

Resitin and adiponectin in the Bahraini population : plasma levels, gene polymorphisms and their association with type 2 diabetes and related metabolic parameters.

AUTHOR(S)

Fatima Al Hannan

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Resistin and Adiponectin in the Bahraini Population: Plasma Levels, Gene Polymorphisms and their Association with Type 2 Diabetes and Related Metabolic Parameters

By

Fatima Al Hannan, B.Sc. (1st class honours).

School of Medicine

Royal College of Surgeons in Ireland- Medical University of Bahrain

A thesis submitted to the National University of Ireland for the degree of Master of Science (Research)

Under the supervision of:

Dr. Kevin Culligan, School of Medicine, RCSI Bahrain

Dr. Maria Morgan, Molecular & Cellular Therapeutics, RCSI Dublin

Dr. Paul O'Farrell, School of Medicine, RCSI Bahrain

Dr. Orna Tighe, Molecular & Cellular Therapeutics, RCSI Dublin

April, 2012

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree <Master of Science>, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed

Fatima Al Hannan

RCSI Student Number

09102221

Date

01/04/2012

*To my parents,
to Hameed,
and to my little angel Hussain
This work is dedicated to you....*

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Abstract

Low concentrations of adiponectin, the protein product of the adiponectin gene (ADIPOQ), and high concentrations of resistin have been reported to be associated with obesity and insulin resistance. Several adiponectin gene polymorphisms have been described, and their association with obesity, type 2 diabetes mellitus (T2DM), and metabolic syndrome, in different populations and sample types, is controversial. The purpose of the present study was to investigate the association of the two most well-known single nucleotide polymorphisms (SNPs) of ADIPOQ (+45T>G and +276G>T) with serum adiponectin concentrations, metabolic parameters associated with diabetes, and risk of T2DM in the Bahraini population. We also aimed to investigate the associations between serum adiponectin and resistin levels with the metabolic parameters and the prevalence of T2DM in Bahraini population.

We performed a cross-sectional study using a representative sample of 140 unrelated Bahraini patients with T2DM and 66 non-diabetic healthy subjects. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to determine the distribution of allele and genotype frequency of the SNP +45T>G polymorphism (exon2) and SNP +276G>T polymorphism (intron 2) in ADIPOQ. Lipid profile was measured by enzymatic methods. An ELISA was used to determine serum adiponectin and resistin levels.

It was observed that obesity, insulin resistance and T2DM are associated with low serum adiponectin levels. Compared with the control group, the T2DM group exhibited lower adiponectin levels and higher resistin levels. The G allele and TG/GG genotype of SNP +45T>G occurred more frequently than the common T allele and TT genotype in T2DM patients compared to the controls ($P<0.05$). Subjects with the GG/TG genotype of SNP +45T>G were associated with lower serum adiponectin levels. There was no statistically significant difference in allele and genotype frequencies of SNP +276G>T comparing control group with T2DM group.

No association with metabolic parameters was detected with either of the SNPs. In summary, our results demonstrated that, adiponectin SNP +45T>G, rather than SNP +276G>T is more associated with adiponectin levels. However, we could not confirm an association of these two SNPs with metabolic parameters of the metabolic syndrome.

Keywords: adiponectin; resistin; ADIPOQ; single nucleotide polymorphisms; SNP +45T>G; SNP +276G>T; metabolic parameters; insulin resistance; T2DM; metabolic syndrome

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List of Abbreviations

%BF	Percentage of body fat
ACRP 30	Adipocyte complement-related protein of 30 kDa
ADIPOQ	Adiponectin gene
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
ADSF	Adipocyte Secreted Factor
AMP	Adenosine monophosphate-activated protein
APM1	Adipose most abundant gene transcript
BAT	Brown adipose tissue
BDF	Bahrain Defense Force hospital
BIA	Bioelectrical impedance analyzer
BMI	Body mass index
bp	Base pair
cm	Centimeter
CV	Coefficient of variation
DBP	Diastolic blood pressure
dH ₂ O	Distilled water
dNTPs	deoxynucleoside triphosphates
ELISA	Enzyme-linked immunosorbent assay
FBG	Fasting blood glucose
FISH	Fluorescence in situ hybridization
FIZZ3	Found In Inflammatory Zone 3
FWD	Forward
GBP28	Gelatin binding protein of 28 KDa
Hb A1c	Glycated hemoglobin
HDL-C	High density lipoprotein- Cholesterol
HMW	High molecular weight
HOMA- IR	Homeostatic model assessment – insulin resistance
IFG	Impaired fasting glucose
IL-6	Interleukin-6
IR	Insulin resistance

IVGTT	Intravenous Glucose Tolerance Test
kDa	KiloDalton
Kg	Kilogram
LD	Linkage disequilibrium
LDL-C	Low density lipoprotein cholesterol
LE	Low electroendosmosis
LMW	Low molecular weight
m ²	Square meter
mA	miliAmber
MAF	Minor allele frequency
MDD	Minimum detectable dose
MetS	Metabolic Syndrome
MgCl ₂	Magnesium Chloride
MMW	Middle molecular weight
mRNA	Messenger RNA
NEFA	Non-esterified fatty acid
OD	Optical density
OGTT	Oral glucose tolerance test
PCR	polymerase chain reaction
PPAR- γ	Peroxisome proliferator-activated receptor gamma
RBC	Red blood cells
RBS	Random blood sugar
REV	Reverse
RFLP-PCR	Restriction fragment length polymorphisms polymerase chain reaction
SA index	HMW adiponectin/total adiponectin index
SBP	Systolic blood pressure
SDS	sodium dodecyl sulphate
SNP	Single Nucleotide Polymorphisms
T2DM	Type 2 Diabetes Mellitus
TAE buffer	Tris/Acetate/EDTA buffer
TBE buffer	Tris/Borate/EDTA buffer
TC	Total cholesterol

TEMED	N, N, N', N'- Tetramethylethylenediamine
TEN solution	(Tris/EDTA/ NaCl) solution
TG	Triglyceride
TNF- α	Tumor Necrosis Factor-alpha
TZDs	Thiazolidinediones
UV	ultraviolet
V	Volts
WAT	White adipose tissue
WBC	White blood cells
WC	Waist circumference
WHR	Waist-to-hip ratio
WHTR	Waist-to-height ratio

Chapter 1- Introduction

1.1 Type 2 Diabetes Mellitus

Type 2 Diabetes mellitus (T2DM) is a major public health problem worldwide. It is particularly prevalent among Gulf states countries (Haslam and James, 2005). Five Arabian Gulf countries are among the world's ten highest for diabetes prevalence. These countries are Kuwait, Oman, Saudi Arabia, United Arab Emirates and Bahrain which is ranked the third (International Diabetes Federation, 2009). Al-Mahroos and McKeigue performed the first cross sectional survey in Bahrain to detect the prevalence of diabetes mellitus among Bahraini natives. A total of 2128 Bahrainis, aged between 40 and 69-years participated in the study. Based on the study, the total rate of diabetes among the Bahraini population was 30%. The highest numbers of diabetics were seen in the 50-59 years age group in which the total prevalence of diabetes was 29% in men and 35% in women. The results showed that about 35% of Bahraini diabetics between the ages of 40 and 69 years were undiagnosed previously (Al-Mahroos and McKeigue, 1998). In 2002, it was estimated that 9% of total deaths in Bahrain were due to diabetes (WHO, 2002).

Figure 1.1 shows the pathogenesis of type 2 diabetes mellitus. T2DM is characterized by peripheral insulin resistance coupled with a failure of pancreatic β -cells to produce compensatory levels of insulin and typically accompanies advancing age, inactivity, and weight gain (Stumvoll et al., 2005). Low levels of circulating insulin results in decreased glucose absorption into the cells leading to hyperglycemia and eventually to T2DM. In addition to the metabolic and environmental causes, T2DM has a strong genetic component. Kaprio *et al.* studied the cumulative incidence, concordance rate and heritability for diabetes mellitus in a nationwide cohort of 505 type 2 (non-insulin-dependent) diabetes twins. The concordance rate in monozygotic twins was 34% whereas in dizygotic twins it was 16% (Kaprio et al., 1992). In another Japanese study which included 87 pairs of twins, these figures were 83% for monozygotic twins and 40% for dizygotic twins (Committee on Diabetic

Twins, 1988). The authors concluded that these figures imply a high degree of heritability for T2DM as well as the involvement of environmental factors. Further evidence for a genetic role is suggested by the high prevalence of the disease in particular ethnic groups and its modification by genetic admixture (Stern and O'Connell, 1999). The extent to which multiple genes and the environment impact on disease predisposition and progression has been studied widely through association studies, either of individual genes (candidate gene approach) or using high-density single nucleotide polymorphisms (SNPs) randomly spaced across the entire genome (genome-wide association study). Recently, a number of genes and polymorphisms have been extensively studied and reproducibly associated with T2DM in a variety of studies, reviewed in (McCarthy, 2010).

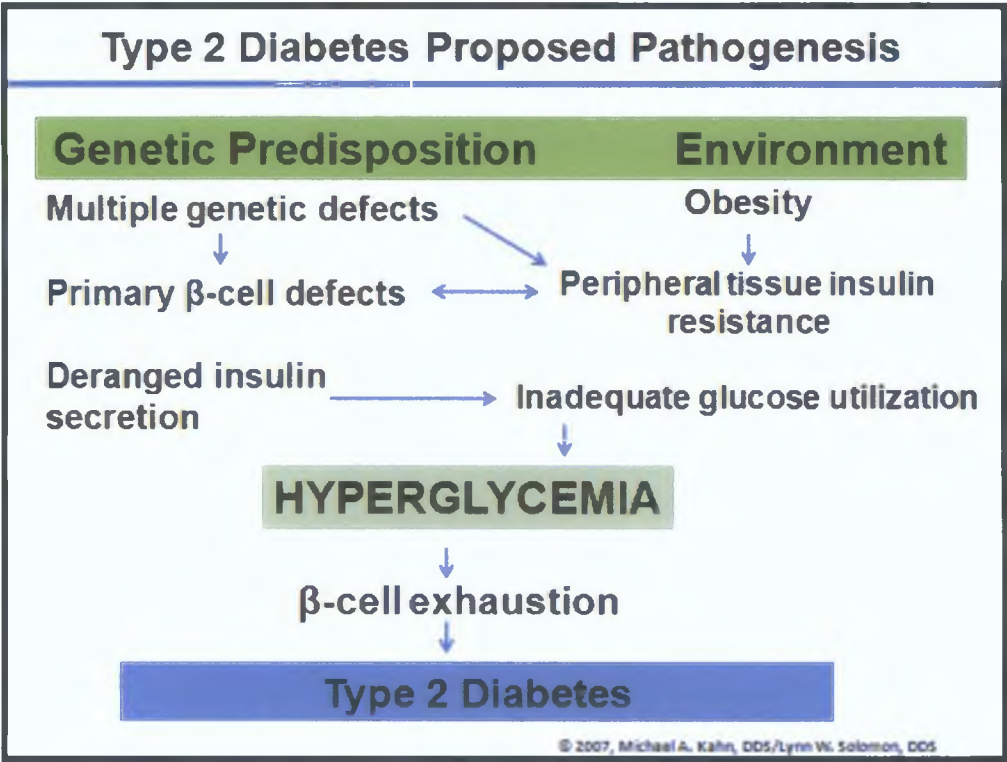


Figure 1.1 Pathogeneis of type 2 diabetes mellitus. Digram adapted from: <http://ocw.tufts.edu/Content/51/lecturenotes/673764/674515>

1.2 Insulin Resistance

insulin resistance is considered the core factor in the pathogenesis of T2DM. It is often associated with a wide array of other pathophysiologic disorders including, obesity, dyslipidemia, hypertension and atherosclerosis. The coexistence of these diseases, termed the metabolic syndrome or syndrome X, was first described in 1988 (Reaven, 1988). Insulin resistance is characterized by the inability of insulin to produce its numerous actions in maintaining a normal blood glucose level, independent of the impaired secretion from the pancreatic β -cells (Flakoll et al., 2004, Reaven, 2004).

Insulin resistance occurs by various genetic and acquired conditions such as a high fat diet, reduced physical activity, and "glucose toxicity" due to hyperglycemia (Hirose et al., 2010). Impairments in cellular events, distal to the interaction between insulin and its surface receptor, through alterations in the activities of signaling molecules, enzymes, and transcription factors are known to be the major cause (Reaven, 1988, Reaven, 1995, Saltiel and Kahn, 2001). Antibodies against the insulin receptor (Kawanishi, 1977, Kobayashi, 1992) or mutations in the insulin receptor gene (Taylor et al., 1990, Taylor et al., 1991, Kadowaki and Kasuga, 1991) have also been reported as possible causes.

Several studies emphasize the importance of insulin resistance in the pathogenesis of T2DM. In a recent prospective study of 6538 British civil servants without diabetes at baseline, 505 subjects were diagnosed with diabetes during a median follow-up period of 10 years (Tabak et al., 2009). Those who developed diabetes showed a marked decrease in insulin sensitivity during the five years prior to diagnosis compared with those who did not develop the disease. Insulin secretion from β - cells increased three to four years prior to diagnosis and then decreased until diagnosis. This highlights the importance of the quantification of insulin resistance in clinical practice since this metabolic state is a treatable precursor of T2DM (Weyer et al., 1999). Established direct methods to

quantify insulin sensitivity, such as the hyperinsulinemic euglycemic clamp have been described. In this procedure, insulin is administered at a constant rate to raise the insulin concentration while glucose is infused to maintain euglycemia. The glucose infusion rate needed to maintain euglycemia is a reflection of insulin action. Insulin sensitivity could be estimated using other methods, such as the minimal model which consists in a mathematical analysis of frequently sampled labeled glucose, given as part of an intravenous glucose tolerance test (IVGTT) (Robert, 1995). The homeostatic model assessment-insulin resistance (HOMA- IR) was also developed as a surrogate measurement of *in vivo* insulin sensitivity by Matthews *et al.* in 1985 (Matthews et al., 1985). HOMA- IR is an empirical mathematical formula based on measuring fasting plasma glucose levels and fasting plasma insulin levels:

$$\text{HOMA-IR} = \frac{\text{fasting plasma insulin } (\mu\text{IU/ml}) \times \text{fasting plasma glucose (mmol/l)}}{22.5}$$

HOMA-IR value of 3 or lower is considered normal. Any values above 7 to 10 is considered insulin resistance (Lavin, 2009).

1.3 Obesity

Obesity poses an emerging global health care problem and is considered a major risk factor in the development of diabetes and cardiovascular disease. Al-Mahroos and Al-Roomi (2001) conducted a cross-sectional national epidemiological community survey involving 2013 Bahraini subjects aged 40-69. The age-standardized prevalence rate among native Bahraini men and women was high. Approximately 32% of women and 25% of men were obese (Body mass index (BMI) $\geq 30.0 \text{ kg/m}^2$) (Al-Mahroos and Al-Roomi, 2001). Obesity has been defined as increased adipose tissue mass due to chronic imbalances between energy intake and expenditure (Tataranni and Ravussin, 2002). There are two types of adipose tissue: white adipose tissue (WAT) which takes up the vast majority of energy and is thought to be the site of energy storage, and

brown adipose tissue (BAT), which is mainly found in human neonates and is important for the regulation of body temperature. White adipose tissue contains adipocytes, which are the most abundant cell type, endothelial cells, fibroblasts, leukocytes and most importantly macrophages whose number directly correlates with obesity (Weisberg et al., 2003). WAT can be further divided, according to its body distribution, into visceral and subcutaneous adipose tissue (Avram et al., 2005). The relative importance of each sub-type of WAT in producing insulin resistance is under debate in current literature (Dolinkova et al., 2008, Gomez-Ambrosi et al., 2004, Murdolo et al., 2008). However, strong clinical evidence indicates that visceral (android) obesity, a condition where adipose tissue preferentially accumulates in the mesenteric region, is more related to insulin resistance and the progression to T2DM than the peripheral (gynecoid) type (Wajchenberg, 2000).

The degree of obesity is usually estimated by the use of simple anthropometric parameters (body mass index (BMI) which is calculated as weight (Kg) divided by height squared (m^2), waist circumference (WC), waist-to-hip ratio (WHR), and waist-to-height ratio (WHTR) or/and the use of percentage of body fat (%BF) mass. WC and WHR have been used to estimate abdominal fat accumulation as components of the metabolic syndrome (Grundy et al., 2005), and WHTR has also been reported as a measurement strongly associated with cardiovascular risk factors (Schneider et al., 2007). However, no consensus exists for specific obesity indicator associated with diabetes and metabolic risk factors. Some studies reported that simple anthropometric parameters were more strongly related to those risks compared to the measurement of body fat composition for example (Bosy-Westphal et al., 2006), while others showed that %BF was a better predictor of risks compared to anthropometry (Kobayashi et al., 2006).

To find the best obesity indicator for obesity-related metabolic risk factors, Lee *et al.* studied a total of 995 healthy Korean women and 577 healthy Korean men. Anthropometric measurements including BMI, WC,

WHR, and WHTR were estimated. Direct body composition measures including the percentage of body fat (%BF) measured by dual-energy X-ray absorptiometry scanners and bioelectrical impedance analyzer (BIA) were also used. They found that WC, WHTR, and BMI were consistently associated with all metabolic risk factors regardless of the subject's gender. Abnormal metabolic risk factors were significantly higher for these three indicators of obesity than for %BF. Their study validates the usefulness of anthropometry over direct body fat measures to predict metabolic risks (Lee et al., 2008). The same results were confirmed in a recent study which included 1,518 Peruvian adults. Knowles *et al.* examined the extent to which measures of adiposity can be used to predict selected components of metabolic syndrome (MetS). WC, BMI, WHR, and WHTR, were examined. They found that for both genders, as adiposity increased, the prevalence of MetS components increased. The authors showed that men and women with high-BMI and high- WC had elevated levels of fasting glucose and triglyceride (TG), high blood pressure, and reduced high density lipoprotein (HDL) compared to individuals with low-BMI and low-WC (Knowles et al., 2011).

Circumstantial and experimental evidence indicate that obesity causes hyperinsulinemia and insulin resistance in different proposed ways. Initially, important observations demonstrated that fat metabolism is extensively impaired with excess weight. Non-esterified fatty acids (NEFA) delivered from adipose tissue by enzymatic cleavage of triglycerides are found at increased concentrations in blood of obese individuals and are associated with a higher risk of developing T2DM (Charles et al., 1997). Experimental elevations of NEFA have been shown to induce insulin resistance in animal models and humans (Roden et al., 1996). In addition, Piro *et al.* have shown that chronic exposure to elevated free fatty acid increases apoptosis in rat pancreatic islets and these cytotoxic effects could be mediated by oxidative stress (Piro et al., 2002). However, the most commonly accepted theory is that obesity is associated with a state of chronic, low grade inflammation, suggesting that inflammation may be a potential mechanism whereby obesity leads

to insulin resistance (Schenk et al., 2008, Hotamisligil, 2006, Rocha and Libby, 2009). Trayhum and Wood have revealed that the initiation of hypoxia, which develops as fat mass within the adipose tissue outgrows its vascular supply, leads to the stimulation of the release of inflammatory cytokines, chemokines and angiogenic factors, the function of which is to increase blood flow and stimulate vascularization (Trayhum and Wood, 2004). Increased levels of markers and mediators of inflammation, acute-phase reactants and white cell count have been seen to correlate with incident T2DM (Tataranni and Ortega, 2005, Spranger et al., 2003) and cause insulin resistance in experimental models (Shoelson et al., 2006).

1.4 Adipokines

The biologic role of the adipose tissue has dramatically changed over the last decade. Once considered an inert energy depot, adipose tissue is now known to serve as an active endocrine and paracrine organ, secreting an ever increasing number of mediators known as adipokines (Trayhum et al., 2006). These secreted proteins, which now amount to more than 50 different molecular entities, influence body weight homeostasis and participate in diverse metabolic processes including glucose and lipid metabolism (Antuna-Puente et al., 2008). Table 1.1 summarizes some of the adipokines secreted by adipose tissue and their metabolic functions.

Table 1.1 Adipokines function and their role in metabolism

(Ikeoka et al., 2010, Rabe et al., 2008, Ronti et al., 2006)

Adipokines	Function, characteristics, target tissues, effects on metabolism
Tumor Necrosis Factor-alpha (TNF- α)	Mainly produced by inflammatory cells and lymphocytes, but also by adipocytes and stromal cells. Stimulates release of free fatty acids by lipolysis, reduces adiponectin synthesis and impairs insulin signaling.
Interleukin-6 (IL-6)	Produced by inflammatory cells, lymphocytes and adipocytes. Reduces sensitivity to insulin by inhibiting gene transcription. Blood levels correlate with body weight.
Adiponectin	Secreted mainly by adipocytes. Circulating levels are reduced in obesity and positively correlate with insulin sensitivity. Suppressed by TNF- α , IL-6. Induces tyrosine phosphorylation of the insulin receptor and reduces gluconeogenesis in the liver. Increases fatty acid oxidation in the liver.
Leptin	Stimulated by insulin and TNF- α . Exclusively synthesized and secreted by adipocytes. Inhibits appetite and reduces food consumption. Is found at elevated levels in obese subjects. Influences the reproductive functions. Reduces insulin- mediated glucose uptake.
Resistin	Induction of insulin resistance in mice. Lack of clear function in glucose metabolism in humans. Elevated in plasma of obese individuals. Induces endothelial dysfunction and can be involved in the genesis of atherosclerosis.
Adipsin	Enhances triglyceride storage in adipose cells through stimulation of glucose transport, enhances fatty-acid re-esterification and inhibits lipolysis.
Visfatin	Expressed and secreted in adipose tissue. Reduces blood glucose levels, acting as an insulin-like hormone.

Adipokines have physiological effects on a multitude of metabolic pathways and their dysregulation caused by hypertrophic adipocytes is known as a potential contributor to insulin resistance in humans (Kershaw and Flier, 2004). Lee *et al.* investigated the effect of adipocyte size on adipokine production and secretion in cultured adipocytes, separated according to cell size, from 30 individuals undergoing elective plastic surgery. The results clearly suggested that adipocyte size is an important determinant of adipokine secretion. There seems to be a differential expression of pro- and anti-inflammatory factors, with increasing adipocyte size. Dysregulation of hypertrophic, very large cells results in a shift toward dominance of pro-inflammatory adipokines (Lee *et al.*, 2009). The discovery of such endocrine function of the adipose tissue has prompted the hypothesis that some adipose tissue-derived hormones, such as adiponectin and resistin, represent a possible connection between obesity, insulin resistance and other components of the metabolic syndrome, such as glucose intolerance, dyslipidemia, and high blood pressure (Steppan *et al.*, 2001, McTernan *et al.*, 2006, Krizova *et al.*, 2008). Serum levels of resistin and adiponectin hormones as well as common variants of the adiponectin gene will be investigated within the framework of this thesis.

1.5 Resistin

Human resistin, also known as Found In Inflammatory Zone 3 (FIZZ3) or Adipocyte Secreted Factor (ADSF), is a cysteine-rich 12.5-kDa protein. It contains 108 amino acids as a prepeptide, and its hydrophobic signal peptide is cleaved before its secretion. Resistin circulates in human blood as a dimeric protein consisting of two 92-amino acid polypeptides that are linked by a disulfide bridge at cysteine residues-26 (Aruna *et al.*, 2003). The resistin gene family and its tissue-specific distribution were first described by Holcomb *et al.* (Holcomb *et al.*, 2000). In later studies, Curat *et al.* have seen that in human adipose tissue, resistin seems to be

produced mainly by infiltrating macrophages (Curat et al., 2006), which could indicate a potential role in inflammation.

Resistin acquired initial attention as a potential link between obesity and glucose regulation. Initial experimental studies in rodents pointed to an important role of resistin as a mediator of obesity-associated insulin resistance. Steppan *et al.* showed that resistin expression was increased in obese animals, and decreased in the presence of thiazolidinediones (TZDs). In particular, administration of recombinant resistin to normal animals produced insulin resistance, whereas resistin immune neutralization by antibody improved insulin sensitivity in obese animals with insulin resistance. In adipocyte cultures, resistin decreased glucose transport in response to insulin, while an anti-resistin antibody produced the opposite effect (Steppan et al., 2001). In a further recent study, Banerjee *et al.* observed a decrease in fasting glucose, improved glucose tolerance and enhanced insulin sensitivity in resistin-gene knockout mice supporting the findings of Steppan *et al.* (Banerjee et al., 2004). The work conducted by Steppan *et al.* was the first to illustrate resistin as a link between obesity and insulin resistance, and quickly led to contradictory publications. In contrast to the Steppan *et al.* findings, various groups have observed a fall in resistin gene expression in obese and insulin resistance animal models. Way *et al.*, Moore *et al.* and Le Lay *et al.* observed lower resistin mRNA in adipose tissue in different models of mouse obesity, such as diet-induced obesity, and in rat models characterized by hyperinsulinemia, hyperglycemia, hypertriglyceridemia, and hypertension (Way et al., 2001, Moore et al., 2001, Le Lay et al., 2001).

The physiologic relevance of resistin for obesity related conditions in humans remains controversial; several studies have examined whether altered circulating resistin levels are associated with T2DM, insulin resistance, and/or obesity. Some of these studies found increased circulating resistin levels and its mRNA expression in adipose tissue in patients with obesity and T2DM, (Degawa-Yamauchi et al., 2003) while

other studies failed to confirm this finding (Lee et al., 2003, Anderlova et al., 2006) and did not show a significant correlation between resistin circulating levels and body mass index or insulin resistance. Regardless of the amount of data presented in previous studies, the true and complete role of resistin in either rodents or humans is still not fully elucidated until the resistin receptor is identified.

1.6 Adiponectin

Adiponectin is a protein of 244-amino acids (30-kDa), secreted mainly from adipocytes and plays an important role in the regulation of lipid and glucose metabolism (Karbowska and Kochan, 2006). Adiponectin was originally identified by four groups in the mid 1990s using different experimental approaches, in both mice and humans. Scherer *et al.* referred to it as adipocyte complement-related protein of 30 kDa (ACRP 30) (Scherer et al., 1995). Hu *et al.* called it ADIPOQ (Hu et al., 1996), Maeda *et al.* used the term adipose most abundant gene transcript (APM1) (Maeda et al., 1996) and Nakano *et al.* named their discovery gelatin binding protein of 28 KDa (GBP28) (Nakano et al., 1996). The circulating plasma range of adiponectin in human is 3-30 µg/ml, accounting for 0.01% of total plasma protein (Scherer et al., 1995, Maeda et al., 1996, Nakano et al., 1996, Hu et al., 1996) thus making it considerably the most abundant adipokine compared to other adipokines such as leptin (2-8 µg/L) or tumor necrosis factor (TNF)-α (<8 ng/L) (Arita et al., 1999). To quantitate the plasma adiponectin concentration, Arita *et al.* have produced monoclonal and polyclonal antibodies for human adiponectin and developed an enzyme-linked immunosorbent assay (ELISA) system (Arita et al., 1999).

Initially, adiponectin was thought to be exclusively synthesized by adipocytes; however, a recent study suggests that adiponectin is also synthesized and secreted by human cardiomyocytes (Pineiro et al., 2005). Despite being produced mainly by adipose tissue, adiponectin

secretion is paradoxically decreased in obesity (Arita et al., 1999, Hajer et al., 2008). This may be attributable to inhibition of adiponectin gene transcription by inflammatory and angiogenic factors secreted by hypertrophic adipocytes as suggested by several *in vivo* studies (Hajer et al., 2008, Bruun et al., 2003) . Maeda *et al.* and Ahn *et al.* showed that the elevated level of TNF- α , which is secreted in states of increased adiposity, inhibits adiponectin secretion leading to reduced plasma levels (Maeda et al., 2001, Ahn et al., 2007).

Currently, adiponectin is among the strongest and most consistent biochemical predictors of T2DM (Sattar et al., 2008). It exerts profound antidiabetic, anti-atherogenic and anti-inflammatory effects (Bik and Baranowska, 2009, Matsuzawa, 2008, Funahashi and Matsuzawa, 2006, Swarbrick and Havel, 2008, Meier and Gressner, 2004, Gannage-Yared et al., 2006, Kadowaki et al., 2006). Using cell ELISA analysis, Ouchi *et al.* determined that adiponectin suppressed TNF- α -induced monocyte adhesion to aortic endothelial cells and suppressed endothelial adhesion molecule expression which interferes with monocyte adhesion/ migration and transition to foam cells. These results indicate that adiponectin may attenuate the inflammatory response (Ouchi et al., 1999). Findings from animal studies and metabolic studies in humans suggest several mechanisms through which adiponectin may decrease the risk of T2DM, including suppression of hepatic gluconeogenesis, stimulation of fatty acid oxidation in the liver, stimulation of fatty acid oxidation and glucose uptake in skeletal muscle, and stimulation of insulin secretion as demonstrated in Figure 1.2.

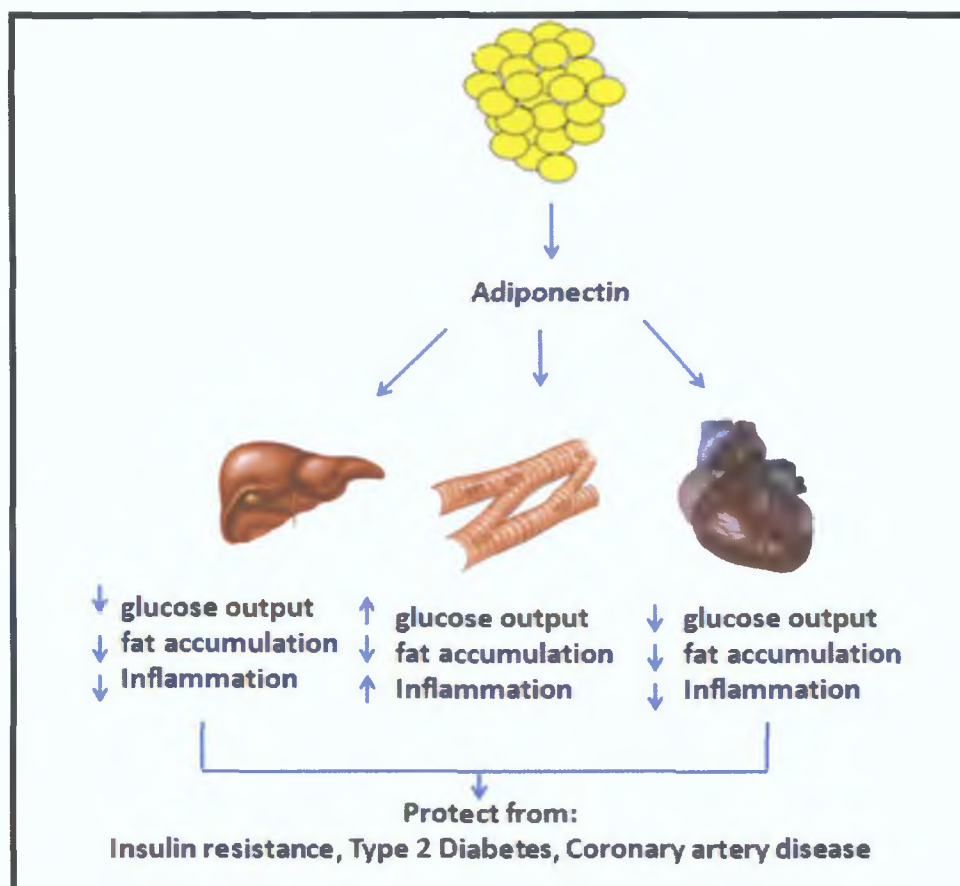


Figure 1.2 Proposed metabolic functions of adiponectin (Menzaghi et al., 2007).

1.6.1 Genomic DNA Structure, mRNA and Protein of Adiponectin

By genomic sequence analysis, Saito *et al.* determined that the ADIPOQ gene spans 16 kb, contains 3 exons and 2 introns and that the promoter lacks a TATA box (a sequence involved in the process of transcription). The exon-intron organization of this gene was very similar to that of obese gene, encoding leptin. Saito *et al.* reported the chromosome mapping of this gene by fluorescence *in situ* hybridization (FISH) using a genomic DNA fragment as a probe. The ADIPOQ gene was located on human chromosome band 3q27 (Saito et al., 1999). Takahashi *et al.* confirmed that the ADIPOQ gene maps to 3q27 using radiation hybrid analysis (Takahashi et al., 2000). A genome wide scan in later studies

indicated that a susceptibility locus linked to T2DM (Kondo et al., 2002), insulin resistance and the metabolic syndrome (Kissebah et al., 2000) may reside in this chromosomal region. Heid *et al.* have combined genome-wide association scans of three population-based studies including 4659 people and found that the ADIPOQ gene is the only major gene responsible for plasma adiponectin (Heid et al., 2010). Northern blot analysis detected a 4.5-kb adiponectin transcript in adipose tissue but not in muscle, intestine, placenta, uterus, ovary, kidney, liver, lung, brain or heart (Ouchi et al., 1999, Das et al., 2001) (Figure 1.3 a). The molecule encoded by ADIPOQ gene has an N-terminal collagen-like motif and C-terminal globular domain and is present in a multimeric form in the plasma (Figure 1.3 b, c). The adiponectin gene has notable homology with collagen X, VIII and complement factor C1q as shown in Figure 1.4.

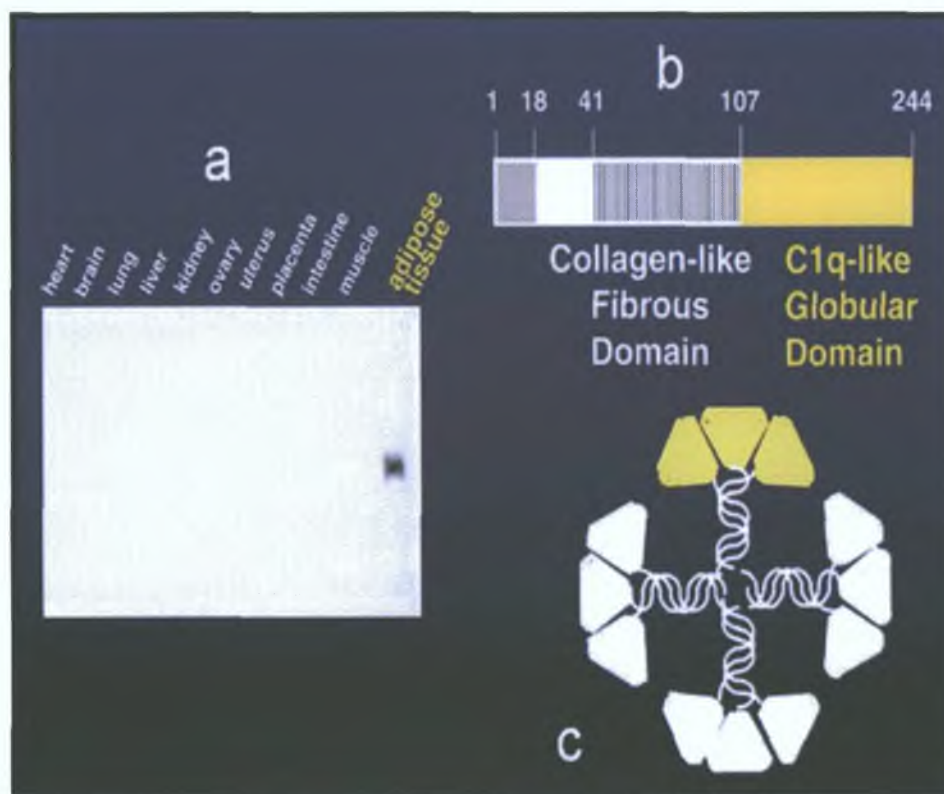


Figure 1.3 Adipose-specific collagen-like protein, adiponectin. **A.** Northern blot analysis detected a 4.5-kb adiponectin transcript in adipose tissue but not in muscle, intestine, placenta, uterus, ovary, kidney, liver, lung, brain or heart. **B.** The molecule encoded by ADIPOQ gene has an N-terminal collagen-like motif and C-terminal globular domain, and has notable homology with collagen X, VIII and complement factor C1q. **C.** Adiponectin is present in plasma in a unique multimeric form (Matsuzawa, 2010).

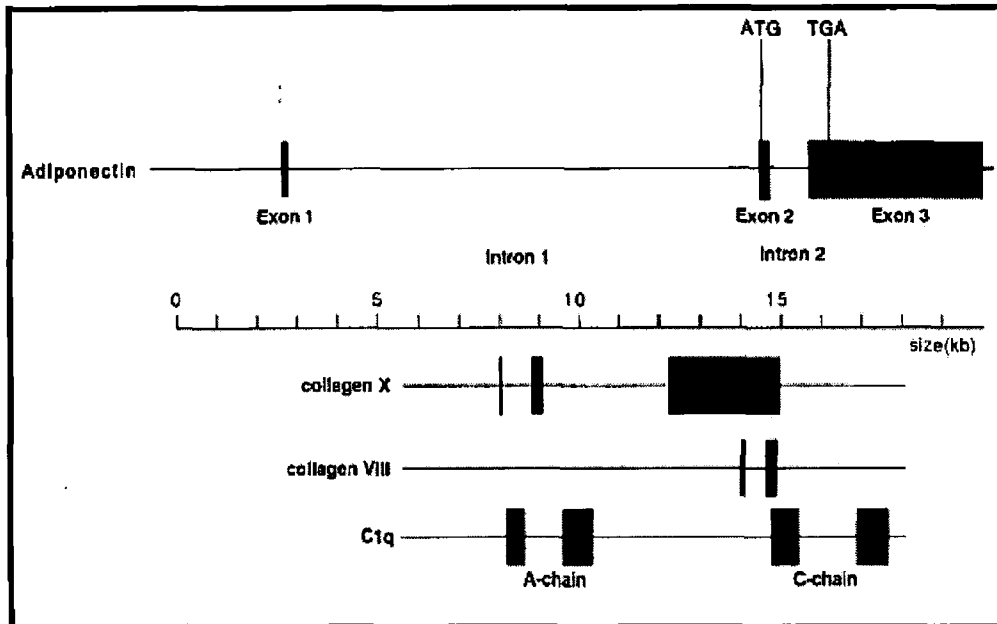


Figure 1.4 Genomic structure of the human adiponectin gene and comparison with homologous genes. Three exons are represented by solid boxes and numbered. The presumed translation initiation sites in exon 2 and the translation stop site in exon 3 are indicated by an arrow. The structures of collagen VIII, X and C1q genes were also shown (Takahashi et al., 2000).

In human plasma, adiponectin exists in a wide range of multimeric complexes and combines via its globular or collagen domains to create various oligomeric complexes. Using SDS-PAGE to analyze human and mouse adiponectin isolated from serum or adipocytes and recombinant adiponectin expressed in mammalian cells, Waki *et al.* detected three different molecular mass species and characterized them as low-molecular weight (LMW) trimer (via globular domain interactions (67 kD), a middle-molecular weight (MMW) hexamer (136 kD), and high-molecular weight (HMW) 12- to 18-multimer adiponectin (via collagenous domain interactions) (greater than 300 kD) as shown in Figure 1.5. A disulfide bond through an N-terminal cysteine was required for the formation of multimers larger than a trimer. The authors demonstrated that simple SDS-PAGE, under *non-reducing* and *non-heat-denaturing* conditions, clearly separates multimer species of adiponectin (Waki et al., 2003).

Also, existing in lower quantities is a smaller form of adiponectin that consists of globular C-terminal domain fragments cleaved from full-length adiponectin (Pajvani et al., 2003). Recently, a novel sandwich enzyme-linked immunosorbent assay (ELIZA) kit has been developed that accurately determines the serum levels of various types of adiponectin without requiring any sample pretreatment (Nakano et al., 2006).

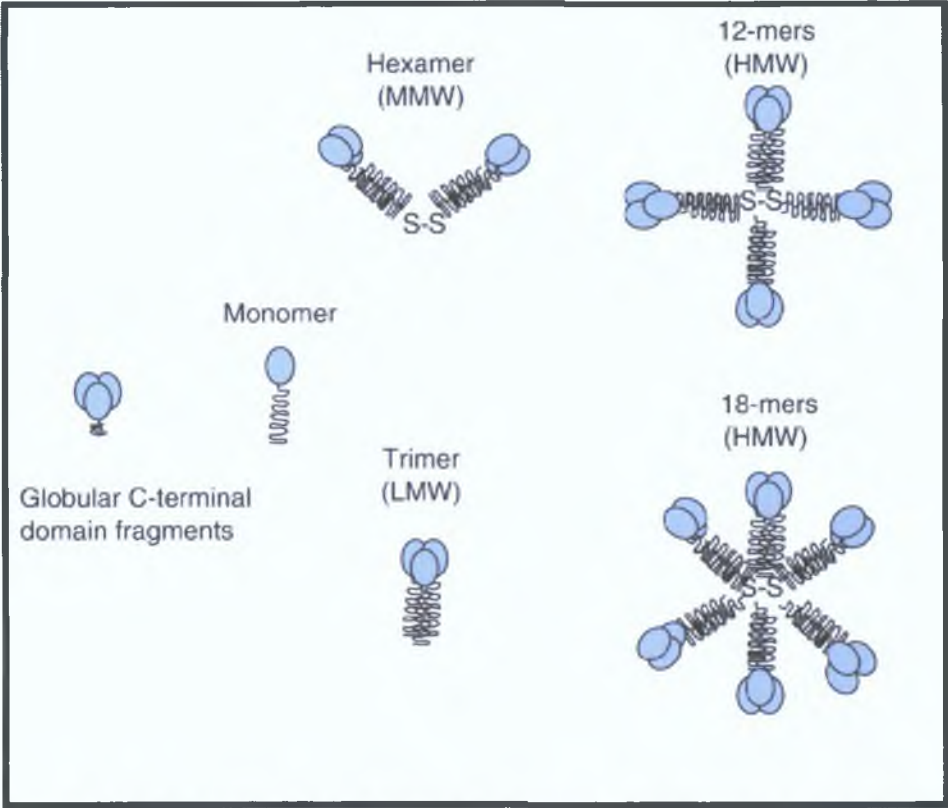


Figure 1.5 Adiponectin isoforms. Adiponectin is present in the plasma as full-length molecules or smaller globular C-terminal domain fragments. The full-length adiponectin in plasma exists as a trimer (low molecular weight, LMW), hexamer (medium molecular weight, MMW), and 12- 18-multimer (high molecular weight, HMW) whereas extremely low amount of the Globular C-terminal domain fragments cleaved from full-length adiponectin is present (Więcek et al., 2007).

The different multimeric forms of adiponectin may potentially have different physiological properties. To investigate this, Waki *et al.* have found that human adiponectin with rare missense mutations (G84R and G90S) didn't form HMW multimers. These mutations were associated with T2DM, hypoadiponectinemia and insulin resistance. The authors have also shown that an amino-terminal (Cys-Ser) mutation, which could not form multimers larger than a trimer, abrogated the effect of adiponectin on the adenosine monophosphate-activated protein (AMP) kinase pathway in hepatocytes. Moreover, R112C and I164T mutants, which are shown to be associated with hypoadiponectinemia, did not assemble into trimers, resulting in impaired secretion from the cell. These data suggested impaired multimerization and/or the consequent impaired secretion to be among the causes of a diabetic phenotype or hypoadiponectinemia in subjects having these mutations (Waki *et al.*, 2003).

HMW adiponectin has been purified and suggested to have more biological activity than either LMW or MMW adiponectin (Pajvani *et al.*, 2004). A very recent study showed that measurements of total and HMW adiponectin have similar utility for the identification of insulin resistance (Almeda-Valdes *et al.*, 2010, Heidemann *et al.*, 2008). To compare the utility of total adiponectin, HMW adiponectin and the HMW adiponectin/total adiponectin index (SA index) for the identification of insulin resistance (IR) and related metabolic conditions, Almeda-Valdes *et al.* performed a cross-sectional analysis in a group of 101 men and 168 women, aged 20 to 70 years, in Mexico City. Total, HMW adiponectin and the SA index were characterized and plotted for the identification of metabolic disturbances. Sensitivity and specificity for the identification of IR were calculated. The data showed that total adiponectin, HMW adiponectin and the SA index all had similar utility for the identification of IR and metabolic disturbances (Almeda-Valdes *et al.*, 2010).

1.6.2 Adiponectin Receptors

The molecules mediating the biological functions of adiponectin were not known until 2003, when adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) were cloned by Yamauchi *et al.* using expression cloning methods. AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver. These two adiponectin receptors are predicted to contain seven transmembrane domains, but to be structurally and functionally distinct from G-protein-coupled receptors. Yamauchi *et al.* have seen that expression of AdipoR1/R2 or suppression of AdipoR1/R2 expression by small-interfering RNA supports their conclusion that they serve as receptors for globular and full-length adiponectin, and that they mediate increased adenosine monophosphate-activated protein (AMP) kinase and peroxisome proliferator-activated receptor- gamma (PPAR- γ) ligand activities, as well as fatty-acid oxidation and glucose uptake (Yamauchi et al., 2003).

Studies aimed at both receptors mRNA and protein expression levels in various diabetic insulin resistant states have produced contradictory results. Civitarese *et al.* have reported that the expression of these receptors is reduced in people with a family history of T2DM, whereas Tan *et al.* have reported that their levels are induced in adipose and muscle tissues from obese and insulin resistant women with polycystic ovary syndrome, (Tan et al., 2006, Civitarese et al., 2004). Nevertheless, the majority of studies support the hypothesis that the expression of these receptors is significantly decreased in muscle and adipose tissue in hyperinsulinemic and hyperglycemic states associated with T2DM, obesity and insulin resistance (Civitarese et al., 2004, Bluher et al., 2007, Nannipieri et al., 2007). Moreover, Weigert *et al.* have shown in a recent study that monocytes from overweight and obese individuals with T2DM have an impaired expression and lower levels of adiponectin receptors (AdipoR1 and AdipoR2) compared to normal-weight controls (Weigert et al., 2008).

Recently, a third adiponectin receptor, T-cadherin has been isolated and found to be capable of binding to the adiponectin molecule. Hug *et al.* performed a series of expression cloning studies using a magnetic bead-based panning method. They identified T-cadherin as a receptor for the hexameric and high molecular weight species of adiponectin but not for the trimeric or globular species. T-cadherin represents extracellular protein and lacks known cellular functions. However, it is expressed in vascular cells, where it is positioned to interact with adiponectin (Hug *et al.*, 2004). These data implicate T-cadherin to be involved in regulation of vascular functioning and remodeling.

1.6.3 Plasma Adiponectin Levels and Disease Associations

Adiponectin levels vary according to sex, body fat distribution, genetic background, dietary factors and metabolic status (Arita *et al.*, 1999, Nishizawa *et al.*, 2002, Salmenniemi *et al.*, 2005, Kadowaki *et al.*, 2006). Arita *et al.* have found total adiponectin concentrations to be higher in females than in males (Arita *et al.*, 1999), Nishizawa *et al.* have estimated that men have approximately 15% lower levels compared with women (Nishizawa *et al.*, 2002). To determine the association of adiponectin level with clinical phenotypes of metabolic syndrome, including glucose and lipid oxidation, energy expenditure, insulin sensitivity, and visceral obesity, Salmenniemi *et al.* performed detailed metabolic studies in a cohort (n = 158) of offspring of patients with T2DM by applying the euglycemic clamp technique and indirect calorimetry. They found that adiponectin levels were lower in offspring of T2DM patients than in control subjects. When the data were analyzed, an elevated adiponectin level was associated with high glucose disposal and high energy expenditure; low levels of free fatty acids and low rates of lipid oxidation; low levels of inflammatory cytokines; and a low amount of intraabdominal fat evaluated by computed tomography. They concluded that adiponectin has multiple effects on glucose, lipid and free fatty acid

metabolism and inflammatory markers in offspring of T2DM subjects (Salmenniemi et al., 2005).

Adiponectin gene expression and circulating adiponectin levels were seen to be lower in patients with T2DM than in nondiabetic individuals. In a prospective study of 3,599 nondiabetic men followed up for 5 years, low adiponectin levels were associated with increased risk of T2DM, even after adjustment for traditional risk factors including BMI, lifestyle factors, preexisting cardiovascular disease, and systolic blood pressure. The inverse relation between low adiponectin and diabetes was significantly stronger in men who were obese (waist circumference > 102 cm or BMI ≥ 30 kg/m²) relative to leaner men (Wannamethee et al., 2007). Similar results have been found in a cohort study representing the ~9-year experience of the 10,275 middle-aged U.S. African American and Caucasian participants where higher adiponectin levels were found to be associated with a lower incidence of diabetes (Duncan et al., 2004). Adiponectin is decreased in obesity and seems to be involved in insulin resistance. Arita *et al.* found a negative correlation between body mass index and plasma adiponectin levels in Japanese men and women. Interestingly, there were marked variations in adiponectin levels even among obese subjects (Arita et al., 1999). Negative correlation between plasma adiponectin levels and body mass index or body fat was further supported by studies of Weyer *et al.* performed on Caucasian and Pima Indian populations (Weyer et al., 2001).

In contrast to decreased adiponectin levels in obese individuals, weight loss was found to enhance adiponectin concentrations by 40–60% (Hotta et al., 2000). Simonyte *et al.* studied the changes in plasma adiponectin levels with body weight reduction among twenty-seven obese women who underwent gastric bypass surgery. Two years after gastric bypass surgery, significant reductions were observed in the mean BMI (from 44.4 to 30.8 kg/m²) and mean waist circumference (from 121.9 to 90.6 cm) with concomitantly increased circulating adiponectin levels. The authors

concluded that body weight reduction increased the plasma levels of adiponectin (Simonyte et al., 2010).

Recent studies have shown that adiponectin levels can be changed through lifestyle and pharmaceutical interventions. Kim *et al.* evaluated the effects of a 10-month lifestyle intervention on blood concentrations of adiponectin and the components of the metabolic syndrome in 130 Koreans with metabolic syndrome. All participants followed a 10-month lifestyle modification interventional program, including dietary counseling, advice on increasing physical activity, and recommendations to stop or limit smoking and alcohol drinking. Blood concentrations of adiponectin and anthropometric and biochemical parameters related to the components of the metabolic syndrome were measured. They found that at baseline, adiponectin concentrations were moderately negatively correlated to insulin concentrations and insulin resistance evaluated by the homeostasis model assessment. In response to lifestyle modification, statistically significant changes were found in adiponectin concentration, postintervention levels were higher compared with the preintervention levels. The authors concluded that lifestyle modification program in Korean patients with metabolic syndrome led to favorable changes in metabolic parameters and adiponectin levels (Kim et al., 2011). Dietary factors may also potentially influence adipokine levels and insulin sensitivity. Several studies showed that higher consumption of foods with high glycemic index/glycemic load values is associated with lower adiponectin levels in both healthy and diabetic individuals (Qi et al., 2005, Pischon et al., 2005).

One obvious pharmaceutical intervention would be the administration of peroxisome proliferator-activated receptor-gamma (PPAR- γ) agonists (Olefsky, 2000). PPAR- γ is the master regulator of adipocyte differentiation and controls many adipocyte genes. Maeda *et al.* have shown that the administration of thiazolidinedione (TZDs) which are synthetic PPAR γ ligands significantly increased the plasma adiponectin concentrations in insulin resistant humans and rodents without affecting

their body weight (Maeda et al., 2001). Yamauchi *et al.* and Berg *et al.* also found separately in studies using various types of obese mice and diabetic mice, that the administration of adiponectin improves both insulin resistance and blood glucose levels (Yamauchi et al., 2001, Berg et al., 2001).

1.6.4 Genetic Polymorphisms of the Adiponectin Gene and Disease Association

The first genetic analysis of adiponectin levels was conducted by Comuzzie *et al.* who assayed serum levels of adiponectin in 1100 adults of predominantly northern European ancestry distributed across 170 families. Quantitative genetic analysis of adiponectin levels detected an additive genetic heritability of 46% (Comuzzie et al., 2001). Mutation screening for the ADIPOQ gene in T2DM established a total of 42 single nucleotide polymorphisms (SNPs) with minor allele frequency (MAF) > 1.5% (Gu, 2009). Genetic association of SNPs in the ADIPOQ gene with T2DM and other metabolic disorders in different ethnic populations that showed significant associations is summarized in Table 1.2.

Table 1.2 Associations between adiponectin genetic polymorphisms and metabolic disorders in different ethnic populations with significant associations (Gu, 2009).

SNP	Clinically associated conditions	Ethnic group
rs16861194 -11426 A>G	Adiponectin levels T2DM	French Caucasians, Swedish, European Caucasians
rs17300539 -11391 A>G	Adiponectin levels T2DM Obesity Insulin resistance Insulin sensitivity Diabetic nephropathy	French Caucasians UK Caucasian women German, Italian Black South Africans Spanish, Polish
rs266729 -11377 C>G	Adiponectin levels T2DM Obesity Insulin resistance Insulin sensitivity Diabetic nephropathy	French Caucasians Swedish, Danish German, Italian Chinese, Spanish Polish
rs2241766 +45T>G	Adiponectin levels Fasting glucose levels T2DM Obesity Insulin resistance Insulin sensitivity Diabetic retinopathy Diabetic nephropathy Atherosclerosis Cardiovascular diseases	Japanese, Chinese Korean, Italian Uygurs, Swedish, Finnish African Americans Spanish, German European Caucasians Polish
rs1501299 +276G>T	Adiponectin levels T2DM Obesity Insulin resistance Insulin sensitivity Diabetic nephropathy Polycystic ovary syndrome Cardiovascular diseases	European Caucasians Japanese, Italian, German Chinese, Korean Swedish, Finnish African Americans Uygurs, Chinese, Spanish Polish
rs17366743 +331T>C	Adiponectin levels Body mass index T2DM	German, Polish Finnish Framingham Americans

Positive and negative numbers refer to residue position from the transcription start site

The linkage disequilibrium (LD) of the ADIPOQ gene is moderate, but there are two small LD blocks, one including SNPs in the promoter region and another one spanning the boundary of exon 2-intron 2 (Gu, 2009). Figure 1.6 shows ADIPOQ gene, locations of the genotyped variants and their pairwise linkage disequilibrium (LD) patterns. Two SNPs at the ADIPOQ locus are a silent T to G substitution in exon 2 (+45T>G, rs2241766) and a G to T substitution in intron 2 (+276G>T, rs1501299) have been extensively studied. However, the association studies of these two SNPs, either independently or as a haplotype, have resulted in conflicting evidences in different populations and sample types. Stumvoll *et al.* reported a positive association between the G allele of the +45T>G polymorphism and obesity traits in a 371 nondiabetic German individuals ($P=0.02$) (Stumvoll *et al.*, 2002), whereas in Taiwanese nondiabetic subjects ($n= 245$), the same allele was related to a lower risk of obesity (Yang *et al.*, 2003). Recently, the G allele at the +45T>G polymorphism has been associated with higher serum adiponectin concentrations (Mackevics *et al.*, 2006, Berthier *et al.*, 2005), higher adiponectin mRNA expression in adipose tissue (Yang *et al.*, 2003), and improved insulin sensitivity in Caucasian and Quebec populations (Menzaghi *et al.*, 2002, Ruchat *et al.*, 2008). On the other hand, Xita *et al.* found that the G allele of the SNP +45T>G was associated with increased risk of IR in a sample of Greek women with polycystic ovary syndrome ($n= 100$ women with polycystic ovary syndrome and 140 healthy controls, ($P <0.05$)) (Xita *et al.*, 2005). Similar findings were observed in a group of 747 unrelated Spanish subjects where the G allele of SNP +45T>G was associated with impaired glucose tolerance ($P=0.020$) (Gonzalez-Sanchez *et al.*, 2005).

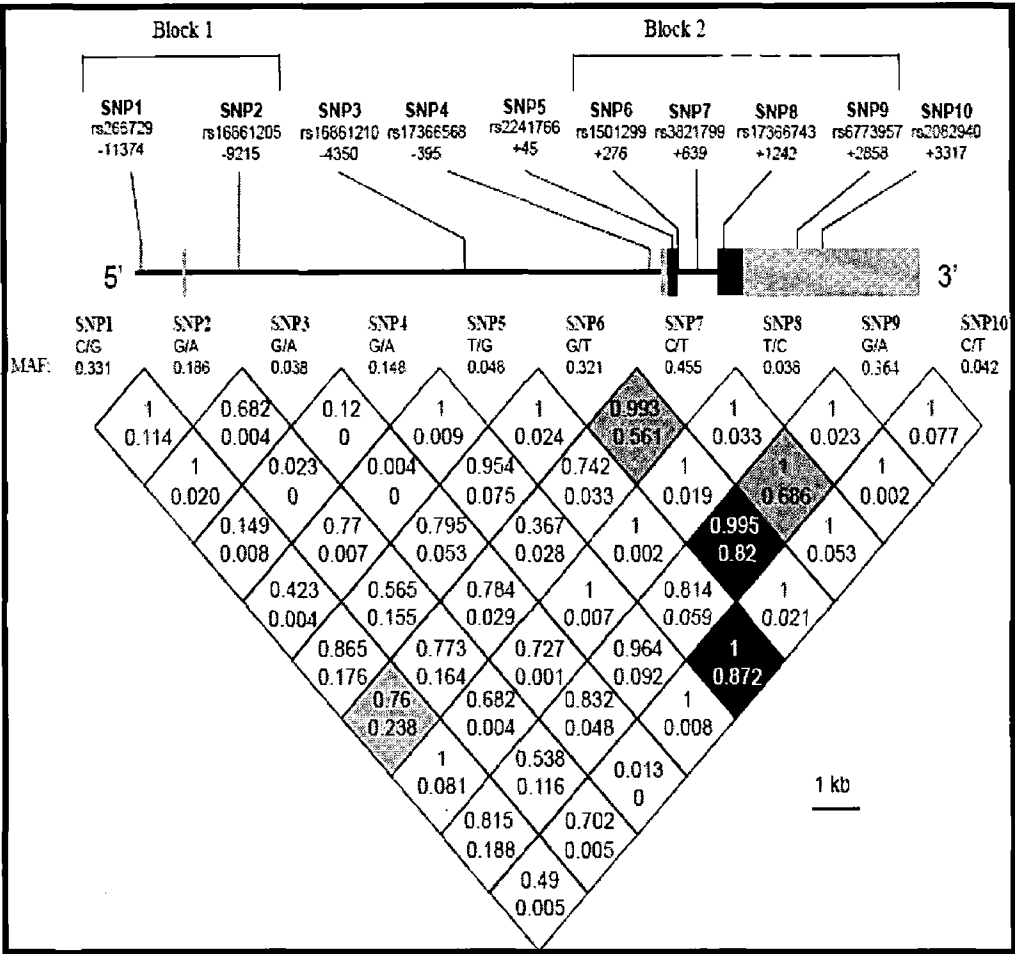


Figure 1.6 ADIPOQ gene, locations of the genotyped variants and their pairwise linkage disequilibrium (LD) patterns. Schematic presentation of the ADIPOQ, indicating the locations of the analysed variants, the two observed haploblocks and the pairwise LD measures D' (above) and r^2 (below). Grey boxes, untranslated region; black boxes, coding region; MAF, minor allele frequency (Siitonen et al., 2011).

The T allele of the SNP +276G>T has been associated either with increased or decreased levels of plasma total adiponectin in different Caucasian populations. Filippi *et al.* have studied the association between SNP +276G>T of the adiponectin gene and adiponectin plasma levels. The SNP was analyzed in a cohort of 595 subjects. They observed a significant association between the SNP +276G>T in the adiponectin gene and adiponectin serum level. The presence of T allele of the SNP +276G>T was a significant determinant of lower levels of serum adiponectin levels (Filippi et al., 2005). Despite that, Hara *et al.*

have observed that the T allele at position +276 was linearly associated with higher plasma adiponectin levels in the Japanese population (n= 480 nondiabetic and 384 diabetic subjects, $P=0.01$). They also showed that subjects with the G/G genotype at position +276 had a significantly increased risk of type 2 diabetes and a higher insulin resistance index compared with those having the T/T genotype ($P =0.002$, OR 2.16, 95% CI 1.22–3.95) (Hara *et al.*, 2002). Kyriakou *et al.* and Gonzalez-Sanchez *et al.* found separately that the T allele of the SNP +276G>T was significantly associated with elevated adiponectin in samples of Caucasian women (the Chingford Study; n = 808, mean age 62.8 ± 5.9 years and Twins UK; n = 2,718, mean age 47.4 ± 12.6 years, $P < 0.05$) and in 747 unrelated Spanish subjects with metabolic syndrome, $P=0.015$) respectively confirming Hara *et al.* findings (Kyriakou *et al.*, 2008, Gonzalez-Sanchez *et al.*, 2005).

However, other studies have not found any effect of either SNPs +45T>G and SNP +276G>T on adiponectin level and risk for type 2 diabetes. For example Vozarova de Courten *et al.* found no association between SNP +45T>G and serum adiponectin or diabetes in Pima Indians (n= 790 nondiabetic and 548 diabetic subjects) (Vozarova de Courten *et al.*, 2005). Similarly, recent studies in French (n= 1373 diabetic and obese subjects) (Vasseur *et al.*, 2002), American (n= 642 incident cases of type 2 diabetes and 995 matching control) (Hu *et al.*, 2004), and Swedish (n= 166 patients with type 2 diabetes) (Kang *et al.*, 2005) subjects have not detected any association of SNP +276G>T with risk of type 2 diabetes.

Haplotypes comprised of the two SNPs have also provided evidence of association with plasma total adiponectin levels, obesity, lipid profile and insulin resistance. Menzaghi *et al.* genotyped 413 nondiabetic individual's phenotypes to assess whether the haplotypes of these two variants contribute to the prevalence of insulin resistance in Caucasians. They detected that the haplotype defined by the two together was strongly associated with many components of the insulin resistance syndrome compared with each polymorphism alone. Homozygotes for

the risk haplotype had higher body weight ($P=0.03$), waist circumference ($P=0.004$), systolic ($P=0.01$) and diastolic ($P=0.003$) blood pressure, fasting glucose ($P=0.02$) and insulin ($P=0.005$) levels, homeostasis model assessment (HOMA) for insulin resistance ($P=0.003$), and total to HDL cholesterol ratio ($P=0.01$). Homozygotes also had significantly lower plasma levels of adiponectin ($P=0.03$), independent of sex, age, and body weight. Menzaghi *et al.* concluded that SNPs +45T>G and +276G>T haplotype at the adiponectin locus is strongly associated with obesity and other features of the insulin resistance syndrome (Menzaghi *et al.*, 2002). Mackevics *et al.* replicated a strong association of ADIPOQ SNPs +45T>G/ +276G>T genotypes and haplotypes with adiponectin levels in 1745 well-phenotyped asymptomatic unrelated Caucasian subjects that was previously reported by Menzaghi *et al.*, however, they did not find significant associations between the metabolic parameters of the insulin resistance syndrome and the SNPs +45T>G /+276G>T haplotype (Mackevics *et al.*, 2006). With all these findings, it is still hard to evaluate the data on these studies. The biggest problem with this field and particularly older studies is lack of power associated with small numbers of subjects studied.

1.7 Hypothesis

Low concentrations of adiponectin have been reported to be associated with obesity, insulin resistance and type 2 diabetes mellitus. However, contrasting reports have been published regarding the effect of genetic variability in ADIPOQ on adiponectin serum concentrations as well as characteristics of the metabolic syndrome among different populations. In Bahrain, the association between adiponectin gene polymorphisms and obesity-related clinical markers is not well researched. Therefore, the purpose of this study was to investigate the association of the two most well-known SNPs of ADIPOQ gene (+45T>G and +276G>T) with serum adiponectin concentrations and metabolic syndrome parameters in a cross-sectional survey in a representative sample of 140 incident cases of type 2 diabetes and 66 control subjects. We also evaluated another adipokine resistin, and assessed its relation with the anthropometric parameters and lipid profile in the study subjects. As far as we are aware, this study is the first to investigate the association of adiponectin, resistin and metabolic parameters in a Bahraini population with diabetes mellitus. This study highlights a potential role for the use of adipokines particularly adiponectin and resistin as a biomarker for the early diagnosis of insulin resistance and type 2 diabetes mellitus.

1.8 Thesis Overview

The research presented in this thesis investigates the association of the two most well-known SNPs of adiponectin gene (+45T>G and +276G>T) with serum adiponectin concentrations and metabolic parameters in a case control study. The overall aims of this thesis are outlined below.

- To investigate the relationship between serum adiponectin levels, type 2 diabetes mellitus and metabolic parameters including lipid profile, glycated hemoglobin (Hb A1c), fasting blood glucose (FBG), body mass index (BMI) and systolic and diastolic blood pressure among Bahraini type 2 diabetic patients and healthy control subjects. To compare gender differences on these associations in both type 2 diabetic patients and control subjects. To identify which among the metabolic syndrome parameters are closely associated with pathological levels of adiponectin. To assess the impact of BMI on adiponectin.
- To investigate the associations between serum resistin levels, type 2 diabetes mellitus and metabolic parameters including lipid profile, Hb A1c, FBG, BMI and systolic and diastolic blood pressure among Bahraini type 2 diabetic patients and healthy control subjects. To compare gender differences on these associations in both type 2 diabetic patients and control subjects. To identify which among the metabolic syndrome parameters are closely associated with pathological levels of resistin. Compare between adiponectin and resistin levels in relation to the metabolic and clinical parameters in the whole study population.
- To examine the genetic variability in the adiponectin gene, characteristics of the metabolic syndrome and adiponectin serum concentrations. To investigate the association of the two most well-known single nucleotide polymorphisms (SNPs) of adiponectin gene (+45T>G and +276G>T), with serum adiponectin

concentrations, metabolic parameters and type 2 diabetes. The SNPs were characterized by detecting restriction fragment length polymorphisms post amplification by polymerase chain reaction (RFLP-PCR).

Chapter 2- Materials and Methods

2.1 Study Subjects

A cross-sectional study was conducted from October 2009 until October 2011 among 140 native Bahraini patients suffering from T2DM (mean age 58.4 ± 11.4 years; 74 women and 66 men; mean BMI 34 ± 7 ; mean HbA1c $8.1 \pm 1.7\%$) and 66 non diabetes control subjects (mean age 41.2 ± 6.3 years; 6 women and 60 men; mean BMI 29 ± 4.5). All subjects were Bahraini unrelated individuals and were randomly recruited from Bahrain Defense Force (BDF) hospital from August 2009 to April 2010.

The inclusion criteria for the diabetic subjects involved patients referred to the Diabetic Clinic in the BDF hospital; with the presumptive diagnosis of type 2 diabetes mellitus. The presence of type 2 diabetes was established according to the American Diabetes Association criteria (American Diabetes Association, 2009). A subject was considered diabetic if he/she exhibited a fasting blood glucose (FBG) value of 7 mmol/l (126 mg/dl) or greater, if his/her 2-hr glucose value during a 75-g oral glucose tolerance test (OGTT) was 11.1 mmol/l (200 mg/dl) or greater, or if antidiabetic therapy (oral hypoglycemic agent such as sulphonylureas or metformin, or insulin) was required. Impaired fasting glucose (IFG) was defined as fasting glucose between 5.6 and 6.9 mmol/l (100 to 125 mg/dl inclusively). Patients with type 1 diabetes mellitus and women with gestational diabetes were excluded.

For the comparison, a total of 66 controls without a personal history of any metabolic abnormalities were recruited to the study. The controls were taken from the same geographic areas as the patients. The following exclusion criteria were applied: fever (temperature $\geq 38^{\circ}\text{C}$), hospitalization in the previous two weeks, known diabetes (FBG ≥ 7 mmol/l), arterial hypertension (systolic blood pressure (SBP) ≥ 130 mm Hg or diastolic blood pressure (DBP) ≥ 85 mm Hg), dyslipidemia [total cholesterol (TC) ≥ 6.21 mmol/l (240mg/dl), triglyceride (TG) ≥ 1.7 mmol/l (150 mg/dl), high density lipoprotein cholesterol (HDL-C) ≤ 1.03 mmol/l (40 mg /dl)], coronary heart disease, congestive heart failure, stroke,

chronic renal disease, active hepatic disease, chronic diseases or any other acute or chronic inflammatory illness. Subjects were also excluded if they were on any medication known to affect the metabolic profile (anti-hypertensives, statins, fibrates, metformin, thiazolidinediones, and salicylic acid). At the time of blood sampling, all controls were free of any pharmacological treatments. These criteria were evaluated through personal interview structured by a questionnaire (Appendix 1) and/or by direct measurements to assess the participants' condition. The questionnaire included questions about date of birth, gender, nationality, smoking habits, history of any diseases particularly metabolic diseases and type 2 diabetes mellitus and characterized if any current medications are being used. In addition, each individual's systolic and diastolic blood pressure, height and weight was measured and recorded.

Samples were collected and analyzed over a two-year period. The experimental design of this study was approved by the ethics committee of the Royal College of Surgeons in Ireland (RCSI Bahrain and RCSI Dublin). This study was also approved by the research and ethics committee of the local hospitals (BDF Hospital and Salmanyia Medical Complex) and informed consent was obtained.

2.2 Patients Medical History

Type 2 diabetic patients were characterized using a patient clinical data sheet (Appendix 2), which included the following four main sections:

- General information section: to characterize patient's gender, date of birth, nationality, smoking state and family history of type 2 diabetes mellitus.
- Clinical parameters section: which measures height, weight, fasting blood glucose, systolic and diastolic blood pressure, glycated haemoglobin (HbA1c) and lipid profiles (triglycerides,

total cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol) for each diabetic case.

- Management section: to assess the current medications used and their doses.
- Complications section: This characterizes any sort of complications associated with the disease.

The clinical data were retrospectively collected from patient's medical records and/or through personal interview. The patients were subjected to direct anthropometric and laboratory measurements to assess the clinical parameters. The data highlighted the clinical manifestations associated with the disease and were essentially used to establish any possible phenotypic-genotypic correlation in type 2 diabetic cases.

2.3 Anthropometric Measurements

Height, weight, random blood sugar (RBS), pulse rate and systolic and diastolic blood pressures were measured for participants by trained nurses. Weight and height were measured in kilogram (Kg) and centimeter (cm) respectively. Body weight was measured by a balance scale to the nearest half-kilogram with the individuals in light clothing and without shoes. Height was measured by a stadiometer to the nearest half centimeter. Body mass index (BMI) or Quetelet index was calculated as weight (Kg) divided by squared height (m^2). Subjects were classified as being obese, overweight or normal on the basis of BMI. BMI cut points chosen to make this distinction were according to WHO standards (WHO, 2004). Obesity was defined as having a BMI of ≥ 30 Kg/m², while overweight was defined as a BMI of 25 to 29.9. Subjects with BMI of 18.5-24.9 were considered as being normal.

RBS was measured by blood glucose level testing kit. The measurement was done by first pricking patient's finger with a sharp lancet designed to

penetrate the skin only as far as needed to draw a drop of blood. A small amount of blood was put on the correspondent strip and placed into the glucose measuring device (Accu-Chek Active Blood Glucose Meter, Roche). The blood glucose level was displayed after about 30 seconds.

Systolic and diastolic blood pressures were measured using standard digital sphygmomanometer with the cuff on the right arm and the subjects in a sitting position. The measurements were taken after resting for at least three minutes. Systolic and diastolic blood pressures were measured in duplicate, the results were averaged, and the average value was used for data analysis. Pulse rate was also recorded. Diagnosis of hypertension was based on the presence of elevated systolic (>130mmHg) and/or diastolic (> 85mmHg) blood pressure, and/or the current use of antihypertensive medications (Alberti et al., 2006).

2.4 Blood Collection

After obtaining an informed consent, study participants were referred to the phlebotomy unit in the BDF hospital. Whole blood collection was conducted by vein puncture in three different blood tubes: EDTA-coated tubes for HbA1c determination and genetic studies, sodium fluoride tubes for glucose estimation, and serum separator tubes without anticoagulant for studying the lipid and hormones profiles. Venous blood was drawn from all subjects after an overnight fast for at least 12 hours. The serum and red blood cells were separated by centrifugation at 3,000 x g for 10 minutes at room temperature and were directly subjected to the laboratory measurements at the biochemistry laboratory of the BDF hospital. After obtaining the biochemical results, the blood and serum samples were then transported to the research laboratory of the RCSI Bahrain for subsequent assays. The samples were received, checked, and the patient's information was confirmed. An internal numbering system corresponding to each patient was allocated to guarantee patient's confidentiality. Samples inadequately labeled, or inappropriately

obtained, handled, preserved, processed, transported or stored were rejected. Accepted blood samples were stored non-frozen at +4°C for DNA extraction and serum samples were stored frozen at -20°C for subsequent hormonal assays. The analysis was performed within three days from receiving the samples.

2.5 Laboratory Measurements

Blood samples were analyzed for biochemical variables at the biochemistry laboratory of the BDF hospital. Serum glycated hemoglobin (HbA1c), fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) were measured. HbA1c was assayed using the latex agglutination reaction on Roche cobas c 111 automatic biochemistry analysis system (Roche Diagnostics, Indianapolis, IN). FBG, TC, TG, HDL-C and LDL-C were assayed using the Roche cobas® 6000 automatic biochemistry analysis system (Roche Diagnostics, Indianapolis, IN). FBG was measured using the hexokinase/glucose-6-phosphate dehydrogenase method (Neeley, 1972), the serum levels of TG, TC and HDL-C were measured by enzymatic colorimetric test, and LDL-C concentration was calculated by the Friedewald formula: $LDL = \text{Total cholesterol level} - HDL - [\text{Triglyceride level} / 5]$ (Warnick et al., 1990).

2.6 Metabolic Syndrome and Its Components

The presence of metabolic syndrome was established using the US National Cholesterol Education Program Adult Treatment Panel III (ATP III) (NCEP, 2001). The five metabolic syndrome criteria were waist circumference (WC), FBG, TG, HDL-cholesterol, and blood pressure. Patients were concluded to have metabolic syndrome if they exhibited at least three of the following criteria: (a) central (abdominal) obesity with a

waist circumference ≥ 120 cm (males) or ≥ 88 cm (females), (b) fasting plasma glucose values of ≥ 6.1 mmol/L (110 mg/dL); (c) TG values of ≥ 1.7 mmol/l (150 mg/dl); (d) HDL cholesterol concentrations below 1.03 mmol/l (40 mg/dl) for males or below 1.29 mmol/l (50 mg/dl) for females; or (d) blood pressure measurements of 130/85 mmHg or higher or use of antihypertensive agents. Since the WC measurements were not available, the criteria used for defining the patient as having the metabolic syndrome were only the values of FBG, TG, HDL-cholesterol, and blood pressure.

Since insulin serum level was not measured either, insulin resistance was evaluated and considered present when the triglyceride/HDL-cholesterol index was ≥ 3 (Almeda-Valdes et al., 2010, González-Chávez et al., 2011).

2.7 Hormone Assay

Adiponectin and resistin levels were detected by immunoassay analysis with a sandwich enzyme-linked immunosorbent assay (ELISA) system from R&D Systems (Abingdon, Oxon, UK).

2.7.1 Adiponectin

Serum levels of adiponectin were measured using a Quantikine Human Adiponectin Immunoassays kit from R&D Systems (Abingdon, Oxon, UK) (Catalogue No. SRP300). The assay was conducted according to the manufacturer's instructions.

2.7.1. 1 Samples Dilution

Serum samples were diluted to 100-fold dilution. 2 μ l of sample was added to 198 μ l of Calibrator Diluent RD6-39.

2.7.1. 2 Adiponectin Standards Preparation

The adiponectin standard was reconstituted with 2.0 ml of Calibrator Diluent RD6-39 to produce a stock solution of 250 ng/ml. The standard was mixed to ensure complete reconstitution and allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. The stock solution was used to produce a dilution series of 125, 62.5, 31.2, 15.6, 7.8 and 3.9 ng/ml (Figure 2.1). Each tube was mixed thoroughly before the next transfer. The 250 ng/ml standard served as the high standard. Calibrator Diluent RD6-39 served as the zero standard (0 ng/ml).

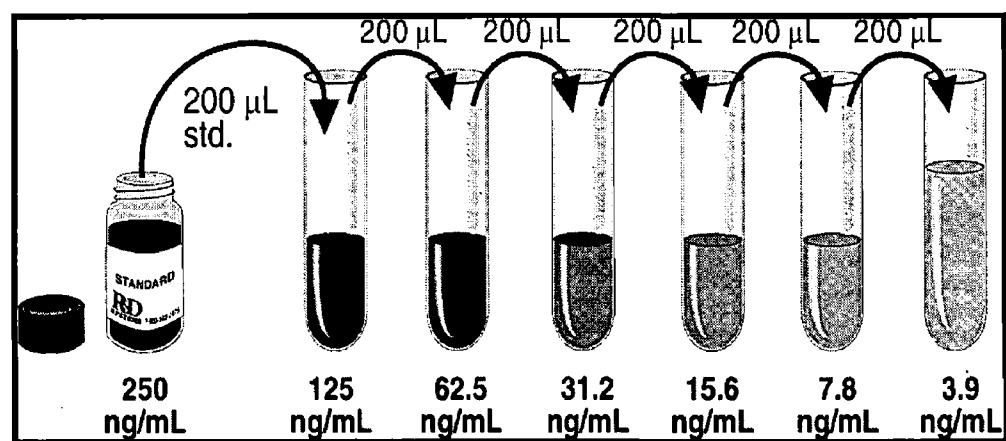


Figure 2.1 Adiponectin standard preparations. Six series of dilution was conducted in 200 μ l Calibrator Diluent RD6-39. In the first dilution, 200 μ l of the stock solution (250 ng/ml) was pipetted into the 200 μ l Calibrator Diluent RD6-39. In subsequent dilution steps, 200 μ l of the previous dilution was added to prepare the next dilution. The dilution series produced were 125, 62.5, 31.2, 15.6, 7.8 and 3.9 ng/ml.

2.7.1.3 Assay Procedure

First, 50 µl of diluted serum sample and standards and 100 µl of Assay Diluent RD1W were pipetted into each of 96 wells of a microplate coated with monoclonal anti-adiponectin antibody. The content of the wells was incubated for 2 hours at room temperature. Following the incubation, the wells were aspirated and washed with 400 µl of diluted wash buffer repeating the process three times for a total of four washes. After the last wash, the plate was inverted and blotted against clean paper towels for complete removal of liquid and un-captured molecules. 200 µl of polyclonal conjugate antibody was added to each well and incubated for 2 hours to allow binding to the captured molecule. A second washing step (like previous) was done following the incubation. 200 µl of substrate solution (equal volumes of Colour Reagents A and B mixed together) was added and incubated for 30 minutes. The reaction was stopped by adding 50 µl of stop solution which bound to the complex and changed the colour in the wells from blue to yellow. The optical density of each well was measured within 30 minutes, using a 680 microplate reader from Bio-Rad Laboratories set to 450 nm with wavelength correction set at 540 nm.

2.7.1.4 Calculation of the Results

Samples were run in duplicate, averaged and the average value was corrected by subtracting the average zero standard optical density. The optical density for the standards was plotted versus the concentration of the standards and the best curve was drawn. The data was linearized and analyzed using the log transformation and regression analysis. The Adiponectin concentration (x) of each sample was determined by using the equation $Y = b + (m * x)$, where y= optical density (OD) of sample, m= slope of the plotted standard curve and b= standard curve intercept. The concentration read from the standard curve was multiplied by the dilution factor of the samples.

2.7.1.5 Precision

The minimum detectable dose (MDD) of the assay ranged from 0.079 - 0.891 ng/ml with a mean value of 0.246 ng/ml. The intra- and inter-assay variations were evaluated by measuring three samples of known concentration twenty times on one plate and in forty separate assays respectively. The intra-assay coefficient of variation (CV) was between 2.5% and 4.7%, and the inter-assay CV of 5.8- 6.9%.

2.7.2 Resistin

Serum levels of resistin were measured using a Quantikine Human Resistin Immunoassays (R&D Systems, Abingdon, Oxon, UK) (catalogue number SRSN00). The assay was conducted according to the manufacturer's instructions.

2.7.2.1 Samples Dilution

Serum samples were diluted to 5- fold dilution. 60 µl of sample was added to 240 µl of Calibrator Diluent RD5K.

2.7.2.2 Resistin Standard Preparation

The resistin standard was reconstituted with 1.0 ml of deionized water to produce a stock solution of 100 ng/ml. The standard was mixed to ensure complete reconstitution and allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. The stock solution was used to produce a dilution series of 10, 5, 2.5, 1.25, 0.62, 0.31, 0.16 ng/ml (Figure 2.2). Each tube was mixed thoroughly before the next transfer. The 10 ng/ml standard served as the high standard. Calibrator Diluent RD5K served as the zero standard (0 ng/ml).

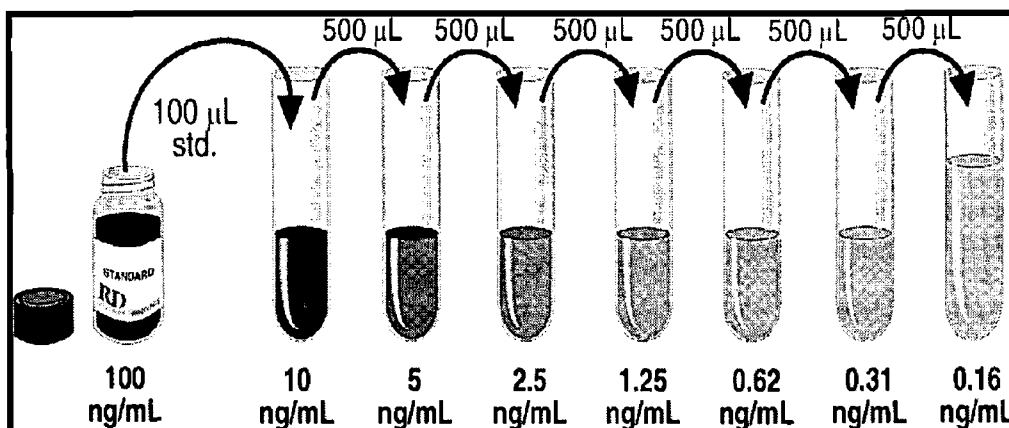


Figure 2.2 Resistin standard preparations. Seven series of dilution was performed in Calibrator Diluent RD5K. 900 µl of the Calibrator Diluent RD5K was pipetted into the 10 ng/ml tube and 500 µl was pipetted into the remaining tubes. The first dilution step was conducted by adding 100 µl of the stock solution (100 ng/ml) to the 900 µl of Calibrator Diluent RD5K. In the subsequent dilution steps, 500 µl of the previous dilution was added to prepare the next dilution. The dilution series produced were 10, 5, 2.5, 1.25, 0.62, 0.31 and 0.16 ng/ml.

2.7.2.3 Assay Procedure

First, 100 µl of diluted serum sample, standards, and Assay Diluent RD1-19 were pipetted into each of 96 wells of a microplate coated with monoclonal anti-resistin antibody. The content of the wells was incubated for 2 hours at room temperature. Following the incubation, the wells were aspirated and washed with 400 µl of diluted wash buffer, repeating the process three times for a total of four washes. After the last wash, the plate was inverted and blotted against clean paper towels for complete removal of liquid and un-captured molecules. 200 µl of polyclonal conjugate antibody was added to each well and incubated for 2 hours to allow binding to the captured molecule. A second washing step (like previous) was done following the incubation. 200 µl of substrate solution (equal volumes of Colour Reagents A and B mixed together) was added and incubated for 30 minutes. The reaction was stopped by adding 50 µl

of stop solution which bound to the complex and changed the colour in the wells from blue to yellow. The optical density of each well was measured within 30 minutes using a 680 microplate reader from Bio-Rad Laboratories set to 450 nm with wavelength correction set at 540 nm.

2.7.2.4 Calculation of the Results

Calculation of adiponectin serum concentrations were performed as previously described in **section 2.8.1.4**.

2.7.2.5 Precision

The minimum detectable dose (MDD) of the assay ranged from 0.010 - 0.055 ng/ml with a mean value of 0.026 ng/ml. The intra- and inter-assay variations were evaluated by measuring three samples of known concentration twenty times on one plate and in forty separate assays respectively. The intra-assay coefficient of variation (CV) was between 3.8% to 5.3% and the inter-assay CV of 7.8% to 9.2%.

2.8 Genetic Studies

2.8.1 DNA Extraction

Genomic DNA was isolated from whole blood sample of each subject using phenol- chloroform DNA extraction method. 1 ml of red blood cells (RBC) lysis solution was added to 1 ml of whole blood in a sterile tube. The tube was mixed gently by inversion and kept on crushed ice for 10 minutes. The sample was then centrifuged at $1,132 \times g$ (3,500 rpm) for 10 minutes and the supernatant was discarded. This step was repeated 2- 3 times until a clean white blood cells (WBC) pellet was obtained. The WBC pellet was suspended in 1 ml of resuspension buffer by gentle inversion. The tube was then vortexed for 30 seconds, centrifuged at $2,310 \times g$ (5,000 rpm) for 5 minutes and the supernatant was gently poured off. 500 μ l of resuspension buffer, 75 μ l TEN (Tris/EDTA/ NaCl) solution 10X, 75 μ l 10% sodium dodecyl sulphate (SDS) and 5 μ l proteinase K (10 mg/ml) were added, mixed well and incubated at 57°C for at least 1 hour to ensure efficient lysis. 500 μ l of phenol was added to the lysed cells and vortexed until the sample became uniformly viscous and rendered milky. The tube was then centrifuged at $2,310 \times g$ (5,000 rpm) for 5 minutes and the aqueous upper layer was transferred to a new sterile eppendorf tube. 500 μ l of chloroform- Isoamyl alcohol 24: 1 was added to the tube and mixed until the sample became homogenous and milky. The tube was re-centrifuged at $2,310 \times g$ (5,000 rpm) for 5 minutes and the aqueous upper layer was transferred to a new sterile eppendorf tube. Following that, 1 ml of cold absolute ethanol was added to the sample and mixed by inversion until a precipitation of DNA forms. The tube was centrifuged at full speed ($18,000 \times g$) for 20 minutes. Ethanol supernatant was completely discarded and the tube was left upside-down for 20 minutes. Finally, 75 μ l of distilled water was added to the tube and kept for 30 minutes at room temperature. The tube was then vortexed, pulse centrifuged and incubated at 65°C for 5 minutes. The tube was re-vortexed, re-centrifuged and kept in rotating position overnight at room

temperature. The quality and the integrity of the extracted total DNA was checked by standard ethidium-bromide 1% agarose gel electrophoresis and quantification was performed spectrophotometrically at 260 nm (Pharmacia Biotech, Ultrospec 3000 UV/VIS Spectrometer, Freiburg, Germany). All samples had a 260/280 nm absorbance ratio between 1.6 and 1.79. The tube containing the DNA was labelled appropriately and stored at -20°C for subsequent characterization of the adiponectin gene SNPs +45T>G and +276G>T.

2.8.2 Assessment of the SNP +45T>G Restriction Fragment Polymorphism

Isolated DNA was used for determination of the single nucleotide polymorphism (SNP) in the adiponectin gene (+45T>G, rs2241766) in exon 2 (Figure. 2.3). The SNP +45T>G polymorphism of ADIPOQ was genotyped using the polymerase chain reaction (PCR) with subsequent restriction analysis of PCR products (RFLP). The method of the polymerase chain reaction-restriction fragmented length polymorphism (PCR-RFLP) was used as described previously by Mackevics *et al.* (Mackevics *et al.*, 2006).

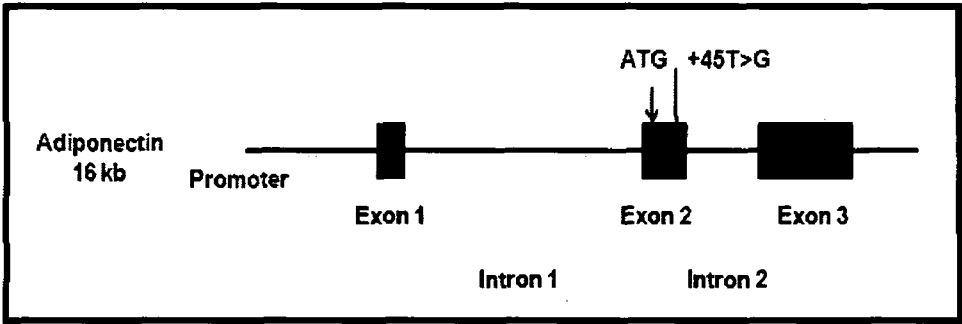


Figure 2.3 Schematic representation of the adiponectin gene SNP +45T>G. Black boxes denote exons and introns are found between the exons. ATG represents translation start site. The polymorphism +45T>G is represented by blue vertical line in exon 2. Adapted from (Heid *et al.*, 2006).

2.8.2.1 Primers

The DNA was amplified by PCR using specific primers for human adiponectin DNA, previously reported by Mackevics *et al.* (Mackevics et al., 2006). A 305-bp DNA fragment containing the polymorphic site was amplified by polymerase chain reaction (PCR) using forward primer 5'-TGT GTG TGT GGG GTC TGT CT-3' and reverse primer 5'-TGT GAT GAA AGA GGC CAG AA-3'. The primers were ordered online from the MWG website. The primer sequences and their details are shown below in Table 2.1.

Table 2.1 The primer sequences used for SNP +45T>G PCR and their details

Primer Name	Primer sequence	GC content	T _m °C	MW (g/mol)	Primer size (bp)	PCR Product size (bp)
FWD	5'-TGTGTGTGTGGGGTCTGTC T-3'	55%	59.4	6217	20	305
REV	5'-TGTGATGAAAGAGGCCAGA A-3'	45%	55.3	6239	20	

FWD represents forward primer and REV represents reverse primer

2.8.2.1.1 Primers Reconstitution and Dilution

Each primer was reconstituted in sterile nuclease-free ultra-pure water from Epicentre (cat no. W7350ML). A stock solution was made of each primer at a concentration of 100 µM (Table 2.2). The primer mixture was vortexed to ensure complete homogenized solution. 10 µl of stock primer solution was diluted into 90 µl of sterile nuclease-free ultra-pure water (final conc. 10 µM) and stored in aliquots at -20°C (repeated freezing and thawing were avoided).

Table 2.2 Reconstitution conditions of the primers of SNP +45T>G

Primer	Description and reconstitution conditions	
	No. of moles	Contained 12.3 nmoles lyophilized primer
FWD	Reconstitution	123 μ l of sterile nuclease-free ultra-pure water was added to give a stock solution of 100 μ M
	No. of moles	Contained 8.1 nmoles lyophilized primer
REV	Reconstitution	81 μ l of sterile nuclease-free ultra-pure water was added to give a stock solution of 100 μ M
	No. of moles	Contained 8.1 nmoles lyophilized primer

FWD represents forward primer and REV represents reverse primer

2.8.2.2 PCR Mastermix

Trials to optimize the PCR reaction were conducted, including troubleshooting with annealing temperature and primer concentrations. PCR-mediated *in vitro* amplification of genomic DNA was carried out with BioTherm™ Taq DNA Polymerase (Catalogue No. GC-002-0500) using a DNA Engine® Peltier Thermal Cycler (PTC-0200 DNA Engine) from Bio-Rad Laboratories. Table 2.3 illustrates the PCR mastermix preparations for the SNP +45T>G. The PCR reaction was carried using 5 μ l of DNA in a volume of 50 μ l containing 5 μ l of 10XPCR BioTherm buffer with a final concentration of 1.5 mM magnesium chloride, 0.2 μ M of each primer, 200 μ M deoxyribonucleotide triphosphate (dNTPs), and 2.5 U of Blotherm®-Taq-Polymerase.

Table 2.3 PCR mastermix preparations for the SNP +45T>G

Stock Conc.	Reagents	Volume	Final conc.
-	Sterile nuclease-free ultra-pure water	36.5 μ i	-
15mM	10x PCR buffer + MgCl ₂ (15mM)	5.0 μ i	1.5 mM
10 mM	dNTPs	1.0 μ i	200 μ M
10 μ M	SNP +45T>G FWD Primer	1.0 μ i	0.2 μ M
10 μ M	SNP +45T>G REV Primer	1.0 μ l	0.2 μ M
5 U/ μ l	Biotherm®-Taq-Polymerase	0.5 μ i	2.5 U
20ng/ μ l	DNA	5.0 μ i	100 ng (1 ng-1 μ g)
Total Volume		50 μ l	

2.8.2.3 PCR Thermal Cycler Program

The PCR thermal cycler program is illustrated in Table 2.4. Thirty five cycles of amplification were performed and each cycle consisted of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, with additional steps of initial denaturation at 95°C for 5 minutes and final extension at 72°C for 7 minutes.

Table 2.4 The thermocycler program of the SNP +45T>G

PCR step	Temperature	Time	No. of cycles
Initial denaturation	95°C	5 minutes	1x cycle
Denaturation	95°C	30 seconds	35x cycles
Annealing	55°C	30 seconds	
Elongation	72°C	30 seconds	
Extension	72°C	7 minutes	1x cycle

2.8.2.4 Estimation of PCR Products

5 µl of PCR amplified product was mixed with 1 µl of 6x loading dye and loaded into 2% low electroendosmosis (LE) agarose gel mixed with ethidium bromide (0.5 µg/ml ethidium bromide was added to the molten Agarose). The loaded gel was electrophorised under 50 V for 90 minutes using 1x Tris/Acetate/EDTA buffer (TAE) buffer along with a standard DNA Molecular Weight Marker (fermentas, DNA marker pBR322/Alu I (Marker 20), Catalogue no. SM0121) until the purple/blue dye markers reached the end of the gel. The stained gel was subsequently visualized under ultraviolet light using Cole-Parmer High Performance UV Transilluminator and the molecular weight of PCR products was estimated. If the gel showed the desired fragment, the remaining 45 µl of PCR products was either subjected to digestion reaction or stored frozen at -20°C for later analysis.

2.8.2.5 Digestion with the Restriction Enzyme Eco88I (*Ava*I) for the SNP +45T>G

10 µl of the PCR product was digested with 10U of Eco88I (*Ava*I) in the recommended 10X Tango™ Buffer (MBI fermentas, ER 0381) for overnight at 37°C. Table 2.5 shows the digestion reaction protocol.

To validate enzyme activity, 1 µg of Lambda DNA as well as 5 µl of PCR product mixed with 1 µg of Lambda DNA were digested using the same protocol shown in Table 2.5. In both cases, the digestion pattern of Lambda DNA was obtained indicating that the enzyme is active and PCR reagents did not inhibit enzyme activity.

Table 2.5 The digestion reaction protocol used to assess the SNP +45T>G

Reagents	Volume
Restriction enzyme <i>AvaI</i> (10U)	1 μ l
10 X Buffer Tango™	2 μ l
Nuclease-free ultra-pure water	17 μ l
PCR product (0.1-0.5 μ g of DNA)	10 μ l
Total	30 μl
Incubate for overnight at 37 °C	

2.8.2.6. Estimation of the Digested Products

After digestion with the restriction enzyme *AvaI*, fragments were loaded onto a 12% native polyacrylamide gel (Table 2.6). Polymerization was initiated by the addition of 10 μ l N, N, N', N'- Tetramethylethylenediamine (TEMED), the gel cast and the lane combs were immediately placed onto the gels. After polymerization, the lane combs were removed, and excess acrylamide removed from the lanes by rinsing with distilled water (dH₂O). For the preparation of DNA fragments for electrophoresis separation, 18 μ l of each sample mixed with 3 μ l of 6x loading dye was loaded in each well. 1x Tris/Borate/EDTA (TBE) was used as electrophoresis buffer. To eliminate false positive results, i.e. eventual contamination, a negative control was included (5 μ l of sterile nuclease-free ultra-pure water instead of the DNA was added to 45 μ l of the master mix). For each sample, the undigested and digested PCR products were loaded in wells beside each other on the gel to further rule out any false positive results and confirm whether the digestion had occurred or not. A known positive control was always included on each gel run to validate the processing. Electrophoresis was performed under 50 mA for 60 minutes. Finally gels were incubated in 1xTBE buffer containing 15-20 μ l of ethidium bromide (12.5 μ g/ μ l) with shaking for 10 minutes. The DNA fragments were

visualized by UV illumination and the sizes were estimated by comparison with known size markers (fermentas, DNA marker pBR322 / BsuRI (Marker 5), Catalog. No. SM0271).

Table 2.6 12% native polyacrylamide gel reagent recipe. Mini-PROTEAN® 3 from Bio-Rad Laboratories was used to produce polyacrylamide gels.

Reagents	Volume
Acrylamide: Bis solution 1:29	4.8 ml
Deionised water	4.8 ml
5xTBE buffer	2.4 ml
10 % APS	100 µl
TEMED	10 µl
Total	Approx. 12.5 ml

TBE buffer: Tris/Borate/EDTA buffer, TEMED: N, N, N', N'-Tetramethylethylenediamine, APS: Ammonium persulfate

Sequence from human adiponectin gene, base pairs spanning 93065918 to 93066252 according to location on NCBI is indicated below. Primers are underlined; restriction site is indicated in bold and highlighted in green.

TAGGTCCCAACTGGGTGTGTGTGTGGGGTCTGTCTCTCCATGGCTG
ACAGTGCACATGTGGATTCCAGGGCTCAGGATGCTGTTGCTGGGAG
CTGTTCTACTGCTATTAGCTCTGCCC**GG**KCATGACCAGGAAACCAC
GACTCAAGGGCCCGGAGTCCTGCTTCCCCTGCCCAAGGGGGCCTG
CACAGGTTGGATGGCGGGCATCCCAGGGCATCCGGGGCCATAATGG
GGCCCCAGGCCGTGATGGCAGAGATGGCACCCCTGGTGAGAAGGG
TGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTCTTTCATCACA
GACCTCCTACACTGA

K= the IUPAC ambiguity code for the T/G alternative bases.

Primers (forward primer: TGTGTGTGTGGGGTCTGTCT, reverse primer: TTCTGGCCTCTTTCATC ACA) were designed to span the region producing a 305 bp product. *Ava*I cuts the 305 bp PCR product at position 105. If the restriction cutting site is present in both alleles (G/G), the PCR product produces fragments of 105 and 200 bp length; if the restriction cutting site is present in only one allele (T/G), the size of the fragments are 305, 200, and 105 bp; if restriction cutting site is absent in both alleles (T/ T), only an undigested 305 bp fragment is visible on the gel. The gel patterns obtained for the (T/ T), (T/G), and (G/G) genotypes are shown in Table 2.7 and Figure 2.4 below.

Table 2.7 The sizes of the fragments obtained after the digestion reactions of SNP +45T>G for the (T/ T), (T/G), and (G/G) genotypes

T/T	T/G	G/G
(homozygote wild type)	(heterozygote)	(homozygote mutant)
305 bp	305 bp	
	200 bp	200 bp
	105 bp	105 bp

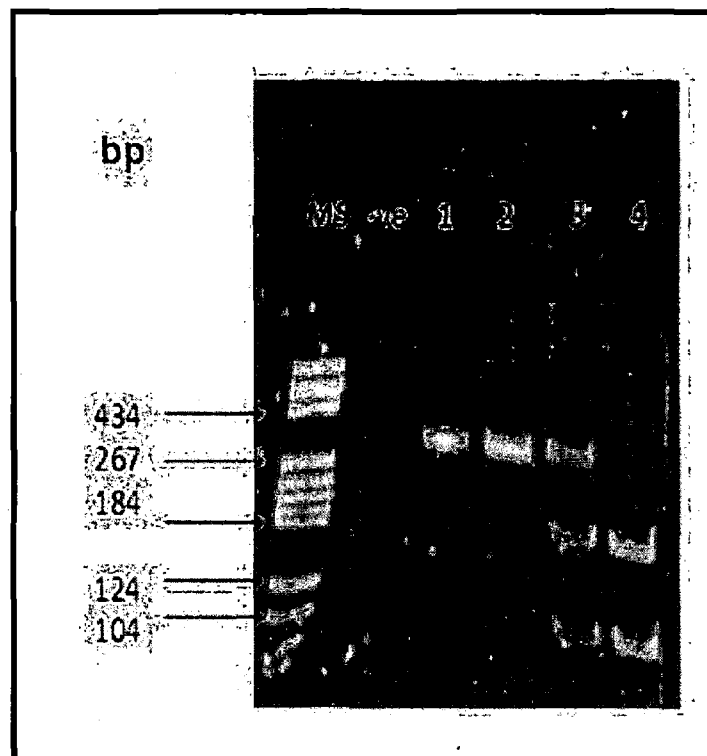


Figure 2.4 The gel patterns obtained after loading the digested fragments for the (T/ T), (T/G), and (G/G) genotypes. M5 represents marker 5, (-ve) represents negative control, (sample 1) represents undigested sample, (sample 2) represents wildtype genotype, (sample 3) represents heterozygous genotype and (sample 4) represents homozygous genotype.

The detection details of the adiponectin SNP +45T>G is summarized in Table 2.8.

Table 2.8 Summary of the detection details of adiponectin SNP +45T>G

Forward (sense) primer* 5'- TGTGTGTGTGGGGTCTGTCT-3'		
Reverse (antisense) primer* 5'-TGTGATGAAAGAGGCCAGAA-3'		
Temperature	Time	No. of cycles
95°C	5 min	1x cycle
95°C	30 s	35x cycles
55°C	30 s	
72°C	30 s	
72°C	7 min	1x cycle
PCR product length= 305bp		
Restriction enzyme Eco88I (<i>Ava</i> I) [‡]		
RFLP conditions 37 °C/ overnight		
RFLP product length		
T/T	T/G	G/G
(homozygote wild type)	(heterozygote)	(homozygote mutant)
305 bp	305 bp	
	200 bp	200 bp
	105 bp	105 bp

*Primer sequences from Mackevics *et al.* (Mackevics et al., 2006)

[‡] Fermentas life science

2.8.3 Assessment of the SNP +276G>T Restriction Fragment Polymorphism

Isolated DNA was used for determination of the SNP in the adiponectin gene (+276G>T, rs1501299) in intron 2 (Figure. 2.5). The +276G>T polymorphism of ADIPOQ was genotyped using the polymerase chain reaction (PCR) with subsequent restriction analysis of PCR products (RFLP). The method of the polymerase chain reaction -restriction fragmented length polymorphism (PCR -RFLP) was used as described previously by Mackevics *et al.* (Mackevics *et al.*, 2006).

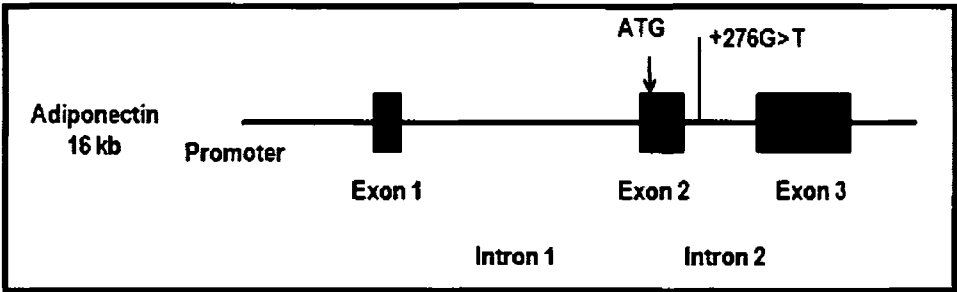


Figure 2.5 Schematic representation of the adiponectin gene SNP +276G>T. Black boxes denote exons and introns are found between the exons. ATG represent translation start site. The polymorphism +276G>T is represented by blue vertical line in intron 2. Adapted from (Heid *et al.*, 2006).

2.8.3.1 Primers

The DNA was amplified by PCR using specific primers for human adiponectin DNA, previously reported by Mackevics *et al.* (Mackevics *et al.*, 2006). Because there was no restriction site in the polymorphic region, one base in the forward primer was exchanged thus artificially creating the restriction cut in the region of the polymorphic site of the PCR product. The primer in which one base is modified is described as a mismatch-primer.

The natural sequence of the wild type allele is AAGGC. In the mutated allele it is AAGTC. By means of the mismatch primer the sequence was modified so that in the wild type allele it was GAGGC, in the mutated allele however it changed to GAGTC. Upon applying the restriction enzyme *HinfI*, which recognizes the GAGTC sequence, both alleles could be distinguished, whereby only the mutated allele of the PCR fragment was recognized and cut by the *HinfI*.

A 110-bp DNA fragment was amplified by PCR using forward primer 5'-CTA CAC TGA TAT AAA CTA TAT GGA G-3' and reverse primer 5'-CCC CAA ATC ACT TCA GGT TG-3'. The primers were ordered online from the MWG website. The primer sequences and their details are shown below in Table 2.9.

Table 2.9 The primers sequence used for SNP +276G>T PCR and their details

Primer Name	Primer Sequence	GC content	T _m °C	MW (g/mol)	Primer size (bp)	PCR Product size (bp)
FWD	5'-CTACACTGATATAAACTATATGGAG-3'	32%	56.4	7673	25	110
REV	5'-CCCCAAATCACTTCAGGTTG-3'	50%	57.3	6036	20	

FWD represents forward primer and REV represents reverse primer

2.8.3.1.1 Primers Reconstitution and Dilution

Each primer was reconstituted in sterile nuclease-free ultra-pure water. A stock solution was made of each primer at a concentration of 100 pM (Table 2.10). The primer mixture was vortexed to ensure complete homogenized solution. 10 µl of stock primer solution was diluted into 90

μl of sterile nuclease-free ultra-pure water (final conc. 10 μM) and stored in aliquots at -20°C (repeated freezing and thawing were avoided).

Table 2.10 Reconstitution conditions of the primers of the SNP +276G>T

Primer	Description and Reconstitution Conditions	
FWD	No of moles	Contained 11.2 nmoles lyophilized primer
	Reconstitution	112 μl of sterile nuclease-free ultra-pure water was added to give a stock solution of 100 μM
REV	No of moles	Contained 16.1 nmoles lyophilized primer
	Reconstitution	161 μl of sterile nuclease-free ultra-pure water was added to give a stock solution of 100 μM

FWD represents forward primer and REV represents reverse primer

2.8.3.2 PCR Mastermix

Trials to optimize the PCR reaction were conducted, including troubleshooting with annealing temperature and primer concentrations. PCR-mediated *in vitro* amplification of genomic DNA was carried out with BioTherm™ Taq DNA Polymerase (Catalogue No. GC-002-0500) using a DNA Engine® Peltier Thermal Cycler (PTC-0200 DNA Engine) from Bio-Rad Laboratories. Table 2.11 illustrates the PCR mastermix preparations for the SNP +276G>T. The PCR reaction was carried using 5 μl of DNA in a volume of 50 μl containing 5 μl 10X Biotherm PCR buffer with a final concentration of 1.5 mM magnesium chloride, 0.2 μM of each primer, 200 μM deoxyribonucleotide triphosphate (dNTPs), and 2.5 U of Biotherm®-Taq-Polymerase.

Table 2.11 PCR Mastermix Preparations for the SNP +276G>T

Stock Conc.	Reagents	Volume	Final conc.
-	Sterile nuclease-free ultra-pure water	36.5 µl	.
15mM	10x PCR buffer + MgCl ₂ (15 mM)	5.0 µl	1.5 mM
10 mM	dNTPs	1.0 µl	200 µM
10 µM	SNP +276G>T FWD	1.0 µl	0.2 µM
10 µM	SNP +276G>T REV	1.0 µl	0.2 µM
5 U/ µl	Blotherm®-Taq-Polymerase	0.5 µl	2.5 U
20ng/ pl	DNA	5.0 µl	100 ng (1 ng-1 µg)
Total Volume		50 µl	

2.8.3.3 PCR Thermal Cycler Program

The PCR thermal cycler program is illustrated in Table 2.12. Thirty five cycles of amplification were performed and each cycle consisted of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, with additional steps of initial denaturation at 95°C for 5 minutes and final extension at 72°C for 7 minutes.

Table 2.12 The Thermocycler Program of the SNP +276G>T

PCR Step	Temperature	Time	No. of cycles
Initial denaturation	95°C	5 minutes	1x cycle
Denaturation	95°C	30 seconds	35x cycles
Annealing	55°C	30 seconds	
Elongation	72°C	30 seconds	
Extension	72°C	7 minutes	1x cycle

2.8.3.4 Estimation of PCR Products

5 µl of PCR amplified product was mixed with 1 µl of the loading dye and loaded into 2.5% LE agarose gel mixed with ethidium bromide (0.5 µg/ml ethidium bromide was added to the molten Agarose). The loaded gel was electrophorised under 50 V for 90 minutes using 1x TAE buffer along with a standard DNA Molecular Weight Marker (fermentas, DNA marker pBR322 / BsuRI (Marker 5), Catalogue. No. SM0271) until the purple /blue dye markers reached the end of the gel. The stained gel was subsequently visualized under ultraviolet light using Cole-Parmer High Performance UV Transilluminator and the molecular weight of PCR products was estimated. If the gel showed the desired fragment, the remaining 45 µl of PCR products was either digested or stored frozen at -20°C for later digestion reaction.

2.8.3.5 Digestion with the Restriction Enzyme *HinfI* for the the SNP +276G>T

10 µl of the PCR product was digested with 10 U of *HinfI* in the recommended 10X Buffer R (MBI fermentas, ER 0801) for overnight at 37°C. Table 2.13 shows the digestion reaction protocol.

To validate enzyme activity, 1 µg of Lambda DNA as well as 5 µl of PCR product mixed with 1 µg of Lambda DNA were digested using the same protocol shown in Table 2.5. In both cases, the digestion pattern of Lambda DNA was obtained indicating that the enzyme is active and PCR reagents did not inhibit enzyme activity.

Table 2.13 The digestion reaction protocol used to assess the SNP +276G>T

Reagents	Volume
Restriction enzyme <i>Hinfl</i> (10U)	1 μ l
10 X Buffer Tango™	2 μ l
Nuclease-free ultra-pure water	17 μ l
PCR product (0.1-0.5 μ g of DNA)	10 μ l
Total	30 μl
Incubate for overnight at 37 °C	

2.8.3.6 Estimation of Digested Products

After digestion with the restriction enzyme *Hinfl*, fragments were loaded onto a 12% native polyacrylamide gel (Table 2.6). Polymerization was initiated by the addition of 10 μ l N, N, N', N' - Tetramethylethylenediamine (TEMED), the gel cast and the lane combs were immediately placed onto the gels. After polymerization, the lane combs were removed, and excess acrylamide removed from the lanes by rinsing with dH₂O. For the preparation of DNA fragments for electrophoresis separation, 18 μ l of each sample mixed with 3 μ l of 6x loading dye was loaded in each well. 1x TBE was used as electrophoresis buffer. To eliminate false positive results, i.e. eventual contamination, a negative control was included (5 μ l of sterile nuclease-free ultra-pure water instead of the DNA was added to 45 μ l of the master mix). For each sample, the undigested and digested PCR products were loaded in wells beside each other on the gel to further rule out any false positive results and confirm whether the digestion has occurred or not. A known positive control was always included on each gel run to validate the processing. Electrophoresis was performed under 50 mA for 60 minutes. Finally, gels were incubated in 1xTBE buffer containing 15-20 μ l of ethidium bromide (12.5 μ g/ μ l) with shaking for 10 minutes. The DNA fragments were visualized by UV

illumination and the sizes were estimated by comparison with known size markers (fermentas, DNA marker pBR322 / BsuRI (Marker 5), Catalogue. No. SM0271).

Sequence from human adiponectin gene, base pairs spanning 1501259 to 1501399 according to location on NCBI is indicated below. Primers are underlined; restriction site is indicated in bold and highlighted in green.

TTCATCACAGACCTCCTACACTGATATAAACTATATGAAGKCATTCAT
TATTAATAAGGCCTAGACACAGGGAGAAAGCAAAGCTTTTTTATGT
TAACCATAAGCAACCTGAAGTGATTTGGGGTTGGTCTTCCAAGGAT
K= the IUPAC ambiguity code for the T/G alternative bases

Primers (Froward Primer: CTACACTGATATAAACTATATGAAG, reverse Primer: CAACCTGAAGTGA TTTGGGG) were designed to span the region producing a 110 bp product. *HinfI* cuts the 110 bp PCR at position 27. If the restriction cutting site is present in both alleles (T/T), the PCR product produces fragments of 27 and 84 bp length; if the restriction cutting site is present in only one allele (T/G), the size of the fragments are 110, 84, and 27 bp; if restriction cutting site is absent in both alleles (G/ G), only undigested 110 bp fragment is visible on the gel. The gel patterns obtained for the (G/G), (T/G), and (T/ T) genotypes are shown in the Table 2.15 and Figure 2.6 below.

Table 2.14 The sizes of the fragments obtained after the digestion reactions of the SNP +276G>T for the (G/ G), (T/G), and (T/T) genotypes

G/G	T/G	T/T
(homozygote wild type)	(heterozygote)	(homozygote mutant)
110 bp	110 bp	
	84 bp	84 bp
	27 bp	27 bp

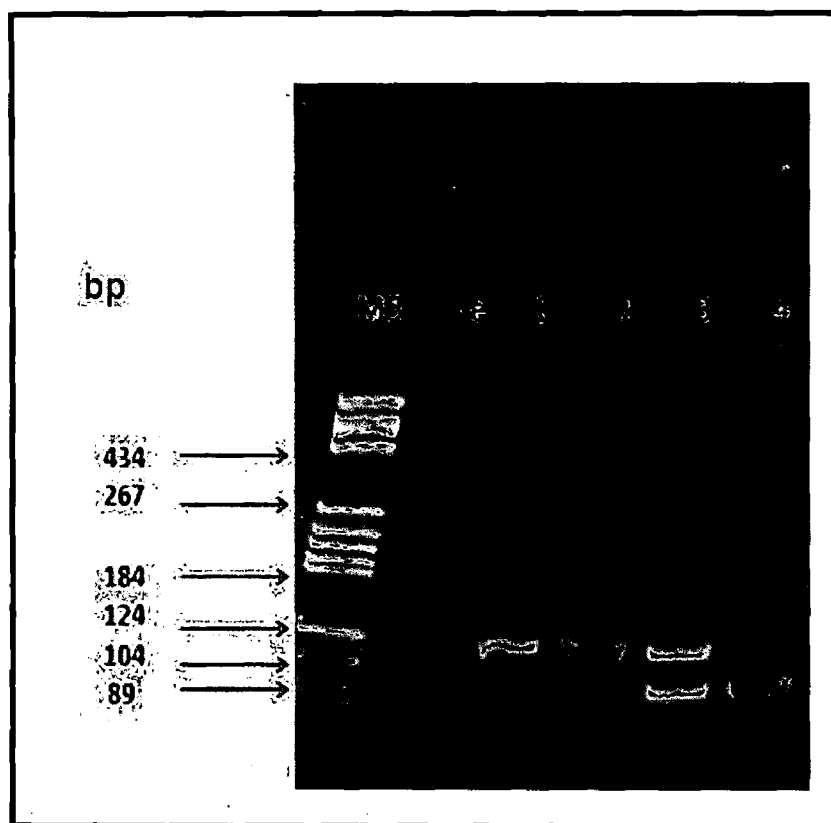


Figure 2.6 The gel patterns obtained after loading the digested fragments of SNP +276G>T for the (T/ T), (T/G), and (G/G) genotypes. M5 represents marker 5, (-ve) represents negative control, (sample 1) represents undigested sample, (sample 2) represents wildtype genotype, (sample 3) represents heterozygous genotype and (sample 4) represents homozygous genotype. To get clear definition between the bigger bands of the marker 5 and the digested samples, the 27bp runs off the gel.

The detection details of the adiponectin SNP +276G>T is summarized in Table 2.15.

Table 2.15 Summary of the detection details of adiponectin SNP +276G>T

Forward primer* 5'-CTACACTGATATAAACTATATGGAG-3'		
Reverse primer* 5'-CCCCAAATCACTTCAGGTTG-3'		
Temperature	Time	No. of cycles
95°C	5 m	1x cycle
95°C	30 s	35x cycles
55°C	30 s	
72°C	30 s	
72°C	7 m	1x cycle
PCR product length 110 bp		
Restriction enzyme <i>Hinf</i> I*		
RFLP conditions 37 °C/ overnight		
RFLP product length		
G/G	T/G	T/T
(homozygote wild type)	(heterozygote)	(homozygote mutant)
110 bp	110 bp	
	83 bp	83 bp
	27 bp	27 bp

*Primer sequences from Mackevics *et al.* (Mackevics et al., 2006)

* Fermentas life science

2.9 Statistical Analysis

Statistical analysis was performed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). All results were presented as mean \pm standard deviation (SD). Comparisons between means were performed using the Student's independent t-test (two-tailed) for normally distributed data or the Mann-Whitney U test for parameters presenting with non-normal distribution. Categorical variables were analyzed by the Chi-square test. Cramer's v test was used as post-test to determine strengths of association after chi-square has determined significance. The z-test was used to compare sample and population proportions to determine if there is a significant difference. P-values less than 0.05 were considered significant. Pearson and spearman coefficient was used to explore the association between total adiponectin, resistin and the metabolic and clinical variables.

The distribution of the alleles of SNP +45T>G and SNP +276G>T was tested for the Hardy-Weinberg equilibrium. The proportions of genotypes and alleles were compared by the Chi-square test analysis. Correlations of SNP +45T>G and SNP +276G>T genotypes with serum adiponectin concentrations and the various metabolic parameters were evaluated by univariate general linear model analyses. All analyses were adjusted for gender, age and BMI. Analyses were performed for the whole sample, as well as for men and women separately, to verify the homogeneity of genetic effect among men and women.

Chapter 3- Results

3.1 Clinical Characteristics of the Study Subjects

The clinical characteristics of type 2 diabetic patients and control participants of the present study are summarized in Table 3.1. The mean age of T2DM patients was 58 ± 11 years, and the mean BMI was 34.2 ± 7 kg/m². The mean age of the control group was 41 ± 6 years, and the mean BMI was 29.0 ± 4.5 kg/m². The diabetic group consisted of 66 male and 74 female subjects, while the control group consisted of 60 male and 6 females. The difference in the number between genders was due to unavailability of female control subjects and issues related to time constraints. In tests where unequal numbers of male/ female subjects are known to affect the final results, a correction for gender and statistical adjustments was made. 80% of T2DM patients are classified as obese, while only 43% of the control subjects are classified as obese. Using the ATP-III definition (NCEP, 2001), 60% of T2DM patients fulfilled the criteria for metabolic syndrome.

Table 3.1 Clinical characteristics of the study subjects

	T2DM patients	Control
	Mean± SD	Mean± SD
n (%)	140 (68)	66 (32)
Male/ Female	66/74	60/6**
Age (yrs)	58± 11	41± 6
Height (cm)	161.2± 8.9	172.9± 5.3
Weight (kg)	85.7± 17.8	87.4± 14.0
BMI (kg/m ²)	34.2± 7.0	29.0± 4.5
Obese (%)	80	43
SBP (mmHg)	147± 22	121 ± 6
DBP (mmHg)	77± 13	70 ±8
HbA1C (%)	8.12± 1.74	6.22± 1.20
FBG (mmol/l)	9.8± 4.1	6.5± 2.0
IR	2.1± 2.4	.
Hypertension (%)	110 (78.6)	.
Smoking (%)	19.0	39.3
MetS (%)	60	.

Data are means ± standard deviation. For the dichotomously coded, obese hypertension, smokers and metabolic syndrome, percentages are given. Number of subjects for each characteristic varies slightly due to occasional missing values. BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; HbA1c glycated hemoglobin; FBG fasting blood glucose; MetS metabolic syndrome; IR insulin resistance value estimated using the triglyceride/HDL-cholesterol index, values of ≥ 3 were considered insulin resistant. *Hypertension is defined as a systolic blood pressure (SBP) ≥ 130 mm Hg or diastolic blood pressure (DBP) ≥ 85 mm Hg or receiving antihypertensive medication therapy.

3.2 Serum Adiponectin Levels in T2DM Patients and in Controls

Samples without measured adiponectin and those which had readings outside the optical density range of the standards (either lower or higher) were excluded from the analysis. Twenty nine samples were excluded (eleven T2DM patient samples and eighteen control samples) for the following reasons: First, no serum sample was obtained from some participants (n= 5); only EDTA-coated tubes was available for the molecular analysis and there was a difficulty in obtaining a second sample from those participants. Second, some serum samples (n= 9) gave readings either below (n=5) or above (n= 4) the linear range of the serum adiponectin standards. Samples with low readings were below the limit of detection for this kit. Unfortunately it was not possible to dilute and re-analyze the samples which fell beyond the linear range because of logistical complications of delivery of additional ELISA kits to Bahrain coupled with funding constraints. Third, some serum samples tubes were not stored or handled properly (for example, 15 number of samples in plain tubes had not been centrifuged to separate the serum, or some were completely hemolyzed.

The serum adiponectin levels were measured in 129 T2DM patients and 48 control subjects. The mean serum levels of adiponectin were significantly lower in subjects with T2DM compared to the control healthy subjects (total adiponectin 5.6 ± 4.1 vs 7.3 ± 4.6 $\mu\text{g/ml}$, $P=0.020$). Adjustment for age and BMI did not change mean adiponectin levels.

Table 3.2 Difference in serum adiponectin concentration between T2DM patients and controls

	T2DM patients n= 129	Control n= 48	P-value*
	Mean \pm SD	Mean \pm SD	
Adiponectin Conc. $\mu\text{g/ml}$	5.6 ± 4.1	7.3 ± 4.6	0.020

Data are means \pm standard deviation

*T-test. P-value is significant at 0.05 level.

To exclude that the results regarding the association of adiponectin with T2DM are subjected to bias due to unequal numbers of males and females in the diabetic and control groups given the higher level of adiponectin in females overall, a second comparison was conducted between male subjects (Table 3.3) and female subjects (Table 3.4) of both group. The mean serum levels of adiponectin were significantly lower in male subjects with T2DM compared to the control healthy subjects (total adiponectin 4.7 ± 3.5 vs 6.7 ± 3.6 $\mu\text{g/ml}$, 0.006) (Table 3.3).

Table 3.3 Difference in serum adiponectin concentration between male T2DM patients and controls

	MaleT2DM patients n= 59	Male control subjects n= 42	P-value*
	Mean± SD	Mean± SD	
Adiponectin Conc. $\mu\text{g/ml}$	4.7 ± 3.5	6.7 ± 3.6	0.006

Data are means ± standard deviation

*T-test. P-value is significant at 0.05 level.

The mean serum levels of adiponectin were significantly lower in female subjects with T2DM compared to the control healthy subjects (total adiponectin 6.3 ± 4.4 vs 11.0 ± 8.5 $\mu\text{g/ml}$, 0.023) (Table 3.3).

Table 3.4 Difference in serum adiponectin concentration between female T2DM patients and controls

	Female T2DM patients n= 70	Female control subjects n= 6	P-value*
	Mean± SD	Mean± SD	
Adiponectin Conc. $\mu\text{g/ml}$	6.3 ± 4.4	11.0 ± 8.5	0.023

Data are means ± standard deviation

*T-test. P-value is significant at 0.05 level.

Consistent with the results of previous studies (Arita et al., 1999, Nishizawa et al., 2002), total adiponectin levels were significantly higher in women than in men (6.3 ± 4.4 vs 4.7 ± 3.5 $\mu\text{g/ml}$, $P=0.024$) in T2DM (Table 3.5) and (11 ± 8.5 vs 6.7 ± 3.6 $\mu\text{g/ml}$, $P=0.032$) in controls (Table 3.6). Further, in men and women pair-matched for age, BMI, adiponectin concentrations remained greater in women (Data are not shown).

Table 3.5 Difference in serum adiponectin concentration between male and female subjects with T2DM

	Diabetic		P-value*
	Male n= 59	Female n= 70	
	Mean± SD	Mean± SD	
Adiponectin Conc. $\mu\text{g/ml}$	4.7 ± 3.5	6.3 ± 4.4	0.024

Data are means \pm standard deviation

*T-test. P-value is significant at 0.05 level.

Table 3.6 Difference in serum adiponectin concentration between healthy control male and female subjects

	Controls		P-value*
	Male n= 42	Female n= 6	
	Mean± SD	Mean± SD	
Adiponectin Conc. $\mu\text{g/ml}$	6.7 ± 3.6	11.0 ± 8.5	0.032

Data are means \pm standard deviation

*T-test. P-value is significant at 0.05 level.

3.3 Association between Serum Adiponectin Levels and Clinical and Metabolic Parameters of the Study Population

The various cross-sectional associations of adiponectin in T2DM patients and controls are shown in Table 3.7. The demographic, clinical and metabolic characteristics of T2DM patients and controls were analyzed with serum adiponectin levels. Considering all diabetic subjects, serum adiponectin level showed significant inverse correlations with HbA1c, triglycerides and IR value estimated by the triglyceride/HDL-cholesterol index ($P=0.039$, $P=0.020$ and $P=0.012$ respectively), and also showed a positive correlation with HDL-C ($P=0.036$). No association was found with BMI, SBP, DBP, TC, LDL-C and FBG ($P>0.05$).

In the control group, there is no statistically significant association in the majority of the clinical and metabolic parameters with serum adiponectin levels. However, a very strong negative significant association was observed between SBP and serum adiponectin levels ($P<0.0001$). Other parameters were less strongly related to adiponectin levels.

Table 3.7 Correlations of adiponectin to various clinical and metabolic parameters in both T2DM patients and control group

	Adiponectin level			
	Diabetic n= 129		Controls n= 48	
	Pearson Correlation	P-value	Pearson Correlation	P-value
Age (yrs)	0.072	0.418	-0.093	0.531
BMI	-0.151	0.236	0.077	0.618
SBP (mmHg)	-0.050	0.582	-0.692	0.000**
DBP (mmHg)	-0.059	0.510	-0.205	0.347
HbA1C (%)	-0.182	0.039*	-0.531	0.279
TC (mmol/l)	0.028	0.764	-0.545	0.342
HDL-C (mmol/l)	0.197	0.036*	-0.531	0.357
LDL -C (mmol/l)	-0.008	0.929	-0.462	0.433
TG (mmol/l)	-0.217	0.020*	0.286	0.641
FBG (mmol/l)	-0.013	0.882	-0.574	0.234
IR	-0.235	0.012*		

Data is presented as pearson coefficients. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed). BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; HbA1c glycated hemoglobin; TC total cholesterol; HDL-C high density lipoprotein cholesterol; LDL-C Low density lipoprotein cholesterol; TG triglycerides; FBG fasting blood glucose; IR insulin resistance value estimated using the triglyceride/HDL-cholesterol index, values of ≥ 3 were considered insulin resistant.

Gender-based analysis was evaluated for both T2DM Patients and controls to assess the impact of the demographic, clinical and metabolic parameters on serum adiponectin levels in men and women.

3.3.1 T2DM Patients

Among T2DM patients, adiponectin had significant positive correlations with HDL-cholesterol levels and an inverse correlation with IR in men ($P=0.015$ and $P=0.018$ respectively). In women however, there were no statistically significant differences in any demographic, clinical and metabolic characteristics (Table 3.8).

Table 3.8 Correlations of adiponectin to various clinical and metabolic parameters among males and females of T2DM patients

	Adiponectin level			
	Male n= 59		Female n= 70	
	Pearson Correlation	P-value	Pearson Correlation	P-value
Age (yrs)	-0.057	0.670	0.204	0.090
BMI	-0.299	0.103	-0.097	0.596
SBP (mmHg)	-0.013	0.921	-0.093	0.449
DBP (mmHg)	0.071	0.599	-0.120	0.327
HbA1C (%)	-0.231	0.078	-0.135	0.263
TC (mmol/l)	0.013	0.929	-0.022	0.866
HDL-C (mmol/l)	0.340	0.015*	0.034	0.794
LDL -C (mmol/l)	-0.044	0.754	-0.012	0.926
TG (mmol/l)	-0.263	0.059	-0.152	0.235
FBG (mmol/l)	-0.049	0.713	0.036	0.768
IR	-0.331	0.018*	-0.124	0.339

Data is presented as pearson coefficients. *Correlation is significant at the 0.05 level (2-tailed). BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; HbA1c glycated hemoglobin; TC total cholesterol; HDL-C high density lipoprotein cholesterol; LDL-C Low density lipoprotein cholesterol; TG triglycerides; FBG fasting blood glucose; IR insulin resistance value estimated using the triglyceride/HDL-cholesterol index, values of ≥ 3 were considered insulin resistant.

3.3.2 Controls

In the control subjects, adiponectin did not show any significant correlations with the majority of the metabolic and clinical parameters in both genders. However, a strong negative association was observed between adiponectin levels and SBP in men (Table 3.9).

Table 3.9 Correlations of adiponectin to various clinical and metabolic parameters among males and females of the control group

	Adiponectin level			
	Male n= 42		Female n= 6	
	Pearson Correlation*	P-value	Spearman Correlation*	P-value
Age (yrs)	-0.034	0.830	-0.696	0.125
BMI	-0.052	0.744	1.000	.
SBP (mmHg)	-0.630	0.002**	-1.000	.
DBP (mmHg)	-0.349	0.121	-1.000	.
HbA1C (%)	.	.	-0.600	0.208
TC (mmol/l)	.	.	-0.791	0.111
HDL-C (mmol/l)	.	.	-0.500	0.391
LDL -C (mmol/l)	.	.	-0.700	0.188
TG (mmol/l)	.	.	-0.359	0.553
FBG (mmol/l)	.	.	-0.771	0.072

*Data is presented as pearson coefficients, because the sample size of female control subjects is only 6, spearman coefficients were used (pearson coefficients could not be applied for female control subjects (n= 6)). **Correlation is significant at the 0.01 level (2-tailed). BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; HbA1c glycated hemoglobin; TC total cholesterol; HDL-C high density lipoprotein cholesterol; LDL-C Low density lipoprotein cholesterol; TG triglycerides; FBG fasting blood glucose; IR insulin resistance value estimated using the triglyceride/HDL-cholesterol index, values of ≥ 3 were considered insulin resistant.

3.4 The Relationship between Serum Adiponectin and BMI

In pearson and spearman correlations, serum adiponectin levels did not show an independent role on BMI in both T2DM patients and controls. The only possible interaction between the serum adiponectin levels and the BMI status was therefore tested by subdividing the study participants according to the universal obesity cut-off point (ie below or above 30 kg/m²). Number of subjects varies slightly due to occasional missing values. Serum adiponectin levels were significantly different between subjects with BMI above or below 30 (obese vs non-obese subjects) in T2DM patients (P=0.0013) (Table 3.10) and in controls (P=0.0008) (Table 3.11).

Table 3.10 Serum adiponectin levels of patients with T2DM according to the universal obesity cut-off point (< or ≥ 30)

		Diabetic		P-value*
		Adiponectin Conc. µg/ml		
		n	Mean± SD	
BMI	< 30	16	6.6± 4.5	0.0013
	≥ 30	99	4.4± 2	

Data are means ± standard deviation. BMI; body mass index.

*T-test. P-value is significant at 0.05 level.

Table 3.11 Serum adiponectin levels of control subjects according to the universal obesity cut-off point (< or ≥ 30)

		Control		P-value*
		Adiponectin Conc. µg/ml		
		n	Mean± SD	
BMI	< 30	21	8± 3.1	0.0008
	≥ 30	18	4.9± 2	

Data are means ± standard deviation. BMI; body mass index.

*T-test. P-value is significant at 0.05 level.

Figure 3.1 demonstrates the significantly lower adiponectin levels among obese (BMI <30) versus non obese (BMI ≥ 30) diabetic and control subjects. It clearly appears from the graph that adiponectin levels in T2DM patients were uniformly low when compared to non- diabetic subjects.

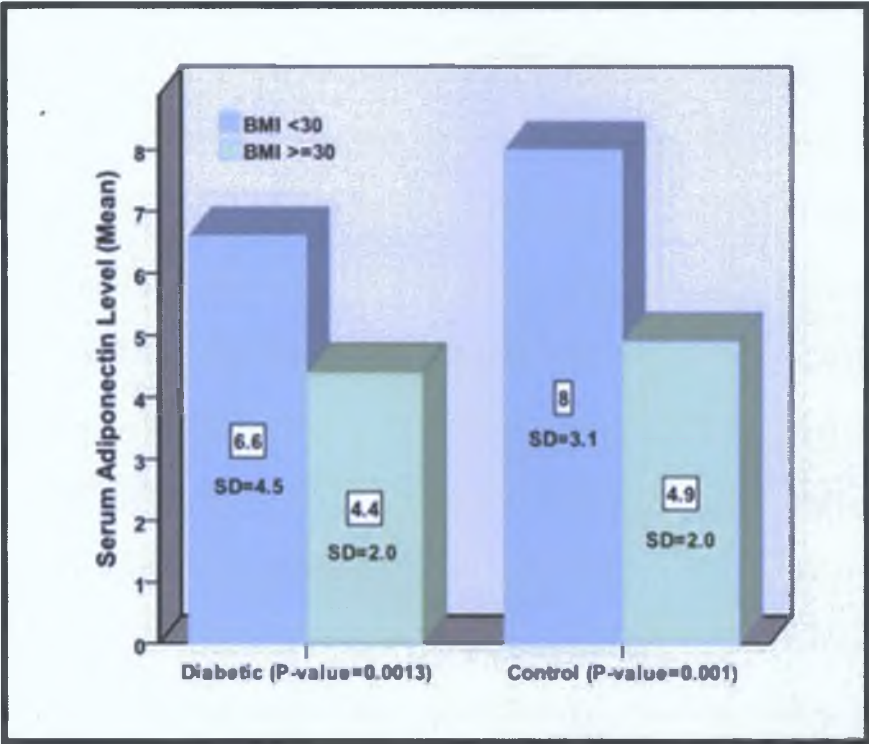


Figure 3.1 Comparison of plasma adiponectin levels in lean (BMI <30) and obese (BMI ≥ 30) diabetic and control subjects. Vertical bars represent the mean adiponectin levels.

3.5 Serum Resistin Levels in T2DM Patients and Controls

Samples which were received with no serum or those which were not stored or handled properly were excluded (for details refer to section 3.3). Six T2DM patients and 14 control cases were excluded from the analysis. As demonstrated in Table 3.12, T2DM patients had significantly higher serum resistin levels than healthy subjects (6.9 ± 6.2 ng/ml in patients vs. 4.9 ± 4.8 ng/ml in controls; $P=0.022$).

Table 3.12 Difference in serum resistin concentration between T2DM patients and controls

	Diabetic n= 134	Control n= 52	P-value*
	Mean± SD	Mean± SD	
Resistin conc. (ng/ml)	6.9± 6.2	4.9± 4.8	0.022

Data are means ± standard deviation

*T-test. P-value is significant at 0.05 level.

Gender-based analysis showed that serum resistin level is higher in male subjects than in females (8.1 ±7.3 ng/ml vs. 5.9 ± 4.9 ng/ml, P=0.033, Table 3.13) in T2DM patients. However, further adjustment for BMI decreased the magnitude of the associations and made it insignificant (P=0.184, Table 3.13). In healthy control subjects, resistin levels did not correlate with gender and their values remained unchanged after further adjustment of BMI and age (Table 3.14).

Table 3.13 Difference in serum resistin concentration between T2DM male and female patients

	Diabetic		P-value*	P-value**
	Male n= 60	Female n= 74		
	Mean± SD	Mean± SD		
Resistin conc. (ng/ml)	8.1± 7.3	5.9± 4.9	0.033	0.184

Data are means ± standard deviation. *P-value unadjusted; **P-value after adjustment for age and BMI.

T-test was used for P-value calculation. P-value is significant at 0.05 level.

Table 3.14 Difference in serum resistin concentration between healthy control male and female subjects

	Control		P-value*
	Male n= 46	Female n= 6	
	Mean± SD	Mean± SD	
Resistin conc. (ng/ml)	4.9± 5.0	5.0± 3.2	0.963

Data are means ± standard deviation

*T-test. P-value is significant at 0.05 level.

3.6 Association between Serum Resistin Levels and Clinical and Metabolic Parameters of the Study Population

The demographic, clinical and metabolic characteristics of T2DM patients and controls were analyzed with serum resistin levels as shown in Table 3.15. Considering all diabetic subjects, serum resistin level did not show significant correlations with any of the clinical and metabolic parameters. BMI index did not show any correlation with serum resistin levels, even after subdividing the study participants according to the universal obesity cut-off point (BMI ≥ or < 30) (data are not shown).

In the control group, however, there was a weak negative association between BMI and serum resistin levels. Other clinical and metabolic parameters did not show significant correlations (P>0.05).

Table 3.15 Correlations of serum resistin levels to various clinical and metabolic parameters in both T2DM patients and control group

	Resistin level			
	Diabetic n= 134		Controls n= 52	
	Pearson Correlation	P-value	Pearson Correlation	P-value
Age (yrs)	0.109	0.202	-0.033	0.793
BMI	-0.084	0.486	-0.266	0.037*
SBP (mmHg)	-0.003	0.972	-0.002	0.992
DBP (mmHg)	-0.078	0.364	0.000	0.999
HbA1C (%)	0.009	0.917	-0.617	0.192
TC (mmol/l)	-0.075	0.403	0.726	0.165
HDL-C (mmol/l)	-0.107	0.238	0.222	0.719
LDL -C (mmol/l)	0.007	0.938	0.797	0.107
TG (mmol/l)	0.127	0.156	0.667	0.218
FBG (mmol/l)	-0.022	0.799	-0.591	0.217
IR	0.069	0.443		

Data is presented as pearson coefficients. *Correlation is significant at the 0.05 level (2-tailed). BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; HbA1c glycated hemoglobin; TC total cholesterol; HDL-C high density lipoprotein cholesterol; LDL-C Low density lipoprotein cholesterol; TG triglycerides; FBG fasting blood glucose; IR insulin resistance value estimated using the triglyceride/HDL-cholesterol index, values of ≥ 3 were considered insulin resistant.

Gender-based analysis was evaluated for both T2DM Patients and controls to assess the impact of the demographic, clinical and metabolic parameters on serum resistin levels in men and women.

3.6.1 T2DM Patients

There was no statistically significant difference in the relationship between the metabolic parameters and resistin level in men and women with T2DM (Table 3.16).

Table 3.16 Correlations of serum resistin levels to various clinical and metabolic parameters among males and females of T2DM patients

	Resistin level			
	Male n= 60		Female n= 74	
	Pearson Correlation	P-value	Pearson Correlation	P-value
Age (yrs)	0.08	0.507	0.11	0.352
BMI	-0.09	0.602	-0.095	0.629
SBP (mmHg)	0.08	0.529	-0.123	0.35
DBP (mmHg)	-0.101	0.433	-0.216	0.10
HbA1C (%)	0.07	0.554	-0.169	0.197
TC (mmol/l)	-0.067	0.617	-0.14	0.266
HDL-C (mmol/l)	-0.18	0.166	0.11	0.369
LDL -C (mmol/l)	0.08	0.562	-0.06	0.644
TG (mmol/l)	0.21	0.103	-0.14	0.245
FBG (mmol/l)	0.06	0.643	-0.15	0.197
IR	0.11	0.397	-0.17	0.175

Data is presented as pearson coefficients. Correlation is significant at the 0.05 level (2-tailed). BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; HbA1c glycated hemoglobin; TC total cholesterol; HDL-C high density lipoprotein cholesterol; LDL-C Low density lipoprotein cholesterol; TG triglycerides; FBG fasting blood glucose; IR insulin resistance value estimated using the triglyceride/HDL-cholesterol index, values of ≥ 3 were considered insulin resistant.

3.6.2 Controls

As shown on Table 3.17, weak negative correlation was observed in the association between BMI and serum resistin in men ($P= 0.033$).

Table 3.17 Correlations of serum resistin levels to various clinical and metabolic parameters among males and females of the control group

	Resistin level			
	Male n= 46		Female n= 6	
	Pearson Correlation*	P-value	Spearman Correlation*	P-value
Age (yrs)	-0.011	0.935	-0.46	0.354
BMI	-0.276	0.033**	0.20	0.747
SBP (mmHg)	0.011	0.952	.	.
DBP (mmHg)	-0.001	0.995	.	.
HbA1C (%)	.	.	0.79	0.111
TC (mmol/l)	.	.	0.30	0.624
HDL-C (mmol/l)	.	.	0.30	0.624
LDL -C (mmol/l)	.	.	0.67	0.219
TG (mmol/l)	.	.	-0.37	0.468
FBG (mmol/l)	.	.	-0.46	0.354

*Data is presented as pearson coefficients, because the sample size of female control subjects is only 6, spearman coefficients were used (pearson coefficients could not be applied for female control subjects (n= 6)). **Correlation is significant at the 0.05 level (2-tailed). BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; HbA1c glycated hemoglobin; TC total cholesterol; HDL-C high density lipoprotein cholesterol; LDL-C Low density lipoprotein cholesterol; TG triglycerides; FBG fasting blood glucose.

3.7 Adiponectin and Resistin Levels in Relation to the Metabolic and Clinical Parameters in the whole Study Population

In an attempt to compare the role of adiponectin and resistin and emphasize their relations with the metabolic parameters, we analyzed the relationships of both adipokines with the various metabolic parameters in the whole study subjects. Adiponectin was found to correlate with a wide number of different biomarkers. Serum adiponectin correlated negatively to HbA1c ($P=0.005$), TG ($P=0.017$), and IR index ($P=0.012$). On the other hand, serum resistin showed no statistical significance in the association with the various metabolic parameters except a weak positive correlation with age (Table 3.18).

Table 3.18 Comparison between the association of adiponectin and resistin with the metabolic and clinical parameter in the whole study subjects

	Adiponectin n= 177		Resistin n= 186	
	Pearson Correlation	P-value	Pearson Correlation	P-value
Age (yrs)	-.0068	0.371	0.165	0.018*
BMI	-0.128	0.188	-0.044	0.613
SBP (mmHg)	-0.130	0.114	0.041	0.596
DBP (mmHg)	-0.096	0.246	-0.048	0.539
HbA1C (%)	-0.238	0.005**	0.014	0.863
TC (mmol/l)	-0.019	0.836	-0.062	0.484
HDL-C (mmol/l)	0.177	0.055	-0.116	0.190
LDL -C (mmol/l)	-0.042	0.649	0.019	0.832
TG (mmol/l)	-0.217	0.017*	0.137	0.120
FBG (mmol/l)	-0.074	0.393	-0.016	0.845
IR	-0.235	0.012*	0.069	0.443

Data is presented as pearson coefficients. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed). BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; HbA1c glycated hemoglobin; TC total cholesterol; HDL-C high density lipoprotein cholesterol; LDL-C Low density lipoprotein cholesterol; TG triglycerides; FBG fasting blood glucose; IR insulin resistance value estimated using the triglyceride/HDL-cholesterol index, values of ≥ 3 were considered insulin resistant.

3.8 Analysis of the Adiponectin Gene SNP +45T>G Polymorphism

3.8.1 Detection of Adiponectin Gene SNP +45T>G Polymorphism

The SNP +45T>G was detected by PCR and enzyme restriction reactions (Mackevics et al., 2006). The allele frequencies of the T and G alleles of the SNP +45T>G were 0.83 and 0.17 in T2DM patients and 0.924 and 0.076 in the controls respectively (Table 3.19). Genotype frequencies were found to be in agreement with the Hardy-Weinberg equilibrium which is described by the binominal distribution: $p^2 + 2pq + q^2 = 1.0$, thus fulfilling this requirement for the present study population.

The Hardy-Weinberg principle is one of the most fundamental principles in human genetics. It is the concept that both allele frequencies and genotype frequencies will remain constant from generation to generation in an infinitely large, interbreeding population in which mating is at random and there is no selection, migration or mutation. According to this principle under the mentioned conditions, distribution of genotypes in a population follows the law of the statistical probability.

3.8.2 Adiponectin Gene SNP +45T>G Distribution in T2DM Patients and Controls

The allele frequencies and genotype distributions of SNP +45T>G in T2DM patients and non-diabetic controls are summarized in Table 3.19. In T2DM group, 100 (71.4%) subjects were homozygous for the wild-type allele (TT), 32 (22.9%) were heterozygous (TG) and 8 (5.7%) were homozygous for the minor allele (GG). In the non-diabetic control group 56 (84.8%) subjects were homozygous for the wild-type allele (TT), 10 (15.2%) were heterozygous (TG) and none of the subjects were homozygous for the mutation (GG) (Table 3.19). The rare G allele of the

SNP +45T>G showed a minor allele frequency (MAF) of 17.1% in T2DM patients and 7.6% in the controls.

There was a statistically significant difference in allele frequencies and genotype distributions of SNP +45T>G in the diabetic and control groups (T frequency 83% vs. 92.4%, and G frequency 17% vs. 7.6%, P=0.009, OR= 1.8) and (TT genotype 71.4% vs. 84.8%, P=0.046 and TG+GG genotype 28.6% vs. 15.2%, P=0.046, OR= 2.52), respectively (Table 3.19). The G allele and T/G + G/G genotype occurred more frequently than the T allele in T2DM patients compared to the controls (P<0.05). Subjects with the G/G genotype were at increased risk for T2DM compared with those having the T/T genotype. This finding is consistent with published data showing that the G allele of SNP +45 is significantly associated with T2DM (Hara et al., 2002, Mohammadzadeh and Zarghami, 2009).

Table 3.19 Differences in genotype and allele frequencies of adiponectin gene SNP +45 between diabetic and control subjects

		Diabetic		Control		P-value*
		n	%	n	%	
Genotype		T/T	100	71.4	56	84.8
frequency	SNP +45	T/G	32	22.9	10	15.2
		G/G	8	5.7	0	0
Allele		T	232	83	122	92.4
frequency	SNP +45	G	48	17	10	7.6

Data are numbers (n) and percentages (%).*Chi-square test. P-value is significant at 0.05 level.

3.8.3 Relationship between the SNP +45T>G Genotypes and Serum Adiponectin Levels in T2DM Patients and Controls

Table 3.20 states the mean serum adiponectin concentrations per SNP +45T>G genotypes. The mean values were divided via a general linear model adjusting for age, gender and BMI. Twenty nine samples without measured adiponectin were excluded from the analysis but their genotypes were detected and calculated in allele and genotypes frequencies. The P-values testing for association of a single nucleotide polymorphism (SNP) genotypes with adiponectin concentrations with and without adjustment are given. All analyses were readjusted for age, BMI and gender. Adjustment for age neither changed mean adiponectin levels nor the gene effect. Adjustment for gender changed the mean adiponectin values as adiponectin plasma levels differ between male and female subjects. Adjustment for BMI did slightly reduce the strength of association the difference of the mean adiponectin levels between the genotype groups, but the effect of adjusting for BMI was relatively small compared to the strong gene effect of adiponectin.

Considering all study population, the mean serum adiponectin levels were significantly different between the three genotype subgroups of the SNP +45T>G. It can be seen that there is a significant association between the SNP +45T>G genotypes and serum adiponectin levels. Further adjustment for BMI, age and gender did not substantially change these results. A clear multiplicative trend of decreasing adiponectin levels per copy of the rare allele (G) can be observed (6.9, 3.7, 2.1 µg/ml for the T/T, the T/G and the G/G genotypes in the +45 locus respectively, P-values < 0.0001), thus suggesting a possible influence of the SNP +45T>G on adiponectin levels. When the male and female subjects were analyzed separately, same gene effects on serum adiponectin levels were observed.

Table 3.20 Serum adiponectin levels in whole study subjects with different genotypes for the +45T>G polymorphism

		Adiponectin Conc. µg/ml		P*	P**
		n	Mean± SD		
SNP +45T>G,	T/T	134	6.9± 4.3	0.000	0.000
	T/G	36	3.7± 3.2		
	G/G	7	2.1± 2.2		
Total					
SNP +45T>G,	T/T	79	6.4± 3.6	0.000	0.001
	T/G	19	2.7±1.9		
	G/G	3	1.4± 2.5		
Males					
SNP +45T>G,	T/T	55	7.5± 5.1	0.035	0.038
	T/G	17	4.9± 3.9		
	G/G	4	2.6 ± 2.1		
Females					

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age, BMI (and gender in case total sample was tested). P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

Further analysis of T2DM patients revealed that the plasma adiponectin concentration of TG+GG genotype carriers were significantly different from that of TT genotype. The G allele was associated with lower serum adiponectin levels compared to the T allele carriers (6.5± 4.1 µg/ml for T/T genotype, 3.7± 3.4 µg/ml for the T/G genotype and 2.1±2.2 µg/ml for the G/G genotype, P<0.001). When male and female subjects with T2DM were analyzed separately, same gene effects on serum adiponectin levels were observed with a more significant association towards the male subjects (P=0.001 vs 0.044) (Table 3.21).

Table 3.21 Serum adiponectin levels in T2DM patients with different genotypes for the +45T>G polymorphism

		Adiponectin Conc. µg/ml		P*	P**
		n	Mean± SD		
SNP +45T>G, Total	T/T	92	6.5± 4.1	0.000	0.005
	T/G	30	3.7±3.4		
	G/G	7	2.1±2.2		
SNP +45T>G, Males	T/T	43	5.7± 3.5	0.001	0.013
	T/G	13	2.2± 2		
	G/G	3	1.4± 2.5		
SNP +45T>G, Females	T/T	49	7.1± 4.5	0.044	0.341
	T/G	17	4.9± 3.9		
	G/G	4	2.6± 2.1		

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age, BMI (and gender in case total sample was tested). P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

In the control group, the frequency of the G/G genotype was zero; therefore the comparison was made between the T/G and T/T genotypes only. As demonstrated from Table 3.22, the plasma levels of adiponectin of TG genotype were significantly lower than that of the TT genotype and the same was observed when analyzing the male subjects separately (Table 3.22).

Table 3.22 Serum adiponectin levels in control subjects with different genotypes for the +45T>G polymorphism

		Adiponectin Conc. µg/ml		P*	P**
		n	Mean ± SD		
SNP +45T>G, Total	T/T	42	7.8± 4.3	0.048	0.09
	T/G	6	3.8± 1.1		
	G/G	0	.		
SNP +45T>G, Males	T/T	36	7.2± 3.7	0.032	0.037
	T/G	6	3.8± 1.1		
	G/G	0	.		
SNP +45T>G, Females	T/T	6	11± 8.5	.	.
	T/G	0	.		
	G/G	0	.		

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age, BMI (and gender in case total sample was tested). P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

3.8.4 Association between the SNP +45T>G Genotypes and Clinical and Metabolic Parameters of the Study Subjects

To assess whether the SNP +45T>G genotypes had any effect on anthropometric and insulin resistance indices; we compared anthropometric and metabolic characteristics among genotype subgroups. A series of metabolic and clinical parameters (Age, BMI, systolic and diastolic blood pressure, HbA1c, total cholesterol, HDL and LDL cholesterol, triglycerides, fasting blood glucose and insulin resistance estimated by triglyceride/HDL-cholesterol index) were analyzed with respect to their association with SNP +45T>G genotypes in T2DM patients (Table 3.23). Due to incomplete clinical data for the control cohort, the correlation was only evaluated for T2DM patients. Considering all diabetic patients, no significant difference in metabolic parameters among T/T, T/G and G/G carriers was observed. The values

of BMI, SBP, DBP, HbA1c, total, HDL and LDL cholesterol, triglycerides, fasting blood glucose and insulin resistance were not different in these three genotype subgroups.

Table 3.23 Clinical and metabolic characteristics of the diabetic subjects according to adiponectin SNP +45T>G genotypes

	SNP +45T>G			P*	P**
	T/T	T/G	G/G		
	Mean± SD	Mean± SD	Mean± SD		
n	100	32	8		
Age (yrs)	59±12	59± 10	52±7.6	0.231	0.489
BMI	34.0± 7.3	35.2±6.9	32.2±3.4	0.690	0.509
SBP (mmHg)	145± 20	150±28	156±19	0.295	0.352
DBP (mmHg)	77±12	74±14	81±19	0.377	0.516
HbA1c %	8.08±1.64	8.39±2.08	7.4±1.4	0.314	0.497
TC (mmol/l)	4.5±1.0	4.7±1.0	4.5±0.7	0.542	0.531
HDL-C (mmol/l)	1.2±0.4	1.2±0.3	1.2±0.4	0.915	0.802
LDL-C (mmol/l)	2.7±0.8	2.9±0.8	2.8±0.6	0.701	0.223
TG (mmol/l)	2.0±1.6	2.4±1.9	1.5±0.6	0.328	0.679
FBG (mmol/l)	9.8±4.1	10.2±4.3	7.7±1.1	0.285	0.649
IR	2.0±2.5	2.5±2.6	1.5±1.0	0.5	0.861

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age, BMI and gender. P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

Further analysis of male and female subjects with T2DM revealed no significant association in the metabolic parameters among the different genotype subgroups of SNP +45T>G (Table 3.24) and (Table 3.25). Tested indices were not different in the three genotype subgroups in both genders (P-value > 0.05).

Table 3.24 Clinical and metabolic characteristics of the diabetic male subjects according to adiponectin SNP +45T>G genotypes

	SNP +45T>G			P*	P**
	T/T	T/G	G/G		
	Mean± SD	Mean± SD	Mean± SD		
n	47	15	4		
Age (yrs)	60± 12	61± 12	52.8±10	0.464	0.946
BMI	32.1± 6.4	36.9± 6.6	34± 5	0.170	0.178
SBP (mmHg)	142± 22	149± 17	153.8±14.7	0.363	0.521
DBP (mmHg)	77± 12	76± 12	79.3±10.1	0.881	0.508
HbA1c %	8.11± 1.87	8.76± 2.28	7.4±1.7	0.369	0.518
TC (mmol/l)	4.4± 1.2	4.5± 1.0	4.6± 0.7	0.886	0.948
HDL-C (mmol/l)	1.1± 0.4	1.0± 0.3	1.3± 0.6	0.529	0.867
LDL-C (mmol/l)	2.7± 0.9	2.7± 0.9	2.9± 0.31	0.885	0.850
TG (mmol/l)	2.2± 2.1	2.7± 1.9	1.8± 0.56	0.635	0.946
FBG (mmol/l)	10.0± 3.8	11.0± 4.9	7.5± 0.8	0.285	0.174
IR	2.4± 3.4	3.1± 2.5	1.7± 1.2	0.690	0.943

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age and BMI. P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

Table 3.25 Clinical and metabolic characteristics of the female subjects according to adiponectin SNP +45T>G genotypes

	SNP +45T>G			P*	P**
	T/T	T/G	G/G		
	Mean± SD	Mean± SD	Mean± SD		
n	53	17	4		
Age (yrs)	58± 12	58± 9	50.8± 5.6	0.486	0.460
BMI	35.9± 7.6	33.2± 7.2	31± 2.5	0.391	0.291
SBP (mmHg)	147± 18	150± 36	158.5± 25.3	0.656	0.721
DBP (mmHg)	77± 13	73± 15	83± 27.1	0.395	0.070
HbA1c %	8.05± 1.43	8.07± 1.90	7.3± 1.4	0.656	0.956
TC (mmol/l)	4.6± 0.8	4.9± 1.0	4.4± 0.7	0.375	0.341
HDL-C (mmol/l)	1.2± 0.3	1.3± 0.3	1.2±0.4	0.832	0.742
LDL-C (mmol/l)	2.8± 0.7	3.0± 0.8	2.7± 0.4	0.631	0.307
TG (mmol/l)	1.8± 0.8	2.1± 1.9	1.3±0.5	0.421	0.327
FBG (mmol/l)	9.7± 4.3	9.5± 3.7	8± 1.3	0.703	0.522
IR	1.6± 0.9	2.0± 2.7	1.3± 0.9	0.625	0.846

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age and BMI. P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

3.9 Analysis of the Adiponectin SNP +276G>T Polymorphism

3.9.1 Detection of the Adiponectin Gene SNP +276G>T Polymorphism

The SNP +276G>T was detected by PCR and enzyme restriction reactions (Mackevics et al., 2006). The allele frequencies of the G and T alleles of the SNP +276G>T were 0.957 and 0.043 in T2DM patients and 0.923 and 0.077 in the controls respectively. Genotype frequencies were found to be in agreement with the Hardy-Weinberg equilibrium which is described by the binominal distribution: $p^2 + 2pq + q^2 = 1.0$

3.9.2 Adiponectin Gene SNP +276G>T Distribution in T2DM Patients and Controls

The genotype and allele frequencies of SNP +276G>T polymorphism in T2DM patients and in controls are shown in Table 3.26. The frequencies of the G/G, T/G, and T/T genotypes of SNP +276G>T were 92.9%, 5.7%, and 1.4% in T2DM patients and 87.9%, 9.1%, and 3% in the controls, respectively. The rare T allele of the SNP +276G>T showed a minor allele frequency (MAF) of 4.3% and 7.6% in T2DM patients and controls respectively. As can be seen from Table 3.26 there was no statistically significant difference in allele frequencies and genotype distributions of SNP +276G>T comparing type 2 diabetic subjects with control group (T frequency 4.3% vs. 7.6%, and G frequency 95.7% vs. 92.4%, $P=0.166$) and GG genotype 92.9% vs. 87.9%, $P=0.480$ and T/G + T/T genotype 7.1% vs. 12.1%, $P=0.480$), respectively.

Table 3.26 Differences in genotype and allele frequencies of adiponectin SNP +276G>T between diabetic and non-diabetic subjects

			Diabetic		Control		P-value
			n	%	n	%	
Genotype	SNP +276	G/G	130	92.9	58	87.9	0.480*
Frequency		T/G	8	5.7	6	9.1	
		T/T	2	1.4	2	3.0	
Allele	SNP +276	G	268	95.7	122	92.4	0.166**
frequency		T	12	4.3	10	7.6	

Data are numbers (n) and percentages (%). *Cramer's V test, **Chi-square test. P-value is significant at 0.05 level.

3.9.3 Relationship between SNP +276G>T and Serum Adiponectin Levels in T2DM Patients and Controls

Table 3.27 displays the mean serum adiponectin concentrations per genotype. The mean values were divided via a general linear model adjusting for age, gender and BMI. Twenty nine samples without measured adiponectin were excluded from the analysis but their genotypes were detected and calculated in allele and genotypes frequencies. The P-values testing for association of a single nucleotide polymorphism (SNP) genotypes with adiponectin concentrations with and without adjustment are given. All analyses we adjusted for age, BMI and gender. Adjustment for age neither changed mean adiponectin levels nor the gene effect. Adjustment for gender changed the mean adiponectin values as adiponectin differ between male and female subjects. Adjustment for BMI did slightly reduce the strength of association the difference of the mean adiponectin levels between the genotype groups, but the effect of adjusting for BMI was relatively small compared to the strong gene effect of adiponectin.

Considering all the study population, there was no significant difference in the mean serum adiponectin levels between the three genotypes subgroups of the SNP +276G>T ($P>0.05$). When the male and female subjects were analyzed separately, no significant difference of serum adiponectin levels were observed in male subjects. Although females showed a slight association with serum adiponectin level in G/G and T/G genotypes of SNP +276G>T, further adjustment for age and BMI changed the results substantially, thus the association can't prove to be significant as the frequency of the T/T genotype among females is zero.

Table 3.27 Serum adiponectin levels in whole study subjects with different genotypes for the SNP +276G>T polymorphism

		Adiponectin Conc. pg/ml		P*	P**
		n	Mean± SD		
SNP +276G>T, Total	G/G	160	5.8±4.7	0.082	0.186
	T/G	13	8.3± 5		
	T/T	4	8± 2.6		
SNP +276G>T, Males	G/G	88	5.3± 3.7	0.273	0.322
	T/G	9	6.5± 3.4		
	T/T	4	8± 2.6		
SNP +276G>T, Females	G/G	72	6.4±2.6	0.016	0.254
	T/G	4	12.4±6.2		
	T/T	0	.		

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age, BMI (and gender in case total sample was tested). P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

Further analysis of T2DM patients revealed that the effects of the SNP +276G>T genotypes on adiponectin concentration did not prove to be statistically significant. When males and female subjects with T2DM were analyzed separately, there was no significant association between the different genotypes of SNP +276G>T with serum adiponectin levels in the male subjects with T2DM. Although females showed an association in the

unadjusted serum levels, but as mentioned earlier, since the frequency of T/T genotype among females was zero, this association could not be confirmed (Table 3.28).

Table 3.28 Serum adiponectin levels in diabetic subjects with different genotypes for the SNP +276G>T polymorphism

		Adiponectin Conc. pg/ml		P*	P**
		n	Mean± SD		
SNP +276G>T, Total	G/G	119	5.3± 3.9	0.042	0.101
	T/G	8	9.1± 5.8		
	T/T	2	5.8± 1.7		
SNP +276G>T, Males	G/G	53	4.6± 3.6	0.732	0.283
	T/G	4	5.8± 3.5		
	T/T	2	5.9± 1.7		
SNP +276G>T, Females	G/G	66	6.0± 4.0	0.004	0.080
	T/G	4	12.4±6.2		
	T/T	0			

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age, BMI (and gender in case total sample was tested). P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

In the control group, as demonstrated from Table 3.29, there was no statistically significant association between the serum adiponectin levels and the different genotypes of SNP +276G>T. No association was observed when analyzing the male subjects separately. The association between the serum levels and SNP +276G>T genotypes can't be evaluated on females as the frequencies of the T/G and T/T genotypes were zero.

Table 3.29 Serum adiponectin levels in control subjects with different genotypes for the SNP +276G>T polymorphism

		Adiponectin Conc. µg/ml		P*	P**
		n	Mean± SD		
SNP +276G>T,	G/G	41	7.15± 4.9	0.694	0.239
	T/G	5	7.1± 3.6		
	T/T	2	10.1± 0.23		
Total					
SNP +276G>T,	G/G	35	6.5± 3.7	0.403	0.389
	T/G	5	7.1± 3.6		
	T/T	2	10.1± 0.23		
Males					
SNP +276G>T,	G/G	6	11±8.5		
	T/G	0			
	T/T	0			
Females					

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age, BMI (and gender in case total sample was tested). P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

3.9.4 Association between SNP +276G>T Genotypes and Clinical and Metabolic Parameters of the Study Subjects

In a secondary analysis, a series of metabolic and clinical parameters (age, BMI, systolic and diastolic blood pressure, HbA1c, total, HDL and LDL cholesterol, triglycerides, fasting blood glucose and insulin resistance estimated by triglyceride/HDL-cholesterol index) were analyzed with respect to their association with SNP +276G>T genotypes in T2DM patients (Table 3.23). Due to incomplete clinical data for the control cohort, the correlation was only evaluated for T2DM patients. Considering all diabetic patients, no significant difference in the metabolic parameters among G/G, T/G and T/T carriers was observed. The values of BMI, HbA1c, total, HDL and LDL cholesterol, triglycerides, fasting blood glucose and insulin resistance were not different in these three genotype subgroups ($P>0.05$). Concerning adiposity, we did not find an

association between any genotypes in SNP +276G>T and adiposity measurements, including BMI.

Table 3.30 Clinical and metabolic characteristics of the diabetic subjects according to adiponectin SNP +276G>T genotypes

	SNP +276G>T			P*	P**
	G/G	T/G	T/T		
	Mean \pm SD	Mean \pm SD	Mean \pm SD		
n	130	8	2		
Age (yrs)	58 \pm 11	59 \pm 16	68 \pm 21	0.530	0.128
BMI	34.0 \pm 7.0	36.6 \pm 7.2	36.7 \pm 7.5	0.673	0.434
SBP (mmHg)	147 \pm 23	143 \pm 20	128 \pm 23	0.447	0.210
DBP (mmHg)	77 \pm 13	77 \pm 8	65 \pm 23	0.423	0.125
HbA1c %	8.12 \pm 1.70	8.65 \pm 2.26	5.93 \pm 0.89	0.143	0.248
TC (mmol/l)	4.6 \pm 1.0	4.3 \pm 1.1	3.6 \pm 0.9	0.266	0.231
HDL-C (mmol/l)	1.2 \pm 0.3	1.1 \pm 0.3	1.3 \pm 0.2	0.691	0.460
LDL-C (mmol/l)	2.8 \pm 0.8	2.8 \pm 0.9	2.1 \pm 0.6	0.445	0.281
TG (mmol/l)	2.1 \pm 1.7	1.8 \pm 0.6	0.8 \pm 0.1	0.479	0.858
FBG (mmol/l)	9.7 \pm 4.0	12.2 \pm 4.4	7.4 \pm 0.8	0.158	0.188
IR	2.1 \pm 2.5	1.7 \pm 0.7	0.6 \pm 0.1	0.642	0.901

Data presented as mean \pm standard deviation, *P-value unadjusted; **P-value adjusted for age, BMI and gender. P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

Further analysis of male subjects with T2DM revealed no significant association in the metabolic parameters among the different genotype subgroups of SNP +276G>T (Table 3.31). However in female subjects, HbA1c showed a significant association in which the T allele carriers showed higher values of HbA1c compared to the G allele carriers ($P<0.05$) (Table 3.32). Other indices were not different in the three genotype subgroups in both genders ($P>0.05$). The results suggested that adiponectin gene SNP +276G>T has a very weak effect on the

metabolic and clinical parameters in the subjects with T2DM of the present study.

It should be acknowledged that there is a large number of significance tests carried out in this study and this is bound to give rise to false positives (multiple testing) which could not be ruled out.

Table 3.31 Clinical and metabolic characteristics of the male diabetic subjects according to adiponectin SNP +276G>T genotypes

	SNP +276G>T			P*	P**
	G/G	T/G	T/T		
	Mean± SD	Mean± SD	Mean± SD		
n	60	4	2		
Age (yrs)	59±12	61±11	68±21	0.595	0.180
BMI	32.9±6.5	40. 3±7.1	36.7±7.5	0.275	0.267
SBP (mmHg)	145±21	142±23	128± 23	0.520	0.136
DBP (mmHg)	78±11	77±9	65± 23	0.299	0.094
HbA1c %	8.3±1.95	7.6±2.03	5.9 0.89	0.192	0.088
TC (mmol/l)	4.4±1.1	4.3±1.4	3.6± 0.9	0.528	0.261
HDL-C (mmol/l)	1.1±0.4	1.0±0.4	1.3± 0.2	0.794	0.599
LDL-C (mmol/l)	2.7±0.8	2.9±1.1	2.1± 0.6	0.489	0.177
TG (mmol/l)	2.4±2.1	1.6±0.6	0.8± 0.1	0.429	0.919
FBG (mmol/l)	10.2±4.1	9.5±3.7	7.4± 0.8	0.599	0.210
IR	2.6±3.3	1.7±0.8	0.6± 0.1	0.585	0.968

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age and BMI. P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

Table 3.32 Clinical and metabolic characteristics of the diabetic female subjects according to adiponectin SNP +276G>T genotypes

	SNP +276G>T			P*	P**
	G/G	T/G	T/T		
	Mean± SD	Mean± SD	Mean± SD		
n	70	4	0		
Age (yrs)	58±11	56±22	.	0.770	0.716
BMI	35.1±7.5	34.2±7.5	.	0.849	0.979
SBP (mmHg)	149±24	145±21	.	0.719	0.918
DBP (mmHg)	76±15	77±7	.	0.996	0.920
HbA1c %	7.9±1.6	9.7±2.2	.	0.024	0.003
TC (mmol/l)	4.7±0.9	4.2±0.7	.	0.358	0.614
HDL-C (mmol/l)	1.2±0.3	1.1±0.1	.	0.557	0.526
LDL-C (mmol/l)	2.8±0.7	2.5±0.6	.	0.497	0.787
TG (mmol/l)	1.8±1.2	2.0±0.6	.	0.815	0.631
FBG (mmol/l)	9.3±4.0	15±3.5	.	0.006	0.000
IR	1.7±1.6	1.8±0.6	.	0.903	0.629

Data presented as mean ± standard deviation, *P-value unadjusted; **P-value adjusted for age and BMI. P-values are calculated from univariate general linear model. P-value is significant at 0.05 level.

It was not possible to perform haplotype analysis for the SNPs +45T>G and +276G>T. Due to the small sample size, the number of cases that harbored the homozygous polymorphic allele of both SNPs was very small; this did not allow us to see the expected variations in haplotypes defined by these two SNPs. For example, none of the subjects harbored the homozygous or heterozygous genotype of both SNPs at a time. The most frequently seen haplotype was of one allele showing the wild type genotype of one SNP and another allele showing the homozygous or heterozygous genotype of the other SNP. Thus, haplotypes analysis does not add any significance to the findings of studying these SNPs separately.

Chapter 4- Discussion

4.1 Analysis of Serum Adipokines Levels

4.1.1 The Relationship between Adiponectin, T2DM and Metabolic Parameters

Over the last 10 years, several clinical and experimental data have radically modified the concept of adipose tissue as an organ devoted to energy storage and release. Adipose tissue is now considered to be an important endocrine organ. It can release hormones into the blood stream in response to specific extra-cellular stimuli or changes in metabolic status. The factors secreted by adipose tissue are actively involved in energy homeostasis. They signal to the brain, the pancreatic β -cells, the liver and skeletal muscle in order to adapt to changes in energy stores through modulating feeding, insulin secretion and insulin sensitivity (Richelsen et al., 2001). Secreted factors include leptin; steroids and glucocorticoids; peptide hormone precursors, e.g. angiotensinogen; complement factors, e.g. adiponectin (which resembles complement factor C1q), adipsin, acylation-stimulating protein (ASP); pro-inflammatory cytokines, e.g. tumor necrosis factor α (TNF- α), interleukin 6 (IL-6); resistin; etc. These adipokines engage, through endocrine, paracrine, autocrine or juxtacrine mechanisms of action, in a wide variety of physiological or pathological processes, including immunity and inflammation (Otero et al., 2005, Karastergiou and Mohamed-Ali, 2010).

Adiponectin is an adipocyte derived hormone expressed abundantly in adipose tissue whose levels are lower in T2DM patients than in non diabetic subjects (Wannamethee et al., 2007). Thus, adiponectin has increasingly been considered to be a potential biomarker and possible therapeutic target for T2DM. Adiponectin has many metabolic actions involving the regulation of energy homeostasis, particularly energy expenditure. In the present study, we examined the serum concentrations of adiponectin in a sample of Bahraini type 2 diabetic

patients and in non diabetic subjects with varying degrees of obesity. The results demonstrate that serum adiponectin levels are significantly lower in subjects with T2DM than in those without diabetes even after correction for age and BMI. However, after dividing the study participants according to the universal obesity cut-off point (ie below or above 30 kg/m²), obese diabetic patients (BMI \geq 30) showed a more decreased adiponectin level than non-obese diabetic patients (BMI < 30). Additionally, when adiponectin levels were compared in lean and obese diabetic and non-diabetic subjects (Figure 3.1), it was evident that adiponectin levels in T2DM patients were uniformly low comparing to non diabetic subjects. These results demonstrate a clear significant relationship between plasma adiponectin concentrations and T2DM.

These results are consistent with findings of a longitudinal study on Japanese population by Daimon *et al.* who indicated hypoadiponectinemia to be a risk factor for the development of type 2 diabetes mellitus (Daimon *et al.*, 2003). Baratta *et al.* and Vendrell *et al.* also found decreased levels of serum adiponectin concentrations in obese and diabetic subjects and significant inverse associations with some measures of insulin resistance (Baratta *et al.*, 2004, Vendrell *et al.*, 2004). There are some possible explanations for the association between T2DM and serum adiponectin concentrations. The lower levels of adiponectin seen in diabetic patients are believed to be associated with the disorder of metabolism of glucose and lipid in diabetes (Lu *et al.*, 2006, Karbowska and Kochan, 2006). Diabetic obese patients demonstrate more deteriorated glucose metabolism exhibited by impaired glucose tolerance, or impaired fasting glucose and had also higher serum free fatty acids, higher total and LDL-cholesterol, higher triglycerides, and lower HDL levels, which could also be contributing factors to the lower adiponectin levels.

Our finding of an inverse relationship between BMI and adiponectin concentrations in Bahraini subjects is in agreement with previous reports in Japanese subjects (Arita *et al.*, 1999), Pima Indians and Caucasians

(Weyer et al., 2001). Arita et al. in 1999 showed that adiponectin was abundantly present in the plasma of healthy volunteers; whereas in obese subjects, the levels were significantly lower, although adiponectin is secreted only from adipose tissue. Weyer et al. in their study in 2001 found that obesity and type 2 diabetes are associated with low plasma adiponectin concentrations in different ethnic groups and indicate that the degree of hypoadiponectinemia is more closely related to the degree of insulin resistance than to the degree of adiposity and glucose intolerance. It is interesting that adiponectin levels are decreased in obese subjects with more fat tissue which could produce more adiponectin. Some researchers speculate that the suppression of adiponectin production in obesity may be mediated by an autocrine negative feedback inhibition in white adipose tissue (WAT), for instance, lower expression of adiponectin mRNA was found in cultured human visceral WAT from obese subjects as compared to visceral WAT from lean subjects. Moreover, microarray studies demonstrated that adiponectin expression is suppressed with the development of obesity and diabetes in mice (Lopez et al., 2004).

Previous reports showed that plasma adiponectin levels are affected by multiple factors including gender, age and lifestyle (Kadowaki et al., 2006). In this study we aimed to elucidate the impact of gender on adiponectin levels in T2DM and control subjects. Female subjects have significantly higher adiponectin levels compared to male subjects in both T2DM patients and controls. Our observations of the influence of gender suggest that adiponectin production is also related to factors independent of body weight. At any particular body size or body weight, adiponectin concentrations are greater in women than in men. Further, in men and women pair-matched for age, BMI, adiponectin concentrations were greater in women. This finding of a sex-based difference in plasma adiponectin concentrations is supported by some (Arita et al., 1999, Zoccali et al., 2002, Nishizawa et al., 2002) but not all other studies (Weyer et al., 2001, Comuzzie et al., 2001). However, the current findings strongly support the concept that gender has an additional

independent effect to modulate adiponectin concentrations. The reasons for this remain speculative. It is suggested that the sex hormones regulate the production of adiponectin, although it is controversial how these hormones are involved in the regulation of plasma adiponectin levels (Kadowaki et al., 2006). Nishizawa *et al.* reported in their study that androgens decrease plasma adiponectin concentrations *in vivo* as well as adiponectin secretion from 3T3-L1 adipocytes *in vitro* (Nishizawa et al., 2002). The variation in adiponectin concentrations can also be explained in terms of body fat variation between genders. The number of fat cells and fat cell size, both of which differ between genders, are possible determinants of adiponectin production rates (Blaak, 2001).

Hypoadiponectinemia has been shown to be closely associated with the clinical phenotype of metabolic syndrome (Ryo et al., 2004, Hulthe et al., 2003). Associations between adiponectin and hypertension (Imatoh et al., 2008), insulin resistance (Hivert et al., 2008, Weyer et al., 2001) and dyslipidemia (Komatsu et al., 2007) have been reported. The present study assessed the association between serum adiponectin and various metabolic parameters. We examined the relationships between plasma adiponectin concentrations, blood pressure, glycemic profile, lipid profile and insulin resistance. The impact of gender was also assessed in these relationships. Our data is consistent with the findings of the above-mentioned studies and indicate that adiponectin plays an important role in metabolic syndrome. While our study is cross-sectional and therefore does not establish cause and effect relationships, we observed a number of interesting associations. We found that in T2DM patients, plasma adiponectin correlate negatively with insulin resistance index, triglyceride and HbA1c and correlate positively with HDL-C. In control subjects, we did not find such associations, but we did find a strong negative correlation between systolic blood pressure and adiponectin levels. These findings agree well with those published by Wang *et al.* in which fasting serum adiponectin values were found to be positively correlated with serum HDL-C and negatively with serum TG concentration in patients with the metabolic syndrome and coronary artery disease (Wang

et al., 2010). Our results are also confirmed by the findings of Eizadi *et al.* who conducted a study to determine the relationship between anthropometrical and lipid profile indices and serum adiponectin in obese men. They found that serum adiponectin level had a significant correlation with total cholesterol, low density lipoprotein, systolic and diastolic blood pressure and age of subjects, suggesting a functional link to insulin resistance (Eizadi et al., 2011). Moreover, Asdie *et al.* in their study to determine the relationship between serum adiponectin and lipid profile in obese people found that there was a significant correlation between low levels of adiponectin with triglyceride, HDL cholesterol and Homeostatic model assessment-insulin resistance (HOMA IR) (Asdie et al., 2007).

The current finding support the hypothesis that adiponectin has a profound effect on lipid metabolism (Tsao et al., 2002, Karbowska and Kochan, 2006). One mechanism by which adiponectin mediates its effect is through activating 5'-AMP-activated protein kinase (AMPK), which plays a central role in the regulation of cellular energy metabolism (Carling, 2004). AMPK activates hepatic FFA oxidation and ketogenesis, inhibits triglyceride synthesis, and stimulates skeletal muscle FFA oxidation and glucose uptake (Winder and Hardie, 1999). In accordance with these mechanisms, we found that total triglyceride level was negatively associated with adiponectin concentration. In contrast, the HDL cholesterol level was positively associated with the adiponectin level. Interestingly, among several proposed lipid markers, TG and HDL-C are considered key metabolic markers in patients with insulin resistance (Kannel et al., 2008). Kannel *et al.* found that triglyceride versus high-density lipoprotein cholesterol ratio is useful for predicting insulin resistance and cardiometabolic risk in 3,014 patients from Framingham. The results of this study support our findings that the association between adiponectin and TG and HDL-C is considered as a major predictor not only of insulin resistance but for metabolic syndrome as a whole.

The fact that HbA1c, an index of blood glucose control, was identified as a statistically significant variable in our results confirms the relationship between diabetes and serum adiponectin levels. Therefore, we can conclude that blood glucose control in diabetic patients is associated with serum adiponectin levels. This can be partially explained by adiponectin playing a role in glucose metabolism as previously mentioned.

Our finding of a negative relationship between adiponectin and insulin resistance supports the hypothesis that adiponectin concentrations could modulate insulin action. It is well known that adiponectin improves insulin sensitivity of the whole body (Fu et al., 2005); therefore we suggest the decreased adiponectin in type 2 diabetes may be one of the factors leading to insulin resistance among diabetic patients. Similar to our findings, a recent study in Japanese subjects has demonstrated a strong relationship between serum adiponectin and insulin resistance index, lipids and blood pressure (Yamamoto et al., 2002), showing, as previously described, lower levels of adiponectin in insulin-resistant subjects. Soebijanto and Waspadji have also found that individuals with low adiponectin concentration have a 10.5 times greater risk of insulin resistance than individuals who have normal or high adiponectin concentration. They concluded that low adiponectin concentration may be a predictor for the occurrence of insulin resistance (Soebijanto and Waspadji, 2010).

4.1.2 The Relationship between Resistin, T2DM and Metabolic Parameters

Resistin is another protein identified recently as a hormone implicated with insulin resistance *in vivo* and *in vitro* and might therefore be an important link between obesity and diabetes. Studies in rodents suggest that resistin is up-regulated in obesity and participates in the pathogenesis of insulin resistance (Steppan et al., 2001, Gabriely et al., 2002, Levy et al., 2002). However, studies in humans have been

controversial. The apparent differences between rodents and humans in the role of resistin in the pathogenesis of T2DM could in part reflect different expression and regulatory mechanisms of resistin in these species. For example, murine resistin is almost exclusively expressed in adipocytes, whereas human resistin is expressed at substantially lower concentrations in adipocytes but is readily detectable in other cell types, especially macrophages (Pang and Le, 2006). Furthermore, mouse and human resistin share only 59% homology at the amino acid level (Pang and Le, 2006). The majority of the studies investigating resistin regulation have analyzed the mRNA adipose tissue expression in different models of rodents with insulin resistance or in adipose tissue from obese and type 2 diabetic patients (Ukkola, 2002, McTernan et al., 2002, Shuldiner et al., 2001, Kern et al., 2003). In this study, we examined serum resistin concentrations in a cohort of Bahraini non diabetic subjects and diabetic patients with varying degrees of obesity. We found that serum resistin concentrations were increased significantly in the diabetes group compared to healthy subjects. Our results are in agreement with the findings of a Saudi study by Al-Harithy and Al-Ghamdi, who found that resistin concentrations are elevated in patients with type 2 diabetes and are associated with obesity and insulin resistance. These data indicate that resistin might be involved in the development of diabetes in humans (Al-Harithy and Al-Ghamdi, 2005). Similarly, several studies demonstrated higher resistin concentrations in insulin-resistant or diabetic subjects compared with their healthy counterparts (Al-Harithy and Al-Ghamdi, 2005, Chanchay et al., 2006, Lu et al., 2006, Tokuyama et al., 2007, Youn et al., 2004), whereas other studies did not find such a difference (Heilbronn et al., 2004, Utzschneider et al., 2005, Norata et al., 2007, Vozarova de Courten et al., 2004, Lee et al., 2003, Yaturu et al., 2006). Although the previous studies did not perform any sort of adjustment, these conflicting data may result from lack of adjustment for potential confounding variables such as age and body fat distribution, limited power due to small sample sizes, and cross-sectional design. Population variation and ethnicity issues could also be considered as potential causes. To our knowledge, this study is the first investigation of

resistin and the associated risk of type 2 diabetes in a Bahraini population.

In contrast to adiponectin, resistin levels did not exhibit a sexual dimorphism. Gender-based analysis showed that serum resistin levels were higher in male subjects than in females ($P=0.033$, Table 3.13) in T2DM patients. However, further adjustment for BMI decreased the magnitude of the associations and made it insignificant ($P=0.184$, Table 3.13). It is noteworthy that resistin level variation associated with gender which was seen in T2DM patients was entirely accounted for by the difference in BMI rather than a real variation among genders. This agrees with the majority of studies which showed that resistin levels don't correlate with gender (Li et al., 2009).

The association between resistin and insulin resistance in humans has been controversial. Many studies did not find an association between resistin and measures of insulin resistance (Lee et al., 2003, Vozarova de Courten et al., 2004); whereas others found a relationship that was weakened when adjusted for adiposity (Heilbronn et al., 2004, Degawa-Yamauchi et al., 2003). A lot of those studies included small numbers of participants, which limited their power. Our findings are consistent with several previous studies, which reported no evidence supporting a role for serum resistin in mediating insulin resistance or reflecting metabolic parameters in humans. Specifically, we found no associations between resistin and insulin resistance, hypertension, hyperlipidemia, hyperglycemia parameters, in healthy and diabetic male and female subjects. Additionally, we found no difference in resistin levels between obese and non-obese subjects in T2DM patients. Similar findings were published by Heilbronn *et al.* who found that serum resistin concentrations were not different among nonobese, and obese diabetic subjects, and were not significantly correlated to glucose disposal rate during a hyperinsulinemic glucose clamp across groups (Heilbronn et al., 2004). Surprisingly and interestingly, we found a weak negative correlation with resistin levels and BMI in healthy male subjects. The

present findings deviate from those in the study by Degawa-Yamauchi *et al.* who found that there was a significant positive correlation between resistin and BMI. They found that there was more serum resistin protein in the obese than that in the lean subjects (Degawa-Yamauchi *et al.*, 2003). The reason for the lack of stronger association of resistin with BMI is not clear. However, plenty of studies failed to confirm a significant positive correlation between resistin and BMI and found that circulating resistin levels are not associated with obesity or insulin resistance in normal, insulin-resistant, and diabetic subjects (Lee *et al.*, 2003, Anderlova *et al.*, 2006). Although this could be due to obviously a false negative association, a possible explanation for the discrepancy may be due to the fact that in humans, the main sources of this hormone are probably monocytes and macrophages residing in the adipose tissue and not the adipocytes themselves. Several reports indicated that resistin may also be involved in inflammatory processes suggesting that inflammatory status rather than body fat content itself may be the major regulator of serum resistin levels (Lehrke *et al.*, 2004). Some pro-inflammatory agents, such as tumor necrosis factor- α (TNF- α), interleukin 6 (IL-6) and lipopolysaccharide (LPS), can regulate resistin gene expression (Pang and Le, 2006, Lehrke *et al.*, 2004). Resistin mRNA was strongly increased by TNF- α in human peripheral blood mononuclear cells (PBMC) (Kaser *et al.*, 2003). It was also shown that serum resistin is directly correlated with inflammatory markers (C-reactive protein (CRP)) and oxidative stress (nitrotyrosine (NT)) (Bo *et al.*, 2005). An intriguing hypothesis, which needs to be tested, is that resistin is secreted in response to a chronic low-grade inflammation, and has antioxidant properties. Since patients inflammatory condition was not characterized, this hypothesis could not be ruled out. Our data does not reveal a significant association between circulating resistin and degree of obesity in humans and thus do not support a role for resistin in mediating the effect of obesity on insulin resistance or diabetes. Our findings are in accordance with a previous report which showed that serum resistin is not associated with metabolic abnormalities in overweight/obese

patients, perhaps due to the higher fat content in these subjects (Bo et al., 2005).

Despite these discrepancies, it should be emphasized that our finding of a negative association between resistin and BMI in controls is not necessarily in conflict with previous reports that resistin concentrations are positively correlated with the degree of obesity. However, our study design permitted us to demonstrate only that resistin levels are independent of BMI; and not all measures of obesity, such as waist circumference and percentage body fat, which were included in the framework of the present study. Finally, identification of the receptor system for resistin, relevant signaling pathway(s), and their sensitivity state are needed for a complete evaluation of the role of the resistin system in humans (Hotamisligil, 2003).

4.2 Adiponectin and Resistin Levels in Relation to Metabolic and Clinical Parameters

In the present study, we observed clustering of the metabolic risk factors with decreased adiponectin levels. In our study, models including adiponectin as the dependent variable showed significantly higher correlations with the clinical and metabolic parameters compared with a model of resistin as the dependent variable, suggesting that adiponectin is a stronger marker for insulin resistance in humans compared to resistin. This finding would be consistent with prior literature on adiponectin (Baratta et al., 2004, Vendrell et al., 2004, Eizadi et al., 2011). Adiponectin demonstrated slightly stronger associations with IR, TG and HbA1c. Our study results are in line with previous studies demonstrating that adiponectin but not resistin is associated with insulin resistance-related phenotypes (Tejero et al., 2004). Adiponectin showed a significant negative correlation with the parameters related to insulin resistance, such as IR, TG and HbA1c, in contrast to resistin, which showed only positive correlation with age.

This data suggests that the relationship between adiponectin and resistin and insulin resistance is probably not one of direct cause and effect in all instances. It is possible that in some situations their inverse relationship may be mediated in part by insulin levels, by other hormones such as catecholamines or androgens (Fasshauer et al., 2002, Yu et al., 2002, Fasshauer et al., 2001), by proinflammatory cytokines (Maeda et al., 2001, Fasshauer et al., 2003), by medications (Fasshauer et al., 2003, Yu et al., 2002), or possibly by changes in clearance of adiponectin and resistin. An alternative explanation may be related to the different forms of adiponectin and its receptors now identified in animals and humans and the possibility that the biological action of adiponectin may differ among these structural variants and isoforms (Wang et al., 2002, Yamauchi et al., 2003). Although our data indicated a less robust relationship of resistin with insulin resistance and obesity, some or all of these factors may also be influenced by the extent and location of obesity. Although resistin was firstly postulated to contribute to insulin resistance, more and more evidence indicates that it may also be involved in inflammatory processes. Resistin levels are markedly increased by endotoxin administration in humans suggesting that inflammatory status may be the major regulator of serum resistin levels (Lehrke et al., 2004). Additionally, the molecular mechanisms modulating insulin resistance may vary between individuals and in different population and circumstances (e.g., obesity and exercise), and thus the relevance of adiponectin and resistin in these situations may also vary. The results of the current study do not permit us to choose between these alternate explanations, but do indicate that elucidating the relationship between adiponectin, resistin and insulin resistance may be more complex than initially thought.

This is the first study to investigate the relationship between adiponectin, resistin and metabolic parameters in a Bahraini population. We have observed that resistin is not associated with insulin resistance. However, previous observations of relationships with weight, T2DM, insulin

resistance-related phenotypes and adiponectin are confirmed. In humans, the metabolic role of resistin is even less clear since receptors have not been identified yet. More work is needed to characterize its target tissues and its receptor, to define its role in signal transduction especially that of insulin, and to delineate a possible role in diabetes and insulin resistance. In contrast to resistin, the adipocyte-hormone adiponectin is considered an important link between insulin resistance and disorders of metabolism of lipid and glucose in diabetes (Cederberg and Enerback, 2003, Soebijanto and Waspadji, 2010). Given the high incidence of diabetes and obesity in Gulf States, particularly in Bahrain (Al-Mahroos and McKeigue, 1998), studies of the roles of adiponectin and resistin in these populations remain a research priority. These studies will shed new light on the prevention and treatment of type 2 diabetes mellitus, and open new avenues for the development of novel therapeutics to treat insulin resistance-related phenotypes.

4.3 Analysis of Adiponectin Gene SNPs +45T>G and +276G>T Polymorphisms

Several susceptibility loci for type 2 diabetes have been identified by genome wide linkage scans (McCarthy, 2003). Among these regions, 3q27 appears to be of special interest, because it harbours the ADIPOQ-gene-encoding adiponectin, which is known to modulate insulin sensitivity and glucose homeostasis (Kissebah et al., 2000, Kondo et al., 2002). It has been demonstrated that serum adiponectin level is highly heritable (Vasseur et al., 2002, Comuzzie et al., 2001) and the plasma level, expression, and biological effects of adiponectin are associated with polymorphisms in the ADIPOQ gene. A series of SNPs in the adiponectin gene have been reported in various ethnic populations. Among these SNPs, two common SNPs, a silent T>G substitution in exon 2, SNP +45T>G (rs2241766) and a G<T substitution in intron 2, SNP +276G<T (rs1501299) have been studied in several populations including Europeans, Asians, and Americans (Gable et al., 2006,

Vasseur et al., 2006). Although there is a lack of consistency among studies, the results indicate that genetic variation in the ADIPOQ gene is associated with insulin resistance and T2DM (Hara et al., 2002, Menzaghi et al., 2002, Stumvoll et al., 2002, Vasseur et al., 2002, Xita et al., 2005, Filippi et al., 2004, Jang et al., 2006). Moreover, the strong association with T2DM, insulin levels, and metabolic syndrome, found in the chromosomal region where the ADIPOQ gene is located, suggests that somewhere in this locus, common genetic variant(s) may have a measurable effect on insulin resistance-related phenotypes.

Gonzalez-Sanchez *et al.* found that the SNPs +45T>G and +276G>T of the adiponectin gene are associated with decreased serum adiponectin levels and risk for impaired glucose tolerance in 747 unrelated Spanish subjects (Gonzalez-Sanchez et al., 2005). Also in the Japanese population, Hara *et al.* found that both SNPs +45T>G and +276G>T had strong associations with T2DM, serum adiponectin level and insulin resistance in patients with T2DM (Hara et al., 2002). However, similar associations were not completely revealed either in a Chinese population (Xia et al., 2004) or in a Korean population (Xia et al., 2004, Lee et al., 2005). In the Korean study, plasma levels of adiponectin were not statistically different according to SNP +45T>G and SNP +276G>T, in either control or type 2 diabetic subjects. The genotype distributions of these SNPs had no association with the risk of type 2 diabetes and metabolic parameters of insulin resistance. In the Chinese study however, only variation at position +45 was demonstrated to be associated with T2DM. However, in Arabs, no data is available regarding the genetic contribution of adiponectin gene polymorphisms on serum adiponectin level, T2DM and metabolic parameter. The present study investigates whether or not SNPs +45T>G and +276G>T in the adiponectin gene are associated with serum adiponectin levels, type 2 diabetes mellitus and insulin resistance in Bahraini patients with T2DM.

4.3.1 Association of Genetic Variants in the Adiponectin Gene with Adiponectin Level and T2DM

To our knowledge, this is the first study that investigates the association between the SNPs +45T>G and +276G>T of the adiponectin gene and serum adiponectin level and its relation to T2DM and metabolic syndrome in the Bahraini population. We assessed whether SNPs +45T>G and +276G>T in the ADIPOQ gene are associated with total adiponectin concentrations, T2DM, metabolic parameters, measures of insulin sensitivity, and obesity. Genotypes data from 206 individuals were available for analysis in our study. In the current study we observed that the G allele and TG/GG genotype of SNP +45T>G occurred more frequently than the T allele and TT genotype in Bahraini T2DM patients compared to the controls. Subjects with the G/G + TG genotype of SNP +45T>G were associated with lower serum adiponectin levels and were at increased risk for T2DM compared with those having the T/T genotype. Our findings are in agreement with the previous reports published separately by Zacharova *et al.* and Mohammadzadeh and Zarghami, who found that the G allele of SNP +45T>G was associated with lower serum adiponectin levels and increased risk for development of T2DM compared to the common T-allele in the STOP-NIDDM trial (which was conducted in Canada, Germany, Austria, Norway, Denmark, Sweden, Finland, Israel, and Spain) and in an Iranian population respectively (Zacharova *et al.*, 2005, Mohammadzadeh and Zarghami, 2009).

The associations of the SNP +276G>T alleles and genotypes with T2DM were also assessed, however, no significant association was found in Bahraini men and women. For the SNP +276G>T, no significant difference was found in allele or genotype frequencies between T2DM patients and the controls. Furthermore, no significant association between the genotypes and serum adiponectin levels was seen. These results were similar to some previous studies in French (Vasseur *et al.*, 2002), American (Hu *et al.*, 2004), and Swedish (Kang *et al.*, 2005)

populations, but contradicted other studies that showed there was a significant association of SNP +276G>T with T2DM. The study in the Japanese population demonstrated that SNP +276G>T was significantly associated with type 2 diabetes, and decreased levels of circulating adiponectin (Hara et al., 2002). SNP +276G>T was also reported to be associated with T2DM and low adiponectin levels in Italian subjects (Gu et al., 2004). The conflicting results indicated that strong racial and regional variations in these different populations could play a key role in the distribution of these SNPs and determining their related phenotypes.

Prior data on adiponectin gene variation, serum adiponectin levels and risk of type 2 diabetes have been inconsistent. In a Japanese study that included 384 type 2 diabetic subjects and 480 nondiabetic control subjects, subjects with the G/G genotype at position +45 or the G/G genotype at position +276 had a significantly increased risk of diabetes compared with those with the T/T genotype at these loci (Hara et al., 2002). In a study conducted in Germany, subjects with G/G + G/T genotypes at position +45 had higher BMI, lower insulin sensitivity and lower serum adiponectin level, but these associations were significant only among subjects without a family history of diabetes (Stumvoll et al., 2002). Moreover, Menzaghi *et al.* reported that a haplotype defined by the SNPs +45T>G and +276G>T was associated with several components of the insulin resistance syndrome. However, neither the two individual SNPs nor their haplotype were associated with risk of type 2 diabetes (Menzaghi et al., 2002). Similarly, a study conducted in French Caucasian families did not detect any significant associations between these two SNPs and risk of type 2 diabetes (Vasseur et al., 2002).

Cross-sectional studies have yielded controversial findings concerning the association of SNP +45T>G in particular. The G-allele of SNP +45T>G has been reported to be associated with an increased risk of type 2 diabetes in Japanese subjects (Hara et al., 2002). However, other studies have reported that the T-allele is a risk allele for the insulin resistance syndrome and type 2 diabetes in Italian (Menzaghi et al.,

2002), French (Vasseur et al., 2002), and healthy Caucasians subjects (Mackevics et al., 2006). Other studies have not found any effect of SNP +45T>G on adiponectin level and risk for type 2 diabetes, an example being the study published by Vozarova de Courten *et al.* who found no association between SNP +45T>G and serum adiponectin or diabetes in Pima Indians (Vozarova de Courten et al., 2005). These conflicting findings could be due to true differences in allelic association with the disease phenotype in various ethnic populations which is believed to be caused by variation in the response to environmental and genetic influences. In agreement with this hypothesis are differences in allele frequencies of SNPs in the adiponectin gene and its related phenotypes in various populations (Takahashi et al., 2000, Yang et al., 2003, Ukkola et al., 2003, Hara et al., 2002).

Mechanisms explaining the effect of the G-allele of SNP +45T>G on the risk of type 2 diabetes have not yet been demonstrated. T2DM is characterized by lower levels of serum adiponectin compared to non-diabetic subjects. Therefore, the role of the G/G genotype of the SNP +45T>G in increasing the risk for T2DM in some populations, as demonstrated, is consistent with its role in decreasing serum adiponectin levels. This could also be due to the effect of adiponectin on both glucose and lipid metabolism (Tsao et al., 2002). Fumeron *et al.* found that the G/G genotype of SNP +45T>G was associated with the risk of hyperglycemia in a 3-year prospective study in approximately 4,500 French Caucasian subjects (Fumeron et al., 2004).

The association of adiponectin gene variants with adiponectin levels remains unclear; therefore, other mechanisms must be considered to explain our findings. It has been recently reported that SNP +45T>G is in strong linkage disequilibrium with other SNPs of the adiponectin gene or other close genes having an effect on adiponectin expression, secretion, structure, or action that might by itself explain the observed association with adiponectin concentrations. It is interesting to note that the SNP +45T>G is in almost complete linkage disequilibrium with an

insertion/deletion polymorphism in the 3' UTR (position 2019) (Menzaghi et al., 2002). As the 3' UTR region plays a key role in the control of gene expression by binding proteins that regulate mRNA processing, translation or degradation, it could have a strong influence on the investigated protein and related diseases (Conne et al., 2000). Though the SNP +45T>G is located in exon 2 of the adiponectin gene and does not cause an amino acid change (GGT to GGG, Gly15Gly) (Takahashi et al., 2000), Yang *et al.* showed that the silent +45T>G mutation may alter RNA splicing or stability, suggesting an allele-specific differential expression of adiponectin (Yang et al., 2003).

Other factors rather than adiponectin SNPs have been shown to regulate adiponectin levels. A Mediterranean diet or a diet rich in whole grain and fat was shown to produce increased adiponectin levels (Mantzoros et al., 2006, Mantzoros et al., 2005). Physical activity was also shown to influence adiponectin, with high levels of physical activity shown to elevate adiponectin levels (Yu et al., 2009). There is also a consensus report by Nelson *et al.* in which adiponectin levels are altered independently of ADIPOQ polymorphisms after dietary supplementation with α -linolenic acid (Nelson et al., 2007). The small sample size (n=57) in the previous study cannot allow interpretation of the genetic effect, thus a possible assumption can be made that specific effects of dietary factors (i.e., high omega-3 fatty acids) can mask the genetic effects in relatively insulin sensitive healthy subjects. Because we did not evaluate diet and exercise interventions in our study, the effect of these factors on serum adiponectin levels could not be excluded. In addition, direct comparisons between studies are difficult, considering the differences in the characteristics of the subjects populations studied (healthy, young, primarily female, versus hyperglycemic patients, middle-aged, fewer female, different ethnicities) and study intervention program.

4.3.2 Adiponectin Gene Variations and Aspects of the Metabolic Syndrome

Although we observed an association between genetic variations in the ADIPOQ gene and adiponectin levels, we could not detect an association between the SNPs +45T>G and +276G>T with the anthropometric and metabolic parameters related to insulin resistance. In the current study, a reasonable implication is that the genetic effect of SNPs +45T>G on the circulating adiponectin levels is mediated through an alteration of gene expression that would eventually affect some of the metabolic parameters. Nevertheless, statistical analysis did not reveal any significant association of SNPs +45T>G and +276G>T with the majority of the clinical and metabolic parameters. This discrepancy has also been shown in other studies where both SNPs did relate to adiponectin concentrations but not to the metabolic parameters. Mackevics *et al.* found a strong association of the adiponectin SNPs +45T>G and +276G>T genotypes with adiponectin levels in healthy Caucasians. However, no association of this gene locus with metabolic parameters of the insulin resistance syndrome could be confirmed (Mackevics *et al.*, 2006). Although adiponectin levels vary significantly with sex, prospective genotype effects on the metabolic parameters were very similar between men and women. This confirms that a mechanism other than an effect on adiponectin concentrations could be involved.

Previous studies showed that the association between ADIPOQ +45T>G and ADIPOQ +276G>T polymorphism with the obesity and metabolic syndrome is conflicting (Laclaustra *et al.*, 2007). Although these two SNPs have been associated with obesity in Japanese subjects, Caucasians residents and Taiwan residents (Hara *et al.*, 2002, Menzaghi *et al.*, 2002, Yang *et al.*, 2007); Gonzalez-Sanchez *et al.* have shown no association between these polymorphisms and BMI in Spanish subjects (Gonzalez-Sanchez *et al.*, 2005). This would agree with our data which did not show an evidence for linkage between BMI and ADIPOQ SNPs and detected no correlation between the genotype and clinical data.

The reasons for not finding a positive association between SNPs +45T>G and +276G>T and the metabolic parameters are not clear, however, several possibilities should be considered. First, metabolic parameters of the insulin resistance syndrome are not only determined by serum adiponectin levels, but are influenced by other multiple genetic and environmental factors or lifestyle intervention (Corpeleijn et al., 2007, Dedoussis et al., 2007). Second, there might be several genes, which have an influence on adiponectin levels and the ADIPOQ gene is only one gene regulating adiponectin concentration (Maeda et al., 2001, Yang and Chuang, 2006). Third, different patterns of linkage disequilibrium and ethnicity among populations could be a reason. It should be noted that the study sample included only Arab subjects and in particular Bahraini. Therefore, our findings might be unique to the Bahraini population and it may not be applicable to other ethnic and racial groups. Fourth, the disparity between our results and expectations may be due to lack of power caused by the relatively small number of patients tested. Large, varied populations of patients should be tested to avoid a statistical false negative. This study is the first report that clarifies the association between ADIPOQ and clinical and metabolic data in Bahraini diabetic patients. Further large-scale genome-wide association studies are recommended to resolve the relationship between ADIPOQ gene variations and the metabolic syndrome.

4.6 Study Limitations

4.6.1 Analysis of Serum Adipokines Levels

The limitations of this study need to be addressed. First, the antihypertensive, antidiabetic and lipid-lowering medications might have influenced the results from the study. The association analysis with related traits has been carried out using samples of diabetes and a group of them were on current medications. The result may be influenced by medical care, therefore, further studies are required to determine whether the association of T2DM with serum levels is more general and whether other variants explain the association. Second was the difference in gender distribution between the groups. To minimize the confusion, we demonstrated and compared the level of adipocytokines according to gender in both diabetic and control subjects. Third, the study sample only included Bahraini, and it is possible that results from the present study may not be applicable to other ethnic and racial groups. Limitations of the present study also include its cross-sectional design, so we cannot exclude potential biases. The possibility of selection bias from the hospital-based case-control study is a relevant issue. Although the study subjects were a general population, they might have better health-related behavior or higher socioeconomic status than others because they chose to take a routine health examination. In addition, the results were based on single measurements of the adipokines and therefore may not reflect long-term levels of these hormones. However, single measurements of resistin and adiponectin have been suggested to be reliable for the risk assessment in longitudinal studies for adiponectin over 3 years (Kaplan et al., 2007). Further long-term prospective studies with a larger sample size are required to reveal the predictive value of the adipokines in association with metabolic syndrome and to establish a causal relationship between adiponectin gene and metabolic syndrome in patients with T2DM.

4.6.2 Analysis of Adiponectin Gene SNPs +45T>G and +276G>T Polymorphisms

Our study is limited by relatively low numbers of subjects; therefore our results of genetic analyses need to be interpreted with caution, and should be replicated in future studies with much larger sample numbers. Some minor genotype-induced differences in metabolic factors may have been missed due to lower statistical power. Further additional studies with a larger sample size are needed to confirm our findings. Despite these limitations, we showed that ADIPOQ genetic variants can impact the inter-individual differences in adiponectin levels. We found the greatest decrease in adiponectin levels in obese diabetic subjects who carry the GG/TG genotype of ADIPOQ +45T>G. However, long-term prospective studies are needed to examine the relationship between adiponectin levels and clinical outcomes in these subjects. Limitations of the present study also include the limited amount of data available about the genetic contribution of ADIPOQ on serum adiponectin levels and metabolic syndrome among Arabs; thus we were unable to compare our results with individuals from same ethnicity but rather with different ethnic and racial groups.

Conclusion

In conclusion, this study confirmed that obesity and type 2 diabetes are associated with low serum adiponectin concentrations in a Bahraini diabetic population. We have also observed that resistin levels correlate positively to the risk of diabetes. The genetic analysis revealed that SNP +45T>G polymorphism is associated with lower serum adiponectin levels in a Bahraini population, and it might contribute to the genetic risk for hypoadiponectinemia in T2DM but no association with metabolic parameters of the insulin resistance syndrome was detected. SNPs that are associated with T2DM or insulin resistance vary in different ethnic populations, therefore, the possibility of other SNPs that playing a major role in the developing of T2DM or insulin resistance requires further study. Additional investigations are also needed to explore the complex interaction between environmental factors and ADIPOQ gene polymorphisms in terms of susceptibility to the metabolic syndrome.

Further studies aimed at screening for polymorphisms within the entire adiponectin gene along with other candidate genes located in the 3q27 region and subsequent functional studies of the identified SNPs are warranted to understand the molecular mechanisms of the observed associations.

Future Plans

The initial results have been presented in poster presentation at the RCSI-Bahrain research day on 13th Dec 2011. The poster information is as follow:

Poster Title

The Influence of SNPs +45T>G and +276G>T at the Adiponectin Gene Locus on Serum Adiponectin Levels, Type 2 Diabetes Mellitus and Related Metabolic Parameters in the Bahraini Population

Authors and affiliations

Al Hannan FA¹, Culligan KG¹, O'Farrell PA¹, Morgan MP², Tighe O²
¹School of Medicine, RCSI Bahrain, ²Molecular & Cellular Therapeutics, RCSI Dublin

Since the final data has just been analyzed and results obtained, it is the intention to present and publish the data locally and internationally.

Appendices

Appendix 1

Royal College of Surgeons in Ireland- Medical University of Bahrain Research Clinical Data Sheet- Controls

Title: Resistin and Adiponectin in the Bahraini Population: Plasma Levels,
Gene Polymorphisms and Association with Type 2 Diabetes and Related
Metabolic Parameters

Personal Specifications

Patient Name: -----
Patient CPR: -----
Date of Birth: -----
Nationality: -----
Telephone No.: -----

History

Are you smoker	Yes	No	Ex-smoker
Do you suffer from the following illnesses?			
Hypertension	Yes	No	
Dislipdeima	Yes	No	
Type 2 diabetes	Yes	No	
Metabolic disease	Yes	No	
Coronary heart disease	Yes	No	
Congestive heart failure	Yes	No	
Stroke	Yes	No	
Chronic renal disease	Yes	No	
Active hepatic disease	Yes	No	
Chronic diseases	Yes	No	
Any other acute or chronic inflammatory illness	Yes	No	
If yes, specify			
Are you on medication?	Yes	No	
If yes, specify			
Have you been admitted to hospital in the last 2 weeks?	Yes	No	
If yes, specify			

Clinical Parameter

Height (cm): -----
Weight (Kg): -----
Systolic blood pressure (mmHg): -----
Diastolic blood pressure (mmHg): -----

Subject ID -----

Appendix 2

**Royal College of Surgeons in Ireland- Medical University of Bahrain
Research Clinical Data Sheet- Diabetic cases**

Title: Resistin and Adiponectin in the Bahraini Population: Plasma Levels,
Gene Polymorphisms and Association with Type 2 Diabetes and Related
Metabolic Parameters

General Information

1.1 CPR -----
1.2 Gender:-----
1.3 Date of birth: -----
1.4 Nationality: -----
1.5 Smoker? -----
1.6 Family History of T2DM -----

Clinical Parameters

2.1 Height (cm) -----
2.2 Weight (Kg) -----
2.3 Fasting blood glucose (FBG) (mmol/l) -----
2.4 Systolic Blood pressure (mmHg) -----
2.5 Diastolic Blood pressure (mmHg) -----
2.6 HbA1c %-----
2.7 TC (mmol/l) -----
2.8 HDL (mmol/l) -----
2.9 LDL (mmol/l) -----
2.10 TG (mmol/l) -----

HbA1c: glycated heamoglobin, TC: Total cholesterol, HDL: high density lipoprotein, LDL: low
density lipoprotein, TG: Triglycerides

Management

Insulin	Yes	No
If yes, specify dose and frequency		
Oral Antidiabetic Therapy	Yes	No
Name of the medication/s		
Specify dose and frequency		
Diet and Exercises	Yes	No
If yes, specify dose and frequency		
Other, Specify		

Complications

Have you ever had the following?

Complications	Yes	No
Macrovascular		
	PVD	
	IHD	
	TIA	
Microvascular		
	Eye	
	Kidney	
	Nerve	

PVD: Peripheral vascular disease, IHD: Ischemic heart disease, TIA: transient ischemic attack

Subject ID -----

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