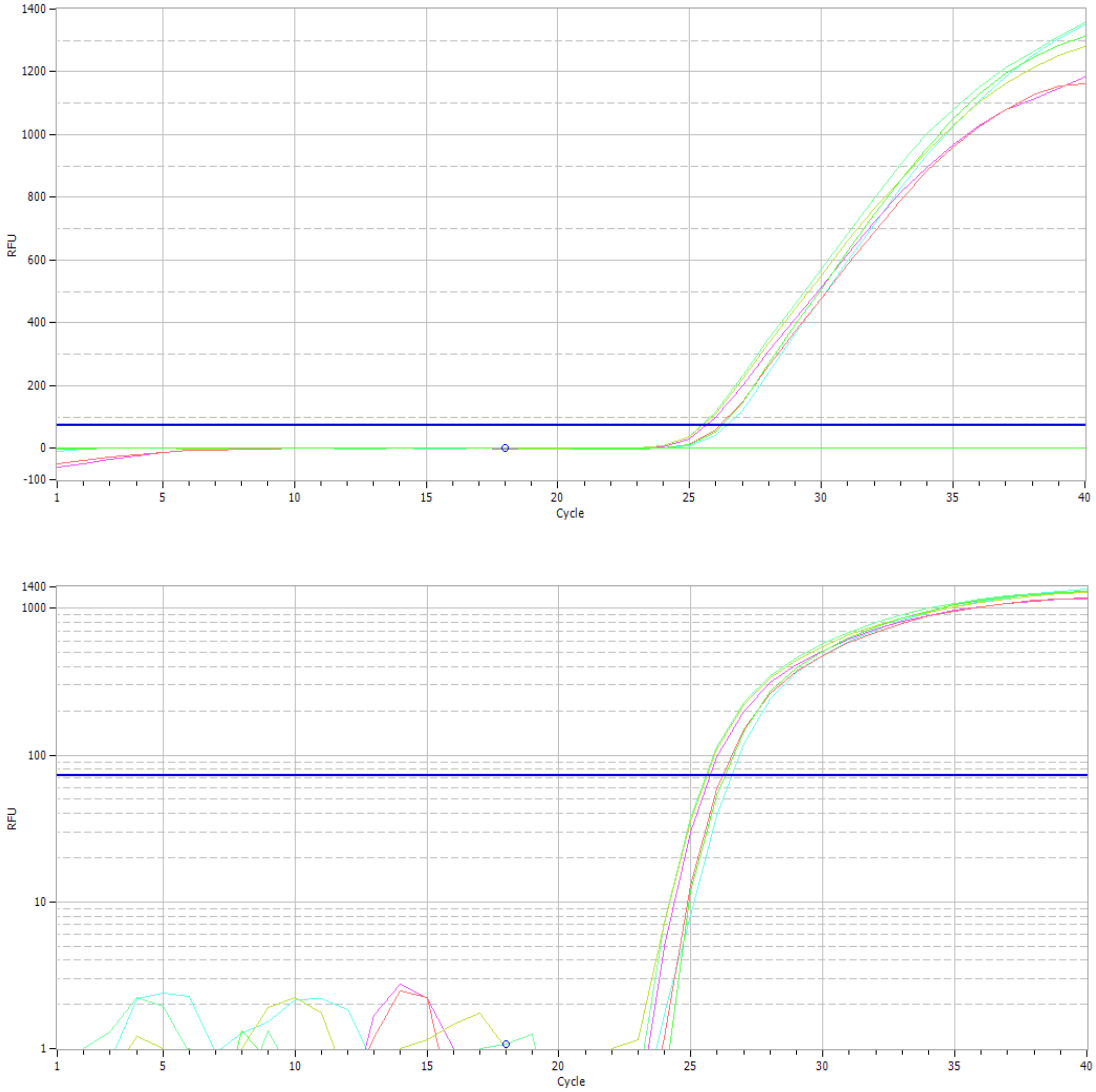


Fig.9.The linear (upper) and log (lower) amplification curves representing the Ct values of *AdipoR2* genes in different groups as following table 8 :

Group	CT value
Control	26.41
GDM	25.45

Effect of GDM on the relative expression of *Retn* gene

The obtained qPCR results revealed significant (P≤0.05) upregulation (2.35 fold increase) of *Retn* gene expression level in blood of GDM group as compared to control group (Table 9 and Fig. 10).

Table 9. Changes in relative expression of *Retn* gene in blood of control and GDM groups.

Group	Fold change mean ± SEM
Control (normal)	1.00 ± 0.08
GDM	2.35± 0.10***

Data expressed as mean ± standard error of mean (SEM) Means within the same column carrying different superscript symbols are significantly different.

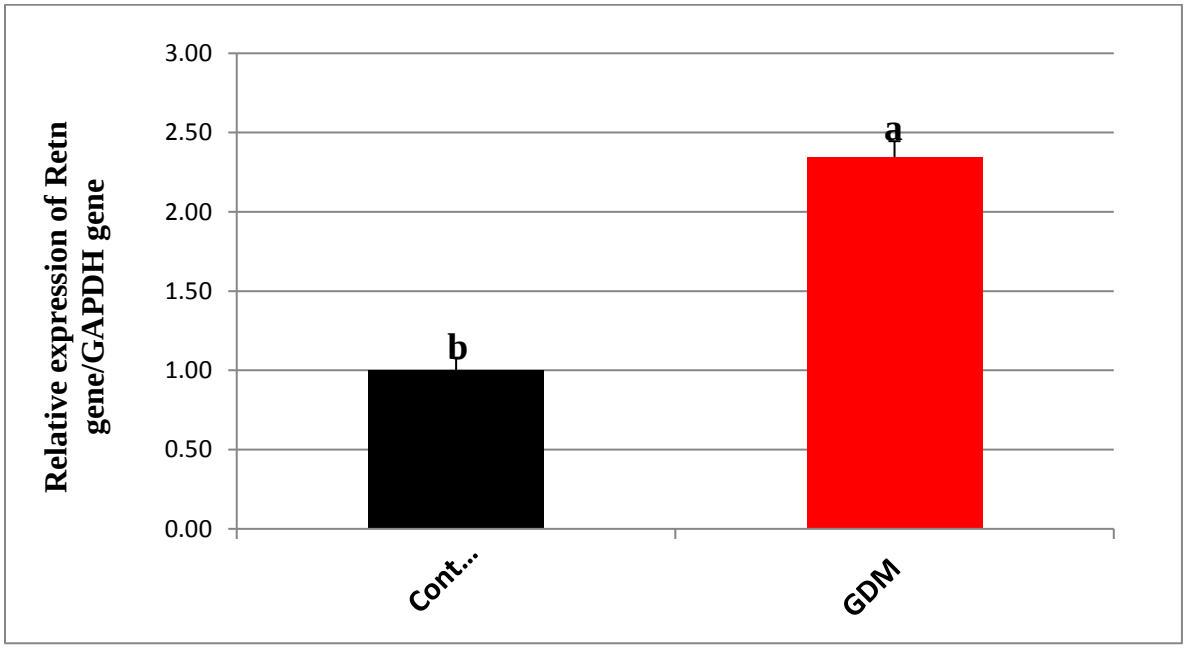


Fig.10. Graphical presentation of real-time quantitative PCR analysis of the expression of *Retn* gene in blood of the GDM group as compared to the control group.

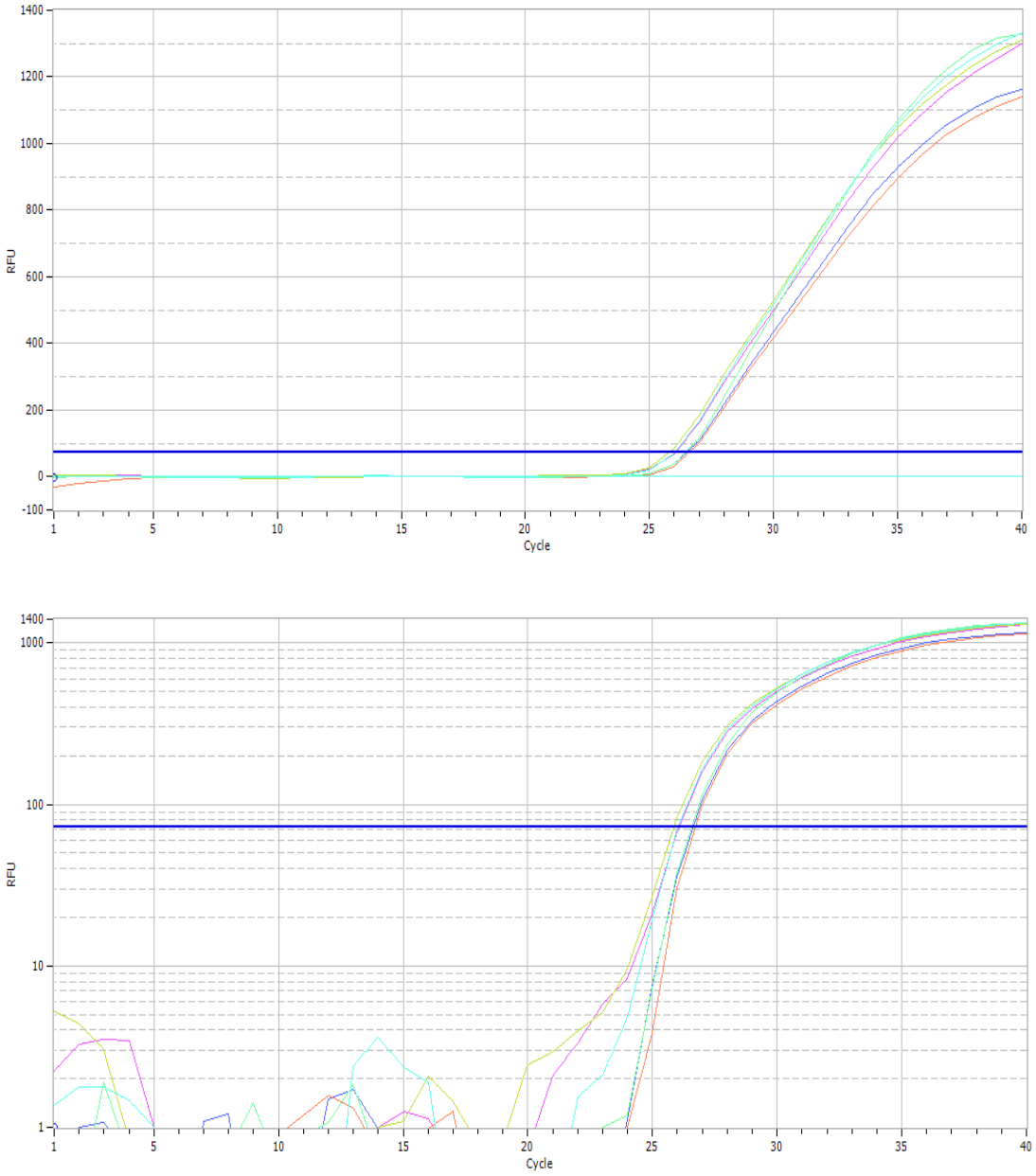


Fig.11.The linear (upper) and log (lower) amplification curves representing the Ct values of *Retn* genes in different groups as following in table 10 :

Group	**CT value**
Control	26.60
GDM	25.82

Discussion

Discussion

Gestational diabetes mellitus (GDM) is one of the most frequent complications of pregnancy that may lead to considerable risk for both the fetus and the mother [*Soheilykhah et al. 2011*].

Like all forms of hyperglycemia, GDM is characterized by insulin levels that are insufficient to meet insulin demands [*Metzger et al. 2007*]. The pathogenesis of GDM has not been clearly explained. The most common theory is that GDM is caused by decreasing insulin sensitivity and increasing anti-insulin hormones [*Gao et al. 2008*].

Diabetes complicating pregnancy is associated with perinatal outcomes [*Ali s et al. 2011*]. Lesser degrees of glucose intolerance have also been shown to be harmful [*Yogev et al. 2010*].

In our study settings, all pregnant women were screened at 24 to 28 weeks of gestation using a 75 g glucose challenge test [*Carpenter et al. 1982*]. Those patients who have abnormal response accordingly (glucose concentrations of 140 mg/dL or higher) underwent a standard 75 g, 3-hour oral glucose tolerance test (OGTT).

If two or more of the results are higher than normal, it diagnosed with gestational diabetes [*Janzen, C.; Greenspoon, J.S. 2006]*.

All results in our study are the average results for all women who have made the study on them which are separated into two groups according to their results which are control (normal) group and GDM group for patients with gestational diabetes mellitus.

These results exhibited a significant increase in FBS levels in GDM as compared to control (healthy) group (Table 1 and Fig.1). The level of OGGT was also significantly elevated in GDM, except at the first hour which showed insignificant increase, as compared to control group. Comparing the results obtained from the two tests within each subject (either control or GDM), we found a significant elevation in blood sugar level after oral administration of sugar in both control and GDM as compared to during fasting (Fig.1). Previous reports have suggested that offering treatment to women with modest degrees of hyperglycemia in pregnancy results in a reduction in birthweight of 100–140 g [Cundy T, Ackermann E, Ryan EA 2014].

However, this increase was either inversely related to time (first hour reading is the highest and the third hour reading was the lowest) as in the control group or proportionally related to time (first hour reading was the lowest and the third hour reading was the highest) as in the GDM group.

The prevalence of GDM in our population was relatively low. Higher incidence rates for hyperglycemia in pregnancy have been reported in other regions worldwide [Guariguata L, Linnenkamp U, et al., 2014].

Fig.2. shows graphical presentation of OGGT levels in control (normal) and GDM pregnant women between 24-28 weeks in pregnancy showed comparison between the results of the two tests in each group. Means (columns) within the same groups carrying different superscript letters are significantly different ($P \leq 0.05$) based on results of one-way ANOVA and Duncan's multiple range test (DMRT). In fact, a large international cohort study designed to have the power to define risks associated with various ranges of hyperglycemia was recommended and is currently in the field [Blank A, Grave BE, et al.,1995].

These tests are made for patients to exhibit the significant increase in OGGT to obtain healthy control group and GDM group for detecting gene expression on each group of them.

The reliability of any relative RT-PCR experiment is improved by including an invariant endogenous control (reference gene) in the assay to correct for sample-to-sample variations as well as errors in sample quantification which can result in misinterpretation of the derived target genes expression profile [*Pfafflet al. 2001*]. Therefore, the abundance of each gene of interest was normalized relative to endogenous reference genes, known as housekeeping genes, which by definition have a relatively higher expression than other genes of interest maintaining a constant level of expression across samples, physiological states, and treatments [*Silver, N., et al. 2006*].

However, reference genes are not stably expressed in all scenarios as numerous studies have been shown as certain experimental conditions can influence their expression leading to incorrect findings if inappropriate housekeeping gene is used [*Jain, M., et al.2006*]. This discovery pointed out the critical importance of validating them within the specific tissue and biological conditions.

Real time PCR (qPCR) was used to detect the relative expression of adipokine genes such as Leptin (*Lep*), Adiponectin (*Adipo*), Adiponectin receptor 1 (*AdipoR1*), Adiponectin receptor 2 (*AdipoR2*), and Resistin (*Retn*) genes in the blood that reflects the changes in transcription levels of these genes in pregnant women with GDM as compared to control (normal).

To conduct real time PCR, we first isolated total RNA from blood of all subjects. After extraction, the quality and concentration/ of total RNA were assessed

by Nanodrop which revealed presence of pure RNA with a considerable higher concentration (ranged from 1490 to 2160 ng/µl) (Fig. 3).

Nanodrop curve in fig.3 showing concentration and purity of extracted RNA from a representative sample which is 1810 ng/µl. In this curve the upper top presents at 260 and the bottom at 230 which indicates the presence of pure RNA.

The isolated total RNA was reverse transcribed into cDNA which was used as a template for qPCR. Throughout the whole real time PCR experiment, the housekeeping gene encoding *GAPDH* was used as an internal reference for normalization and data was expressed as mean ± SEM (n = 3 in triplicate in each group). The expression level of the target gene in the control group was considered the base line.

As a starting we talk about leptin for gene expression. Leptin is an adipocyte-derived hormone, which is produced by some tissues, including the stomach, intestine, and the placenta in humans [*Masuzaki H, et al. 1997*], and acts on the receptors of the hypothalamus to decrease food intake and increase energy consumption [*Zhang Y,1994*].

Some studies said that leptin also has a main effect on the regulation of whole body glucose homeostasis [*Ceddia RB, et al. 2002, Al-Dahhri N,et al. 2002*].

Some clinical studies imply that increasing maternal plasma leptin may result from an upregulation of adipocyte leptin synthesis in the presence of increasing insulin resistance and hyperinsulinemia in the second half of pregnancy [*Laivuori H, et al. 2000*]. The findings of some studies indicate that leptin has an acute inhibitory effect on secretion of insulin [*Ceddia RB, et al. 2002*].

The obtained qPCR results revealed significant (P≤0.05) upregulation (3.41 fold increase) of *Lep* gene expression level in blood of GDM group as compared to control group (Table 3 and Fig. 4).

Table 3. shows changes in relative expression of *Lep* gene in blood of control and GDM groups. Data expressed as mean ± standard error of mean (SEM). Means within the same column carrying different superscript symbols are significantly different at **** P≤ 0.0001 as revealed by student *t* test.

The fold change shows that the control gene always equals one because we have an equation for them calculates this fold change have been described in materials and methods because they are normal it shows an equivalent result true one and for GDM patients shows higher change and SEM shows that this result is significant for the study and the Leptin gene expression increases in GDM patients and higher than normal ones.

We meant to explore the relationship of leptin gene expression in GDM women and healthy pregnant women which is have shown it in figure (4) and showed the relative expression of leptin gene and the high difference between control group and GDM group which showed the increased expression in GDM group.

Women with GDM had higher serum leptin concentrations than non-GDM women. Our results are generally agreed with studies that assessed leptin expression in pregnancies complicated by GDM higher than control group. In a case-control study [*Kautzky-Willer A, et al. 2001*] reported that leptin is higher in GDM women compared with the control group.

Such a relation was also found in the [*Vitoratus et al 2011*] and [*Qiu et al 2004*] studies. Liu [*Liu et al. 2003*] showed that serum leptin level is correlated with glucose tolerance during pregnancy.

Most studies have found increased leptin concentrations in GDM [*Chen D, et al 2010, Highman TJ et al.1998*] . Leptin increases insulin sensitivity by influencing insulin secretion, glucose utilization, glycogen synthesis and fatty acid metabolism, regulates gonadotrophin releasing hormone secretion from the hypothalamus and activates the sympathetic nervous system [*Chen D, et al 2010, Hauguel-de Mouzon S, et al.2006*] .

GDM is characterized by an amplification of the low-grade inflammation already existing in normal pregnancy [*Ouchi N, et al. 2011*].

The increased leptin may adversely affect the outcome of pregnancy and women who have gestational diabetes mellitus (GDM) are at a greater risk of developing type 2 diabetes mellitus (type 2 diabetes) within 10-20 years of their index pregnancy [*Zavalza-Gómez AB, et al. 2008]*.

However, Festa et al [*Festa, A., et al. 1999*], in an earlier case-control study, noted that maternal third-trimester leptin is significantly lower in GDM cases as compared with controls after adjusting for possible confounding factors, such as BMI and insulin concentrations.

Maghbooli [*Maghbooli, Z., et al. 2007*] found that leptin concentration was positively associated with insulin level and HOMA index.

Liu [*Liu ZJ, Liu PQ, et al. 2003*] showed a positive and significant correlation between the maternal leptin and fasting insulin levels.

Our results indicate that Leptin gene expression are higher in GDM compared with the normal group. However, Mohiti et al [*Mohiti J, Afkhami M, Babaei A. 2005*] reported that the serum leptin had a negative correlation with insulin in obese diabetic patients.

Liu et al [*Liu ZJ, Liu PQ, et al. 2003*] also has shown that leptin predicts the development of GDM independent of maternal BMI and other risk factors. The findings of this prospective study are generally consistent with the reports by Kautzky-Willer et al [*Kautzky-Willer A, Pacini G, et al. 2001*], Lappas et al [*Lappas M, Yee K, Permezel M, Rice GE 2005*] and Qiu et al [*Qiu C, Williams MA, et al. 2004*].

In conclusion, we found that patients with GDM had higher leptin expression than non-GDM group.

These results show the presence of an association between leptin expression and glucose metabolism in GDM.

As to whether the high leptin gene expressions can serve as a predictive factor for GDM development, larger prospective studies are needed to address this issue.

Table (4) shows relative expression of genes between Adiponectin gene expression results in normal control group and GDM group and standard error of the mean (SEM) and figure (5) shows relative expression of Adiponectin gene to GAPDH gene shows Noticeable decrease in adiponectin in pregnancy with GDM compared to their matched healthy pregnant female groups.

The obtained qPCR results revealed significant ($P \leq 0.05$) downregulation (3.41 fold increase) of *Adipo* gene expression level in blood of GDM group as compared to control group (Table 4 and Fig. 5).

The levels of adiponectin were lower in GDM patients and it was negatively correlated with blood sugar levels. Adiponectin is considered an insulin sensitizing, anti-inflammatory and anti-atherogenic adipokine [*Lain KY, Daftary AR,et al. 2008*].

In agreement with this finding, lower adiponectin gene expression was noted in GDM [*Chen J, Tan B, et al. 2006*]. Different studies had reported decreased maternal adiponectin levels in GDM independent of maternal BMI and insulin sensitivity [*Lappas M, et al. 2005 , Cortelazzi D,et al. 2007*]. One study [*Lappas M, et al. 2005*] however reported no significant difference in the release of adiponectin between GDM and normal pregnant women.

Other studies had examined adiponectin in GDM and healthy pregnants. Adiponectin was noticeably but not statistically lower in GDM compared to normal pregnancy [*Ranheim T, et al. 2004*]. Thus, insulin might be able to suppress plasma adiponectin concentrations.

Our results indicated agreement with these studies which told that adiponectin gene expression in GDM is lower than normal group.

A previous study reported progressive decline in maternal adiponectin secretion during the course of pregnancy which was associated with a 60 % reduction in adiponectin mRNA in white adipose tissue [*Catalano PM, Hoegh M,et al. 2006*]. Reduced adiponectin levels in second and third trimesters compared to first trimester in healthy pregnant subjects was reported by many authors [*Catalano PM, Hoegh M, et al. 2006, Cseh K, Baranyi É,et al. 2004*], although others reported unchanged values [*Mastorakos G, et al. 2007, Cortelazzi D, et al. 2007*].

Expression of Adiponectin Receptor 1 to GAPDH gene are in the same range slightly high in patients with GDM compared with controls as revealed insignificant ($P \leq 0.05$) upregulation of *AdipoR1* gene expression level in blood of GDM group as compared to control group (Table 5 and Fig. 6) as the SEM shows and in agreement with most studies on AdipoR1 expression.

Adiponectin receptor 1 (AdipoR1) is a protein which in humans is encoded by the ADIPOR1 gene.[*Yamauchi T, et al. 2003*] It is a member of the progestin and adipoQ receptor family (PAQR), and is also known as PAQR1.*[Tang YT, Hu T, et al. 2005*].

Table 6 and figure 7 shows amplification curves comes from the device as an example on our work for Leptin, Adiponectin, and adiponectin Receptor1 in linear (upper) view and log (lower) view which represents CT values of them in different groups which calculate from it the fold change what got from it the expression result and if it significant or not.

Adiponectin receptor 2 (AdipoR2) is a protein which in humans is encoded by the ADIPOR2 gene.[*Yamauchi T, Kamon J,et al. 2003]* It is a member of the progestin and adipoQ receptor (PAQR) family, and is also known as PAQR2.*[Tang YT, Hu T, Arterburn M, et al. 2005*]

Adiponectin Receptor 2 showed in table 7 relative expression of genes between Adiponectin Receptor2 gene expression results in normal control group and GDM group and standard error of the mean (SEM) **which** also explained in Figure 8 in relative expression of AdipoR2 gene/GAPDH gene and explained as non-significant for gene expression and GDM examination.

Figure 9 is for linear (upper) and log (lower) amplification curves representing the Ct values of *AdipoR2* genes in different groups which have got from it the fold change and CT values and also after this the SEM which notice if the result is significant or not as shown in table 8.

The last expression is for Resistin gene to GAPDH gene which upregulation (2.35 fold increase) of *Retn* gene expression level in blood of GDM group as compared to control group (Table 9 and Fig. 10).

Fig.11.The linear (upper) and log (lower) amplification curves representing the Ct values of *Retn* genes in different groups as in table 10. It shows relative expression of genes between Resistin gene expression results in normal control group and GDM group and standard error of the mean (SEM) which explained the significant difference between control group and GDM group which became high level in GDM.

These results are in agreement with many studies. Serum resistin was reported to be higher in GDM compared to normal pregnancy at their 2nd trimester [*Kuzmicki M, et al. 2009*]. Other researchers indicated that despite markedly higher glucose and insulin levels, resistin levels did not differ significantly in pregnant women with GDM and healthy pregnant women at term [*Lappas M, Yee K, Permezel M, Rice GE 2005*].

In human, it was suggested that resistin mRNA expression in adipocytes is increased in obese subjects [*Savage DB, et al. 2001*], but other report was unable to detect any association between obesity and resistin expression [*Janke J, et al. 2002*]. According to this finding it seems that both obesity and increased resistin concentrations may be important factors in the increased insulin resistance accompanying GDM.

A number of studies have found insulin to be a key regulator of resistin gene expression [*Mazaki-Tovi S, et al. 2009, Telejko B, Kuzmicki M, et al. 2007*].

In our study, maternal serum resistin was significantly elevated in GDM women compared to their matched control. These results are in agreement with many studies. Serum resistin was reported to be higher in GDM compared to normal pregnancy at their 2nd trimester [*Kuzmicki M, et al. 2009*]. Other researchers indicated that despite markedly higher glucose and insulin levels, serum resistin

levels did not differ significantly in pregnant women with GDM and healthy pregnant women at term [*Lappas M, Permezel M, Rice GE.2005*].

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Summary

Summary

Expression of adipokine genes associated with gestational diabetes mellitus"

Gestational diabetes is a particular type of diabetes which some women develop during pregnancy. It usually disappears after the birth, and does not mean that the baby will be born with diabetes. It caused by hormonal changes in pregnancy which can change the body's ability to use a substance called insulin. It defined as a carbohydrate intolerance of varying severity, is the most frequent metabolic disorder of pregnancy, affecting 1-10% of all pregnancies . Although most of the women with GDM return to normal glucose tolerance after delivery, they have an increased risk of developing diabetes, mainly type 2 diabetes mellitus (DM2), later on, with an incidence ranging from 6-62 %, depending on the population examined and the length of follow up considered.

Adipokines comprise a variety of proteins with signaling properties produced in body fat. In molecular biology, the polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints

(used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases.

The purpose of this study was to assess the expression profile of genes with potential role in the development of insulin resistance (adipokines).

This study is made on 50 pregnant women classified into two groups:

first group is the control group who are 15 healthy pregnant women and the second group is for pregnant women who are patients of gestational diabetes mellitus (GDM).

All women enrolled into the study underwent the 75 g 3hr oral glucose tolerance test (oGTT) between 24th and 28th week of their pregnancy. Expression of Leptin, Resistin and Adiponectin and Adiponectin receptors 1&2 was determined by real-time polymerase chain reaction (RT-PCR).

Leptin gene expression is higher in patients with GDM than in normal pregnant women. Expression of adiponectin genes in pregnant women with GDM decreases in comparison with their matched healthy pregnant group. Adiponectin Receptor 1 is an the same range in both groups with slight high in GDM compared with controls and seems nonsignificant like Adiponectin Receptor 2 slight decrease in patients with GDM than normal ones. The last expression for Resistin gene is significantly elevated in GDM women compared to their matched controls.

Conclusion And Recommendation

Conclusion and Recommendation

From the present study, it could be concluded that all studied adipocytokines showed remarkable variations in their levels between GDM and control subjects. Increased insulin resistance that accompanies pregnancies complicated with GDM is associated with suppressed leptin and increased Resistin compared to normal pregnancy, suggesting strongly their association with insulin resistance in GDM and are significant for study and decrease in adiponectin also is strong sign but AdipoR1 and AdipoR2 are non-significant because of their converged for their gene expressions.

It is recommended to extend the present study to explore the relationship between levels of the studied adipocytokines and the onset of glucose metabolism disorder and on more number of patients for more variation.

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الملخص العربي

دلائل جينات الاديبوكين وعلاقتها بمرضى سكر الحمل

سكرى الحمل هو نوع خاص من مرض السكرى الذى يظهر على بعض النساء خلال فترة الحمل و عادة ما يختفى بعد الولادة , وهذا لا يعنى ان الطفل سوف يولد مع مرض السكرى .فمن التغيرات الهرمونية التى يمكن ان تسببها فترة الحمل تغير قدرة الجسم على استخدام مادة تسمى الانسولين. داء سكرى الحمل يظهر بنسبة (1-10 %) من جميع حالات الحمل وهو المرض الاكثر شيوعا فى اضطرابات عملية الايض من الحمل.

على الرغم من ان معظم النساء تصاب بداء السكرى من النوع 2 خلال الحمل نسبتهم فى الاصابة اكبر فى وقت لاحق على عكس الطبيعيين يعودون الى النسبة الطبيعية للجلوكوز بعد الولادة و تتراوح هذه النسبة بين(6-62 %) اعتمادا على دراسة السكان وطول متابعة النظر.

الاديبوكينيز هى روابط جزيئية بين البدانة و تصلب الشرايين وهى تضم مجموعة متنوعة من البروتينات في البيولوجيا الجزيئية ، وتفاعل البوليميريز المتسلسل .معاشارة خصائص المنتج للدهون فى جسم الانسان. (الاسترداد) هو تقنية لتضخيم على نسخة واحدة أو عدد قليل من قطعة من الحمض النووي عبر عدة أوامر من وتعتمد الطريقة على الدراجات .حجمها ، وتوليد آلاف إلى ملايين النسخ من تسلسل معين الحمض النووي. الحرارية ، والتي تتكون من دورات متكررة من التدفئة والتبريد في رد فعل للذوبان الحمض النووي وتكرار الأنزيمية من الحمض الريبي النووي.

الاسترداد الآن المشتركة التي لا غنى عنها في كثير من الأحيان ، والتقنية المستخدمة في المختبرات الطبية والبيولوجية لبحث مجموعة متنوعة من التطبيقات، وتشمل هذه استنساخ الحمض النووي للتسلسل ، نسالة المعتمدة على الحمض النووي ، أو تحليل وظيفي للجينات ، وتشخيص الأمراض الوراثية ، وتحديد البصمات .الجينية (المستخدمة في علوم الطب الشرعي واختبار الأبوة) ؛ واكتشاف وتشخيص الأمراض المعدية.

والغرض من هذه الدراسة هو تقييم لمحة تعبير جينات مع دور محتمل في تطوير مقاومة الاديبوكينيز للأنسولين.

هذه الدراسة تمت علي 50 سيدة حامل تم تقسيمهم الي مجموعتين المجموعة الاولي هي مجموعة الفحص و التحكم ل 15 سيدة سليمة و المجموعة الثانية ل 35 سيدة حامل مريضة بسكر الحمل.

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جميع السيدات في هذه الدراسة تم لهم عمل اختبار تحمل الجلوكوز ما بين الاسبوع 24 الى ال28 من الحمل.التعبير عن اللبتين و الريسيستين و بروتين الاديبونكتين ومستقبل الاديبونكتين1 و 2 تم تحديدهم باستخدام الوقت الحقيقى لسلسلة البوليميريز.

النتائج:

التعبير الجيني للبتين اعلي عند مرضي سكر الحمل عن الحوامل السليمة.التعبير الجيني للاديبونكتين جين للسيدات الحوامل المرضي بسكر الحمل يقل مقارنة بالحوامل السليمة. جين مستقبلات الاديبونكتين الاول تقريبا في نفس النسبة للمجموعتين مع زيادة بسيطة في السيدات الحوامل المرضي بسكر الحمل عن المجموعة السليمة اما مستقبلات الاديبونكتين جين رقم اثنين يقل بنسبة بسيطة عن المجموعة السليمة.اخر تعبير جيني هو لجين الريسستين وهو يزيد ايضا بنسبة عالية عند السيدات المرضي بسكر الحمل عن الحوامل السليمة.

جـــامعة مدينة السادات
معهد بحوث الهندسة الوراثية
والتكنولوجيا الحيوية
قسم المشخصات الجزيئية

" دلائل جينات الاديبوكين و علاقتها بمرضى سكر الحمل"

للحصول على درجة الماجستير فى التشخيص الجزيئى لأمراض الأنسان

رسالة مقدمة من

سلمى صلاح محمود يوسف الخولى

بكالوريوس علوم جامعة جنوب الوادى فرع اسوان (2009)

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