Leptin receptor gene polymorphism in obese individuals in the Saudi population

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Approval Sheet

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Dedication

To my dear children Khalid, Deema and Dania, and to all those who are overweight or obese.

Your loving mother

Maha

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My first thanks are to Allah who helped me throughout this work

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List of abbreviations

Ab	**Antibody**
Ag	**Antigen**
ATP	**Adenosine tri phosphate**
BBB	**Blood brain barrier**
BMI	**Body mass index**
bp	**Base pairs**
CHD	**Coronary heart disease**
CSF	**Cerebrospinal fluid**
CVD	**Cardiovascular disease**
EDTA	**Ethylene diamine tetra acetic acid**
FFA	**Free fatty acids**
HDL	**High density lipoprotein**
HDL-C	**High density lipoprotein-Cholesterol**
Kg	**Kilograms**
Kg/m^2	**Kilograms/ meter ^{2}**
LDL	**Low density lipoprotein**
Lep	**Leptin gene**
LepR	**Leptin receptor gene**
MC4-R	**Melanocortin 4-receptor**
mM	**Millimolar**
mmol/L	**Millimole/Litre**
µU/ml	**Microunit/milliliter**
NaCl	**Sodium chloride**
ng/ml	**Nanogram/milliliter**
NPY	**Neuropetide Y**

OB-R	**Leptin receptor**
Ob-Ra	**Leptin receptor type a**
Ob-Rb	**Leptin receptor type b**
Ob-Rc	**Leptin receptor type c**
Ob-Rd	**Leptin receptor type d**
Ob-Re	**Leptin receptor type e**
p	**2 tailed significance**
POCS	**Polycystic ovarian syndrome**
PCR	**Polymerase chain reaction**
PPAR	**Peroxisome proliferators activated receptor**
QC	**Quality control**
RIA	**Radioimmunoassay**
SD	**Standard deviation**
SEK	**Standard error of kurtosis**
SEM	**Standard error of mean**
SES	**Standard error of skewness**
SDS	**Sodium dodecyl sulfate**
Tris	**Hydroxymethyl aminomethyl**
UV	**Ultra violet radiation**

1.0 Summary:

1.2 Summary: (English)

Leptin, the adipose tissue-derived obesity hormone, brings about it`s regulatory action through binding to it`s receptor. It was shown that obesity is a leptin resistant state, where the defect may be at the leptin receptor level, caused by a mutation or polymorphism in the gene. This study focuses on a pentanucleotide insertion polymorphism found at the 3`-UTR of the leptin receptor gene in obese and non-obese Saudis. The study also investigates the effect of leptin receptor polymorphic forms and it`s affect on the development of obesity, as well as comparing the levels of leptin, insulin and lipids in Saudi individuals with the different leptin receptor genotypes and in different weight groups.

The investigation of leptin receptor pentanucleotide polymorphismwas carried out on a group of 99 individuals, 79 were females and 20 were males. It was found that the pentanucleotide insertion/deletion polymorphism does not imply morbid obesity in Saudi individuals, because even normal weight Saudis were homozygous for the insertion. The frequency of the different genotypes and of the alleles in this study on individuals of the Saudi population showed a significant difference between lean and obese Saudis. The frequency of the (+ +) genotype in the obese group (36.66 %) was higher than in the lean group (14.63 %), as well as the frequency of the insertion allele was higher (0.46) in obese Saudis when compared to the frequency in lean Saudis (0.32). The results obtained from this study indicated that in the Saudi population, the BMI mean seemed to increase starting from the (- -) and reaching its highest level at the (+ +) genotype. An interesting finding was the lower insulin levels in the heterozygous carriers of the insertion allele when compared to non-carriers. Another finding was that leptin levels in the different genotypes was 14.27, 15.02 and 17.04 ng/ml respectively. A significant increase was observed form the (- -) to the (+ +) genotypes, but such a difference was not obvious when the males and females were analyzed separately. Lipid levels were not different in the different leptin receptor gene in the Saudi population.

In this study, the plasma levels of leptin, cholesterol, triglycerides and insulin in both male & female invididuals of the Saudi population were measured. The total number

of individuals was 199 (about 31 males and 168 females). Personal data was taken from these individuals and registered into specially created forms, this data included: weight, height, bmi, age and sex, as well as history of disease.

The leptin mean in the total study population of 199 individuals was 15.7 ng/ml, while the leptin mean for Saudi males and females was 11.95 and 16.4 ng/ml respectively. The leptin mean of Saudi females is higher than for Saudi males, which is an expected results due to the difference in the percentage of body fat anf fat distribution between the two sexes. The cholesterol mean for the total study population and for males and females was 4.76, 4.56 and 4.8 mmol/l respectively. The mean triglyceride level in the total study population and in Saudi males and females was 1.25, 1.55 and 1.23 mmol/l respectively, while the mean insulin level for the total study population and for males and females was 13.6, 13.13 and 13.68 μU/ml respectively. When the mean values of leptin, lipids and insulin were measured in different weight groups, the values were higher in obese Saudis, as expected. Correlations were performed for leptin, lipids and insulin in Saudi males and females and they showed that in males, leptin correlated positively with height, BMI and cholesterol (according to t-test). Cholesterol correlated positively with height, BMI and leptin (according to t-test), while triglycerides (according to t-test), correlated positively only with height, whereas insulin correlated positively only with weight. In females, leptin correlated positively with age, weigh, BMI, triglycerides and insulin. Cholesterol correlated positively with age, weight, BMI, triglycerides and insulin (according to t-test), whereas triglycerides correlated positively with age, weight, BMI, leptin, cholesterol and insulin (according to t-test). Insulin correlated positively with weight, BMI, leptin, cholesterol and triglycerides (according to t-test).

In conclusion, this study has revealed the presence of a 3`-UTR pentanucleotide insertion/deletion polymorphism in the leptin receptor gene in the Saudi population, homozygous individuals for the insertion allele were found both in lean and obese individuals of the Saudi populations which means that this polymorphism like in other populations, does not imply the risk of morbid obesity in the Saudi population. This polymorphism was accompanied by lower insulin levels noticed in the carriers of the insertion allele when compared to non-carriers.

1. 1 Summary (Arabic)

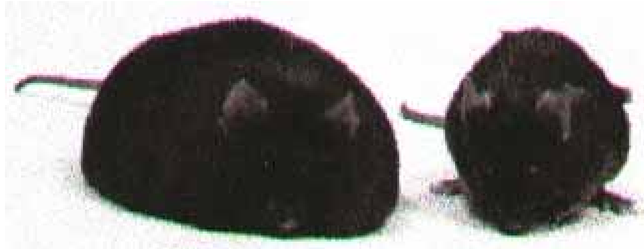
2.0 Introduction

2.1 Obesity

Obesity is a term used to indicate excessive deposition of fat in the body. It is mainly a multifactorial disorder caused by both genetic and environmental factors, where genetic susceptibility is necessary for the environmental factors to precipitate the disease. The genetic factors are mainly multiple genes as seen in the common form of obesity (polygenic), but in a few rare types monogenic obese phenotypes are known (Bougneres et al 2002). More than 300 genes, markers and chromosomal regions have been identified which may contribute to development of obesity. These include peroxisome-proliferator-activated receptor (PPAR) γ2, the leptin gene and the leptin receptor gene (Chagnon et al 2003).

Obesity is characterized by highly elevated intra-abdominal and/or subcutaneous fat deposition. It also occurs in animals and obese mice (Figure 2.1) which have played an important role in the study of genes contributing to obesity development. Obesity is a major risk factor for hypertension, coronary heart disease, and non-insulin-dependant diabetes mellitus (NIDDM), as well as dyslipoproteinemia and cancer (Francke et al 1997). According to the World Health Organization obesity is divided into 3 grades, ie grade 1, grade 2 and grade 3, depending on a corresponding body mass index (BMI) of $>$ 25-29.9, $>$ 30-39.9 and $>$ 40 kg/m^2 respectively (Francke et al 1997). Obesity occurs at a high prevalence in several developed countries and is believed to be the most common nutritional disorder in the World, including Saudi Arabia (El Hazmi et al 1997). According to the definition of overweight and obesity, 6-8 % of the population in many western countries have to be considered obese (Francke et al 1997). In the Kingdom of Saudi Arabia, several studies have been carried out and the numbers of obese individuals is rising in an alarming rate. In a national study on Saudi adults, 13.05 % of the males and 20.26 % of the females were found to be obese, while 27.23 % and 25.20 % respectively were overweight (EL-Hazmi et al 1997), obese individuals with a BMI >30 are subjected to a mortality rate three times as high as normal weight individuals (BMI < 24.9) (Lu et al 2000).

Figure 2.1 Obese and lean mice



2.1.1 Risk factors for obesity

Obesity results from interactions between multiple genes and the environment. It does not follow a clearcut pattern of inheritance, but concentrates in families. Extensive studies on twins have confirmed it`s genetic aetiology, where it has been shown that several genes (polygenic) contribute to susceptibility to development of obesity in presence of predisposing environmental factors (Bougneres et al 2002). Parents obesity is the most important risk factor for childhood obesity. Obesity is on the rise in children and in a study on Saudi children, 1-15 years old, 10.68 % and 12.7 % of the boys and girls were overweight, while 5.9 % and 6.74 % respectively, were reported as obese (EL-Hazmi et al 2001).

Some of the environmental factors which contribute to obesity development, include: eating habits and lack of physical activity, as well as some diseases and drugs that might cause obesity (Chagnon at al 1999). Several genetic risk factors contributing to the development of obesity have been investigated in recent studies, and a number of genetic defects in development of obesity in humans have been identified. These include defects in leptin gene, pro-opiomelanocortin gene, insulin gene and the gene which encodes for the leptin receptor (Arner 2000).

2.1.2 Genetics of obesity

Studies of monozygotic twins and family segregation studies have shown that obesity has a considerable genetic component. A finding based on family studies by Bouchard et al (1993) estimated that up to 75% of the variation of BMI might be explained by

genetic factors in some populations. It has been suggested that inheritance may account for 25 – 40 % of inter-individual difference in adiposity (Oksanen et al 1996). Single gene defects leading to obesity have been discovered in animals and, in some cases, confirmed in humans (Maffeis 2000). These include congenital leptin deficiency or congenital leptin receptor deficiency (Lahlou et al 2002). However, in most cases, genes involved in weight gain do not directly cause obesity but increase the susceptibility to fat gain in subjects exposed to a specific environment (Allison et al 2000). Both genetic and environmental factors promote a positive energy balance which may cause obesity (Bougneres 2002).

The role of the environmental factors in the development of obesity is believed to be the cause of the rapid increase of the prevalence of obesity accompanying the rapid changes in the lifestyle of different populations (Allison et al 2000). In particular, the fat intake is believed to influence the development of obesity (Chagnon et al 1999).

Genetic defects and polymorhisms in the leptin receptor gene have been implicated in the development of obesity. Some of the polymorphisms reported include : Lys109 Arg in exon 4, Gln 223 Arg in exon 6 and Lys 656 Asn in exon 14 (Thompson et al 1997). These mutations have been linked to the studies on obese individuals to indicate that leptin is involved in the regulation of weight gain (Mantzoros et al 1998). A polymorphism in the leptin receptor gene, reported in some obese individuals is the pentanucleotide insertion/deletion polymorphism. This polymorphism occurs in the 3`-untranslated region of the leptin receptor gene, which has been associated with variations in insulin levels and the risk of type 2 diabetes in non-diabetic middle aged men (Oksanen et al 1998).

2.1.3 Leptin and leptin receptor during obesity

Leptin is the adipose tissue peptide hormone which plays an important role in the regulation of body fat and therefore it was called the obesity hormone (Clement et al 1998). Since leptin inhibits food intake by its action on Neuropeptide Y (NPY), initially it was believed that reduced leptin levels may be the cause of obesity (Davies et al 1994) and indeed several individuals were identified with low leptin levels (Mantzoros et al 1998). However, several studies have shown that in most obese individuals, leptin levels are either normal or higher than in normal individuals

(Oksanen et al 1998). These results indicate that in obese individuals there may be a leptin resistance (Igel et al 1997), This resistance is believed to play a role in the development of obesity due to the fact that the excess amount of leptin cannot perform its role in controlling food intake, due to the presence of leptin receptor resistance (Oksanen et al 1998).

The leptin resistance is believed to result from genetic defects in the leptin receptor gene, where the product of the gene is a truncated receptor which lacks most of the cytoplasmic region, Hence, individuals with this mutation, tend to eat more than needed and keep gaining weight (Lahlou et al 2000). Recent studies have shown that suppressor of cytokine signaling 3 (SOCS-3) is a leptin inducible inhibitor of leptin signaling and a potential mediator of leptin resistance in obesity. Leptin resistance can be caused by glucocorticoids which interfere with the interaction of leptin with its receptor (Mantzoros et al 1998). Abnormal leptin receptor, as well as abnormal leptin catabolism and a decreased blood-brain barrier (BBB) leptin transport have been indicated in obesity development Leptin crosses the BBB by a saturable transport system and elevated endogeneous leptin levels during obesity cause an increased competition for binding to the transport system (Burguera et al 2000).

2.1.4 Lipid levels in obesity

Obesity is a risk factor for cardiovascular disease (CVD) which has a high mortality rate in the world (Haffner et al 1998). The link between obesity and CVD are the elevated lipid levels in (especially triglycerides & cholesterol) (Alexander et al 2003). The high levels of cholesterol predispose to several health problems including coronary heart disease, atherosclerosis and gall stones. The main step which triggers the development of atherosclerosis is the deposition of cholesterol-ester filled macrophage foam cells, which contribute to the formation of the atherosclerotic plaque (O`Rourke et al 2001). Gallstones are formed as the bile of obese people is saturated with cholesterol and hence is liable to form stones (Mayes et al 1988). The cause of this saturation is the increased hepatic production of cholesterol as well as low HDL-C levels which cause the accumulation of cholesterol in the bile of the affected individuals, these are accompanied by hypertriglyceridemia (Mendez-Sanchez et al 2002). In addition, the risk of cancer is increased possibly due to the increase in sex hormones (Machinal et al 1999). Elevated plasma levels of

triglycerides are seen in obesity and in type 2 non-insulin-dependent diabetes mellitus which are conditions associated with coronary heart disease development (Szapary et al 2002).

The link between obesity, diabetes and triglycerides is a pancreatic β-cell dysfunction which is caused by the excessive amounts of triglycerides (Shimabukuro et al 1997). A study has revealed the role of leptin in the depletion of triglycerides in all cells which express the leptin receptor at it`s surface via inducing an increase in free fatty acid (FFA) oxidation and a decrease in esterification. This action of leptin is absent in obesity due to leptin resistance (Shimabukuro et al 1997).

2.1.5 Obesity & insulin

Diabetes mellitus widely occurs in the obese individuals due to insulin insensitivity (Meigs 2000). The relation between insulin and obesity lies in the fact that obesity is in most cases accompanied by diabetes especially NIDDM and due to the fact that hyperglycemia especially post-prandial hyperglycemia is a risk factor for atherosclerosis (Haffner 1998). Hence, individuals with NIDDM also have an increased risk of developing cardiovascular heart disease. Hyperinsulinemia may promote atherosclerosis by stimulating smooth muscle proliferation, increasing noradrenaline release through activation of the sympathetic nervous system and stimulating renal sodium and water retention (Haffner 1998). Hyperinsulinemia is also accompanied by elevated triglyceride levels, low levels of HDL, enhanced secretion of VLDL and weight gain (Saltiel 2000).

2.2 Leptin

Leptin is a peptide hormone secreted by adipose tissue. It is made up of 167 amino acids with an amino-terminal secretory signal sequence of 21 amino acids. (Glaum et al 1996).

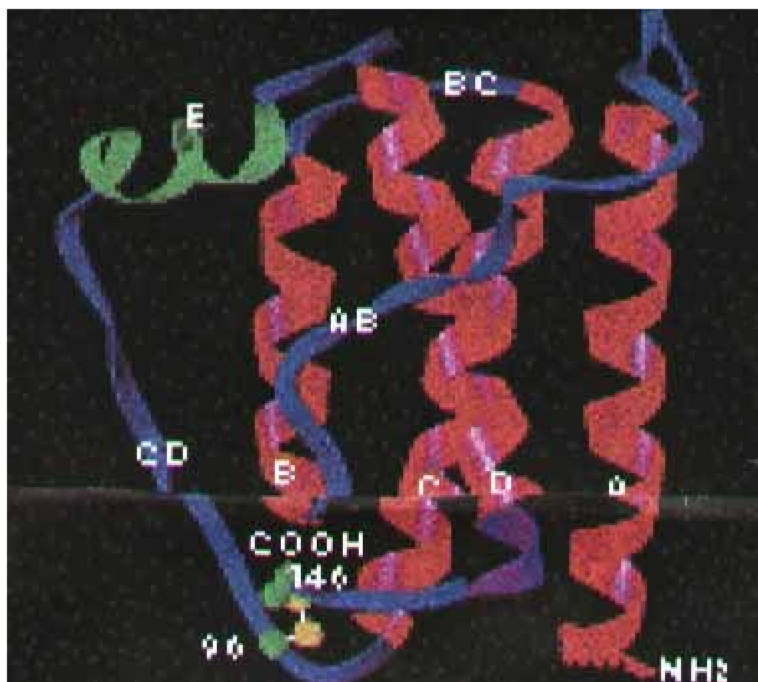
It is a globular protein with a tertiary structure similar to a haemopoitic cytokine (Mantzoro 1998). It regulates food intake, body adiposity and reproductive

competence, and plays a role in fetal growth, gut derived satiety, immune or proinflammatory responses and angiogenesis and lipolysis (Miczke et al 2000).

2.2.1 Structure of leptin

Leptin is a 16 kDa molecule made up of a single subunit of 146 amino acids, It has a four-helical structure which exhibits an up-up-down-down folding pattern Each one of the four helices is about 5-6 turns long, and due to the up-up-down –down arrangement, a two layer packing of the helices is formed (Figure 2.2) (Zhang et al 1997).
There are two long loops connecting helices B to C and the connecting loops wrap around the BD face of the helix bundle and the interhelical angles (Imagawa et al 1998). The features of the loops are similar to those found in the long chain helical cytokine family (Kline et al 1997). Hence, leptin is a globular protein with a tertiary structure (Figure 2.2) (Isse et al 1995).

Figure 2.2 Structure of leptin



2.2.2 Structure of the leptin gene

The leptin gene, referred to as the obesity (Ob) gene, is located on chromosome 7q31.3 (Green et al 1995). It consists of three exons separated by two introns (Thompson et al 1997). The coding region of the Ob gene is 501 nucleotides in length and is contained in exons two and three, which are separated by an intron of 2 kb. (Miller et al 1996). The Ob gene promoter region spans a region of three kb (Chung

et al 1996). Only the first 217 base pairs of the promoter are needed for basal adipose tissue expression of the Ob gene (Mantzoros 1998).

2.2.3 Biosynthesis of leptin

Leptin is synthesized mainly in white adipose tissue and is the gene product of the ob gene (MacDougald et al 1995). White adipose tissue stores energy in the form of triglycerides and releases energy in the form of free fatty acids (Pankov 1996).
On the other hand, brown adipose tissue is responsible for the expenditure of fatty acids derived energy for maintenance of the organism`s thermal stability, dissipating energy as heat (Fielding et al 1996). A direct correlation has been reported between adipocyte tissue mass and leptin levels. Leptin biosynthesis also occurs in other tissues of the body including: the placenta, fetal tissues and gastric mucosa. (Chan et al 2002).

2.2.4 Secretion of leptin

The secretion of leptin is affected by food intake, total body fat and serum levels of several hormones (Mantzoros et al 1998). Insulin, and to a lesser extent other peptide hormones such as pancreatic hormones including glucagon, amylin and pancreatic polypeptide which reduce food intake, have an influence on the secretion of leptin (Mizuno et al 1998). Insulin is the major regulator of leptin production by adipose tissue (Fried et al 2000). Infusions of insulin increases circulating leptin levels in humans (Flier 1998), and leptin levels are markedly decreased in insulin-deficient diabetic rodents (Mueller et al 1998). Based on *in vitro* studies, the effect of insulin in stimulating leptin production appears to involve increased glucose metabolism
(Mantzoros et al 1998). Blockade of glucose transport or glycolysis, inhibits leptin expression and secretion in isolated adipocytes (Mueller et al 1998). Alterations in insulin-mediated glucose metabolism in adipose tissue are likely to mediate the effects of energy restriction to decrease, and refeeding to increase, circulating leptin levels (Havel 2000). Changes in glucose metabolism may also explain the observation that high fat meals lower 24 h circulating leptin levels relative to high carbohydrate meals

in humans, suggesting a mechanism that may contribute to the effects that high fat diets have in promoting increased food intake, weight gain and obesity (Havel 2000). The decreased circulating leptin levels observed during energy restriction is related to increased sensations of hunger in human subjects (Mantzoros et al 1998). Thus, decrease in leptin during energy- restriction and weight-loss regiments might contribute to the strong propensity for weight gain (Flier 1998). Hence, leptin is secreted by adipocytes into the bloodstream and crosses to specific regions in the brain involved in regulating energy balance in the hypothalamus. To reach its target areas, leptin crosses the brain-blood barrier (Burguera et al 2000).

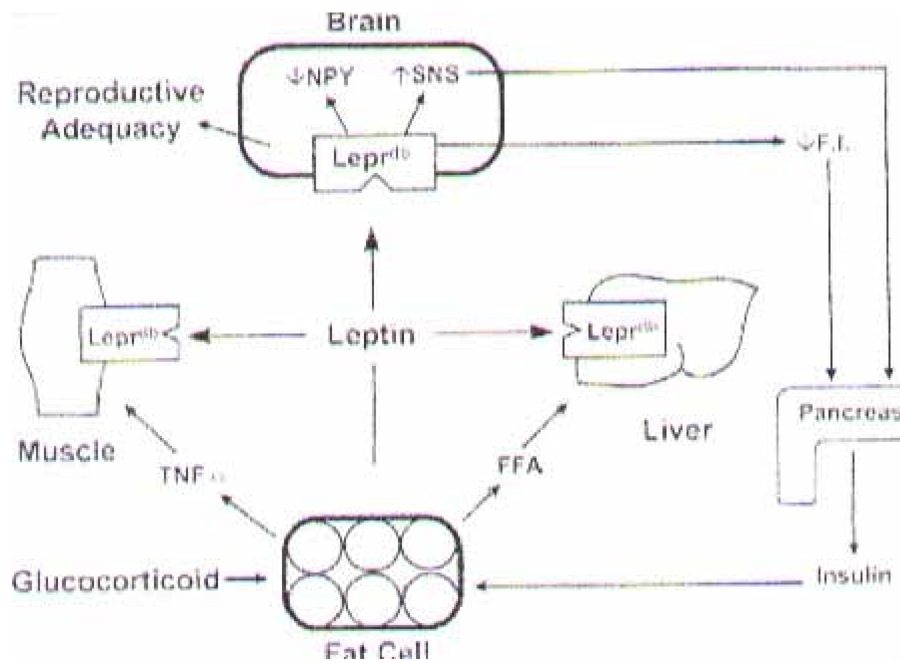
2.2.5 Transport of leptin

Leptin circulates in blood serum in both free and bound forms (Mantzoro 1998). The free form is the biologically active form, while the bound form is bound to a carrier protein. The balance between free and bound leptin is a potential regulator of leptin bioavailability (Lahlou et al 2000).

2.2.6 Mode of action of leptin

The action of leptin is mediated by binding to various isoforms of the leptin receptor (LepR) that are expressed in a variety of tissues including the brain, ovaries and hemopoitic stem cells (Burguera et al 2000). After binding to its receptors, leptin alters the release of several neuropeptides, especially neuropeptide Y (NYP) from the hypothalamus (Baskin et al 1999), and in the hypothalamus leptin inhibits the expression of NPY. Neuropeptide Y is a 36 amino acid protein, from the pancreatic peptide family, which acts as a transmitter in the nervous system. It is important due to its appetite stimulating effect. High hypothalamic concentrations of neuropeptide Y elicit food intake, whereas low concentrations have the opposite effect (Wang et al 1997) (Figure 2.3).

Figure 2.3 Leptin`s mode of action



2.2.7 Signal transduction and mode of action of leptin after binding to its receptor:

Leptin`s signaling occurs through the JAK2/STAT pathway and the phosphorylated residues provide the binding sites for signaling molecules including members of the STAT family (signal transducer and activator of transcription). These molecules are also subject to JAK mediated phosphorylation inducing their release from the receptor complex, and are translocated to the nucleus where they modulate transcription of specific target genes (Fischer et al 1999). So, after leptin binds to its receptor, three conserved tyrosines present in the cytoplasmic domain of the LepR at amino positions 985, 1077 and 1138 become phosphorylated by the activation of (JAK2) which is a tyrosine kinase (Zabeau et al 2003; Lu et al 2000),

2.2.8 Target tissues of leptin

The target tissues for leptin include the hypothalamus as well as peripheral tissues: skeletal muscle, reproductive organs like the placenta, testis and ovaries (Hoggard 2000). In addition, leptin also acts on the lung, stomach and in macrophages and platelets (O` Rourke et al 2001; Lu et al 2000).

2.2.9 Physiological role of leptin

The physiological roles of leptin include the following:
*control of food intake, energy expenditure and lipolysis.
* a role in reproductive competence .
*it plays a role in fetal growth.
*it has a role in immune and proinflammatory responses (Marti et al 1999).

- ***Leptin and the development of obesity:***

Since body weight is regulated by complex mechanisms involving numerous metabolic and hormonal signals, leptin`s mode of action and it`s interactions with other molecules regulating energy homeostasis have been the focus of several studies in order to provide the long needed answers to the pathogenesis of obesity. The result of a recent study was that initially low plasma leptin levels may predispose to future weight gain, due to the lack of control of food intake and energy expenditure by leptin (Mantzoros et al 1998). Other factors which have the potential of causing a positive energy balance which in turn lead to an increase in body weight include a high fat diet and a low level of physical activity, as well as a low resting metabolic rate (Marik 2000). The role of leptin in the regulation of body weight lies in the fact that leptin sends signals to the brain about the body`s energy status, and so has an effect on food intake and satiety (Mantzoros et al 1998). Leptin`s effect on fat metabolism is that it reduces the synthesis of free fatty acids and triglycerides and increases lipolysis. This effect is brought about by an inhibitory effect on acetyl CoA carboxylase, which is the rate limiting enzyme in fatty acid synthesis (Marik 2000).

Since obesity was found to be a leptin resistant state and not a leptin deficient one as first believed (very rare), obese individuals tend to have normal or even higher levels of leptin when compared to normal weight individuals, so this resistance gave reason to believe that the defect lies at the level of the leptin receptor (Saladin et al 1995) (Mantzoros 1999).

The leptin gene and the leptin receptor gene are therefore candidate genes for the development of obesity (Chagnon et al 2003). Mutations in the leptin gene which lead to the development of obesity are reported in a few rare cases, one example are two

morbidly obese children belonging to a Pakistani pedigree with high rate of consanguinity. These children had a mutation in the OB gene which resulted in the deletion of a single guanine nucleotide in codon 133 (Montague et al 1997), another mutation of the leptin gene was found at nucleotide number 26 of the first untranslated exon (Hager et al 1997). Whereas for the leptin receptor gene, it was noticed that a homozygous mutation of the leptin receptor causes early-onset morbid obesity, since there was reduced energy expenditure and no feeling of satiety which leads to increased food intake (Clement et al 1998).

- ***Leptin and its role in reproduction and fetal growth:***

  Leptin has a role in the onset of puberty and the sexual development (Hoggard et al 2001). It was noticed that leptin stimulates the reproductive system in both sexes (Barash et al 1996), this is through an increased release of the pituitary leutinizing hormone and the hypothalamic gonadotropin releasing hormone. Based on these findings it had been suggested that food availability is the most important factor influencing mammalian reproduction (Hileman et al 2000). Leptin has a role in regulating growth and promoting hematopoisis in newborn infants, as well as regulating food intake (Mantzoros 1999).

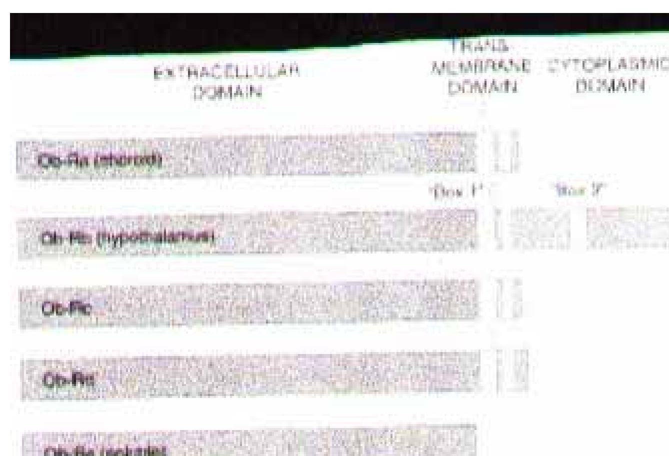
- ***Leptin and its role in immune and pro-inflammatory responses:***

Leptin seems to act as a link between nutritional status and immune function (Esfahani et al 2004; Lu et al 2000), for it has been reported that it elicits a number of immunoregulatory effects including the promotion of T cell proliferative responses and the induction of proinflammatory cytokines (Loffreda et al 1998). Leptin deficiency is associated with an increased susceptibility to infection (Howard et al 1999). It affects the *in vitro* proliferation and cytokine production by naïve and memory T cells (Matarese 2000). Leptin also activates monocytes/macrophages, it was reported that leptin production is acutely increased during infection and inflammation (Fantuzzi et al 2000). Leptin together with ADP, induces platelet aggregation, which suggests the role of leptin as a possible coupling factor between obesity and the cardiovascular disease associated with syndrome X and diabetes (Lu et al 2000).

2.3 The leptin receptor

The leptin receptor (LepR) is a member of the class 1 cytokine receptor family and exists in several alternatively spliced variants which are a result of alternative mRNA splicing at the most C-terminal coding exon (Gotoda et al 1997). In humans there are six isoforms reported so far (Zabeau et al 2003). All of these isoforms are identical in the extracellular binding domain, the transmembrane domain, and the first 29 amino acids of the cytoplasmic domain (Da Silva et al 1998). The longest isoform is Ob-Rb and is made up of 1165 amino acids. It is homologous to mouse Ob-Rb (83%) and is thought to mediate the hypothalamic weight-regulatory actions of leptin (Miczke et al 2000). This isoform is widely distributed in the brain (hypothalamus), while of the four short forms (Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf), Ob-Ra and Ob-Rc can be found in the choroids plexus and brain microvessels suggesting their role in blood-brain barrier transport (Zabeau et al 2003; Tartaglia 1997). The other forms are also expressed in peripheral tissue (Chan et al 2002) especially in the lungs, pancreas, adrenals and testis as well as the ovaries (Burguera et al 2000). Another isoform is Ob-Re and is involved in modulating leptin activity. This is a soluble isoform of the receptor and it is present in serum, and is thought to be the binding protein of the receptor (Chan et al 2002). The isoforms are all generated by alternative splicing from a single gene located on chromosome 1p. (Gotoda et al 1997) (Figure 2.4).

Figure 2.4 Leptin receptor isoforms



2.3.1 Structure of the leptin receptor

The leptin receptor is a transmembrane protein with three domains. These are extracellular, hydrophobic transmembrane and a short intracellular domains (Mantzoros
1998). The long extracellular domain contains the leptin binding domain which was recently identified and localized to residues 323-640. The extracellular domain consists of two barrel-like domains each approximately 100 amino acids in length, which are immunoglobulin like (Haniu et al 1998), two conserved disulphide bridges are found in the N-terminal domain. The intracellular domain contains two sequences for binding of janus kinase, the short form lacks the intracellular domain (Burguera et al 2000).

2.3.2 Structure of the leptin receptor gene

The leptin receptor gene was localized at chromosome 1p between the anonymous microsatellite markers D1S515 and D1S198. The genomic structure of the human leptin gene spans over 70 kb and includes 20 exons (Thompson et al 1997). The first 2 exons are non-coding and are capable of forming several alternative secondary structures. Exon 1 has a GC content of 70% and includes two 8 bp palindromes beginning at positions 28 and 5 (Thompson et al 1997). Exon 3 contains the initiation codon and the putative signal sequence that is also present in exon 4. A newly described imperfect dinucleotide repeat is located in the third intron 230 nucleotides downstream of exon 3. The single putative transmembrane region lies on exon 18, the intracellular domain is encoded in exons 19 and 20, whereas exon 20, the largest exon spans over 900 nucleotides and encodes the last 270 amino acids of the leptin receptor (Thompson 1997).
Several mutations have been identified in the leptin receptor gene. Any mutation in the receptor may result in defective signal transduction and hence altered action of leptin in the target tissue, but not for leptin binding (Lahlou et al 2000). Many of these

mutations are associated with early-onset obesity, hyperphagia, hyperinsulinemia and infertility (Mantzoros 1998). It is unlikely that homozigosity for gross alterations in the leptin receptor gene can explain the variation in human adiposity, but heterozygozity for the loss of leptin function and some common variants could determine susceptibility for increased adiposity within a permissive environment (Chung et al 1998). Leptin resistance can be caused by alterations in the Ob-Rb expression, for this receptor is present in the hypothalamus where it signals various mediators like neuropeptide Y, and the sympathetic nervous system which alters food intake and energy expenditure. It can also be caused by a post-receptor signaling (Zabeau et al 2003). SOCS-3 (suppressor of the cytokine family signaling family) is upregulated following leptin exposure and this results in the inhibition of JAK2 phosphorylation. (Stahl et al 1995). Leptin resistance can be caused by hypercortisolism due to obesity as well as an impaired blood-brain barrier transport which is caused by an increased leptin threshold.

- ***Leptin receptor polymorphisms***

  Some contradictive studies have revealed the effect of some polymorphisms of the leptin receptor on human body mass index (BMI) and fat free mass (FFM). An example of these polymorphisms are the single nucleotide substitutions which cause a single amino acid substitution, two of the more common polymorphisms are: (Lys 109 Arg and Gln 223 Arg). Another polymorphism of the leptin receptor is the pentanucleotide insertion/deletion polymorphism which has been linked to obesity and a variation in insulin levels.

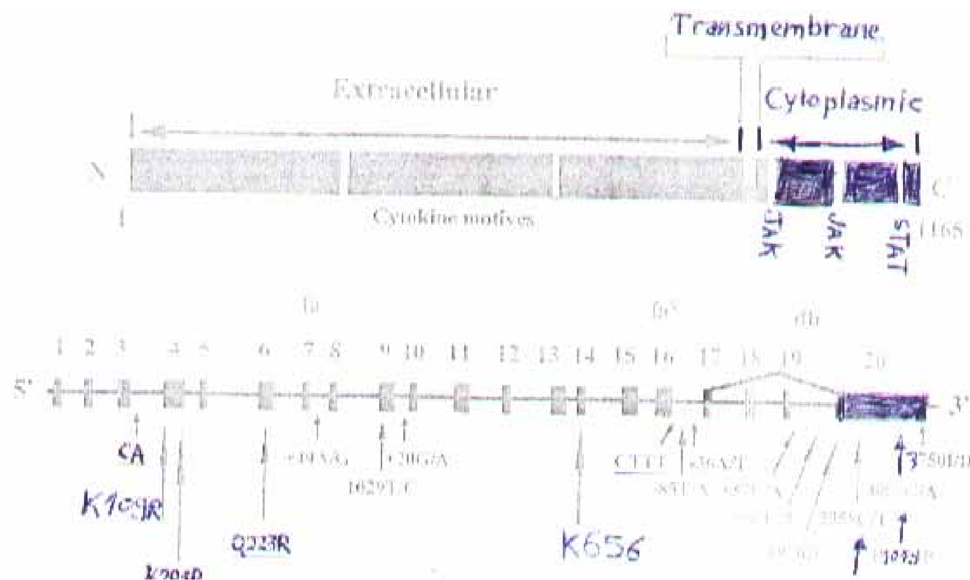
- ***Relationship between tissue expression of the leptin receptor, serum leptin levels and body mass index (BMI):***

A recent study designed to examine the relationship among tissue expression of leptin receptor, serum leptin and body mass index and was performed on 57 non diabetic women who underwent surgery for either myoma of the uterus or ovarian cysts. The findings suggest that there are 3 leptin receptor isoforms in the human omental adipose tissue Ob-Rb, HuB219.1 & HuB219.3) (Huang et al 1999). HuB219.1 is the major isoform of the leptin receptor expressed in human omental adipose tissue and the shorter leptin receptor isoforms in human omental adipose tissue might play an

important role in body weight control. As most obese patients have elevated leptin levels, leptin resistance due to a genetic defect of the leptin receptor remains an attractive explanation for some forms of human obesity. A defect in the leptin-mediated signaling pathway including binding to the leptin receptor could be responsible for this observation (Jacob et al 1997).

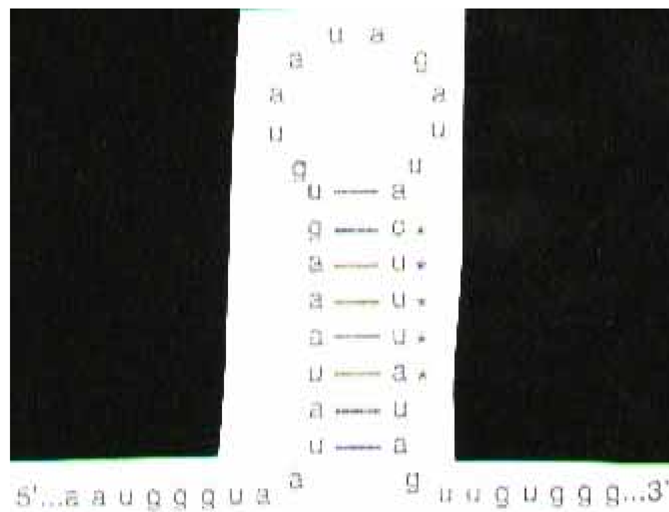
To date, about 19 polymorphisms of the leptin receptor are known, these include 4 amino acid variants of the coding region ie: Lys109 Arg and Gln 223 Arg these cause amino acid substitutions in the extracellular domain of the receptor, studies have related the Gln 223 Arg polymorphism with a strong affect on fat mass, fat free mass and BMI, and adiposity. Carriers of this allele had less fat free mass and higher BMI values compared to non-carriers. Four silent mutations i.e the G→A mutation at position 2145 (Leu 715) of exon 13 (Oksanen et al 1998 ; Chagnon et al 2000), 9 intronic variants not in the coding region of the receptor, like for the example the variants at position 20 (G→A) and at position 31 (T→C) of intron 7, another polymorphism is a CTTT repeat at intron 16 which together with the Lys 656 Asn is related to a change in the cytoplasmic domain properties of the human leptin receptor gene (Chagnon et al 1999), as well as a 3`-untranslated region pentanucleotide insertion /deletion polymorphism (Thompson 1998), (Oksanen et al 1998) (Chagnon et al 2000) (Figure 2.5)

Figure 2.5 Structure of the leptin receptor gene and the location of some polymorphisms



This polymorphism is located 60 nucleotides after the stop signal. It causes the formation of a stem loop in the mRNA which affects it`s stability by rendering it liable for regulatory protein binding, which affects the rate of degradation and /or translation of the mRNA (Oksanen al 1998) (Figure 2.5). This polymorphism has been associated with lower insulin levels (28.7 %) in the carriers of the insertion allele when compared to non-carriers, and the risk of type 2 diabetes in non-diabetic middle aged men (Lakka et al 2000). A Finnish study had shown that carriers of the insertion allele had a 79 % reduced risk of diabetes when compared to non-carriers, this is due to the lesser extent of leptin`s action on the pancreatic β-cells to inhibit insulin secretion. The study revealed that this 3`-UTR insertion was common in the healthy population since the carrier frequency was as high as 23.5 % amongst the controls (Lakka et al 2000).

Figure 2.6 Stem loop-motif in the leptin receptor mRNA resulting from the 3`-UTR pentanucleotide polymorphism



In a recent study on a consanguineous family of kabilian origin, a mutation was discovered which results in a leptin receptor which lacks the transmembrane and intracellular domains, this is caused by a skipping of exon 18 (Lahlou 2000). So the truncated receptor is secreted into blood and binds the majority of serum leptin markedly increasing bound and total leptin. Free serum leptin was similarly correlated with BMI in the mutated and non mutated obese individuals, providing evidence that the relationship between BMI & circulating free leptin is preserved in this family. Family members who were homozygous for the mutation developed early onset morbidity in the first months of life accompanied by hyperphagia and hypogonadotropic hypogonadism (Chagnon et al 1999) (Lahlou 2000).
Another study of obese men indicated that leptin has a role in the regulation of blood pressure, for some variants of the leptin receptor seem to protect from hypertension (Rosmund et al 2000), while other variants resulted in an increased blood pressure.

These include polymorphism Lys 109 Arg in exon four. Two other mutations in coding sequences have also been reported these include:
i) T to C substitution in the first position of codon 105.
ii) a polymorphism in the promoter region of the gene which simply blocks the transcription of the gene (Karvonen et al 1998).
Recent studies on different populations (British, Danish, French, American and Japanese), made clear that there was no significant difference in allele frequencies or genotype distributions between lean and obese subjects (Chagnon et al 1999). These studies made it obvious that different combinations of allelic variants from different

genes may predispose to obesity, and the studies also made it clear that the allelic variants show a significant race and/or population component. Listed below, is table (2.1) which gives us a picture about the frequency of the three most common previously mentioned polymorphisms in different populations in lean and obese subjects (Chung et al 1997; Matsuoka et al 1997).

Table 2.1 Allele frequencies of variant alleles of LepR producing amino acid variations

Allelic position	**Asian**	**Black**	**Caucasian**	**Hispanic**	**Lean**	**Obese**
Percentage of subjects	1 %	11.3 %	67.5 %	20.1 %	13.9 %	86.1 %
Lys 109 Arg	25	21.9	21	22.9	26.3	20.2
Gln 223 Arg	50	37.3	33.6	36.6	42.7	32.7
Lys 656 Asn	0	11.4	16.8	14.8	19.5	14.6

A study performed on 47 obese and 68 non-obese Japanese uncovered 7 nucleotide variants in the leptin receptor gene, 4 of these variants (Lys 109 Arg, Gln 223 Arg, Ala 976 Asp and Pro 1019 Pro) occurred at a frequency of 79, 91, 100 and 85 % respectively, in the obese subjects. The allele frequencies did not differ between lean and obese japanese subjects, ruling out that these variants could be used as markers for other mutations involved in the development of obesity (Matsuoka et al 1997).

2.4 Dietary lipids, lipid digestion and transport

An adult ingests about 60-150 g of lipids per day, of which more than 90% is triacylglycerols, the remainder is made up of cholesterol, cholesteryl esters, phospholipids and free fatty acids (Champe and Harvey, 1994).
The digestion of lipids begins in the stomach by an acid stable lipase and continues in the small intestine (duodenum) through emulsification by bile salts and degradation by pancreatic enzymes. Triacylglycerols are degraded by the action of an esterase

which removes the fatty acids at carbons 1 and 3. Cholesteryl esters are hydrolyzed by pancreatic cholesteryl ester hydrolase.

2.4.1 Dietary lipid transport:

Triacylglycerols and cholesterol are transported from the intestine to tissues by packaging them into lipid droplets surrounded by a thin layer of protein, phospholipids and unesterified cholesterol, called chylomicrons (Toplak et al 2000). Most lipids are transported in the blood as triglycerides within lipoproteins. Lipoproteins are particles containing a core of hydrophobic lipids surrounded by a shell of proteins, phospholipids and cholesterol. The four major groups of lipoproteins are: chylomicrons, high density lipoprotein (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL). Chylomicrons transport lipids away from the small intestine, while VLDL transports triglycerides from the liver to extrahepatic tissues, cholesterol is carried by both HDL and LDL. HDL transports cholesterol from tissue to the liver for excretion or bile acid synthesis, while LDL transports cholesterol to the tissues.

2.4.2 Lipids related to obesity

The lipids which are related to obesity are mainly triglycerides, cholesterol and their transport forms HDL and LDL-cholesterol.

2.4.2.1 Triglycerides

Triglycerides are the chemical form of lipids which exists in food as well as in the body. They are also present in blood plasma and in association with cholesterol form the plasma lipids. Triglycerides in plasma are derived from fats eaten in food (triglycerides are found in both plant and animal sources mainly in meat and fats like crisco and puritan oil) or are made in the body from other energy sources like carbohydrates (Gurr 1993). Calories ingested in a meal and not used immediately by

tissues are converted to triglycerides and transported to adipocytes to be stored (Mayes 1988).

2.4.2.1.1 Structure of triglycerides:

Triglycerides are the so called neutral fats, and are esters of the alcohol, glycerol with fatty acids. They are nearly all mixed acylglycerols, with three different fatty acids. The proportion of triglycerol molecules containing the same fatty acid residue in all 3 ester positions is very small (Mayes 1998).

2.4.2.1.2 Regulation and processing of triglycerides:

There are two sources for the triglycerides: dietary and endogenous sources. The level of triglycerides is regulated by hormones, which regulate the synthesis and release of triglycerides from fat tissue so they meet the body`s needs for energy between meals. Dietary triglycerides mainly come from eating animal products and saturated fat. In the body, triglycerides are mainly synthezised in the liver by two pathways. The first is initiated by the synthesis of glycerol phosphate from glucose and in the second glycerol is converted to glycerol phosphate by glycerol kinase (Champe and Harvey, 1994). This includes the conversion of fatty acids to their active form using coenzyme A, then three sequential additions of two fatty acids from fattyacyl coA , and the removal of phosphate. The addition of a third fatty acid produces a molecule of triglycerol. These dietary lipids are absorbed through the gut, assembled there into chylomicrons, and are delivered through the bloodstream to the liver where they are processed (Mayes 1998). One of the main functions of the liver is to make sure that all the tissues of the body receive the cholesterol and triglycerides they need to function. Whenever possible, the liver takes up dietary cholesterol and triglycerides from the chylomicrons produced in the intestine particularly, during times when dietary lipids are not available. The liver and other tissues, produce cholesterol and triglycerides themselves. The liver then packages the cholesterol and triglycerides , along with special proteins, into spheres called lipoproteins. The lipoproteins are released into the circulation, and are delivered to the cells of the body. The cells remove the needed cholesterol and triglycerides from the chylomicrons. The

chylomicrons are released from the intestinal mucosal cells by exocytosis (Champe and Harvey 1994). Triglycerides are released from the chylomicrons in skeletal muscle and adipose tissue and degraded to free fatty acids and glycerol by lipoprotein lipase. Cholesterol in the form of cholesteryl esters in chylomicrons is taken up by the liver and hydrolyzed to its component parts.

2.4.2.2 Cholesterol

Cholesterol is a waxy- like compound that belongs to a class of molecules called steroids. It is widely distributed in all cells of the body, but particularly in nervous tissue. It is a major component of the plasma membrane and of plasma lipoproteins. Cholesterol has two sources: dietary and endogenous. It is present in animal products, like eggs, meat and cheese and is synthezised in the human body by all tissues mainly the liver, intestine adrenal cortex and reproductive tissues.

2.4.2.2.1 Structure of cholesterol:

Cholesterol is a 27 carbon atom compound designated as 3-hydroxy-cholestene. It consists of four fused rings with the carbons numbered in sequence and an eight-membered branched hydrocarbon chain attached to the D ring (Mayes 1988).

2.4.2.2.2 Functions of cholesterol:

Cholesterol is a lipid which has no role in providing energy unlike triglycerides.

However, it is essential for:

- formation and maintenance of cell membranes (helps the cell to resist changes in temperature and protects and insulates nerve fibers).

- formation of sex hormones (progesterone, testosterone, estradiol) (Champe and

Harvey, 1994)

- production of bile salts, which help to digest food.

- conversion into vitamin D in the skin when 7-dehydrocholesterol is exposed to sunlight. (Mayes 1988)

2.4.2.2.3 Synthesis of cholesterol:

Cholesterol is mainly synthsized in the liver (1000 mg/day), but also in cells lining the small intestine. The precursor of cholesterol is acetyl CoA and is converted to 27 carbon cholesterol by a series of complex reactions involving the following steps: Acetyl CoA (C2) → mevalonate (C6) → isopentenyl pyrophosphate (C5)→ sqalene (C30) → cholesterol (C27).

All the carbon atoms in cholesterol are provided by acetate and NADPH provides the reducing equivalents Champe and Harvey 1994)). The pathway is driven by the hydrolysis of the high- energy thioester bond of acetyl CoA and the terminal phosphate bond of ATP.

- ***Relation between cholesterol and obesity***

Elevated cholesterol levels are a major risk factor for coronary heart disease. There are two kinds of cholesterol: good cholesterol which is carried in HDL, and bad cholesterol which is carried in LDL. Cholesterol levels and LDL –cholesterol are increased in obesity due to the high fat intake of these individuals and their lack of movement, so they are at a high risk of heart disease. When LDL levels are too high,

the LDL tends to stick to the lining of the blood vessels, leading to the stimulation of atherosclerosis or hardening of the arteries. Atherosclerosis plaques cause narrowing of the arteries and lead to heart attacks and strokes, whereas, increased levels of HDL cholesterol are associated with a lower risk of heart disease. Thus, HDL cholesterol appears to be a good protector against heart disease (Anderson et al 1997). There is some evidence that the HDL molecule scours the walls of blood vessels, and cleans out excess cholesterol which is transported to the liver for further processing. Elevated cholesterol levels can be caused by several factors including:

(i) hereditary, eg familial hypercholesteanemia.

(ii) a diet high in saturated fat and cholesterol.

(iii) being overweight increases LDL cholesterol and decreases HDL cholesterol.

(iv) being sedentary increases LDL cholesterol and decreases HDL cholesterol.

(v) age: cholesterol levels increase with age, beginning at about 20 years (Mayes 1988).

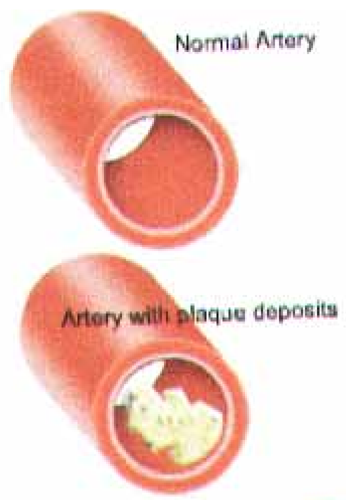
(vi) gender: females prior to menopause have cholesterol levels lower than men at the same age but when menopause occurs their LDL cholesterol levels increase as does the risk of heart disease. There are also some secondary causes for elevated levels of cholesterol. i.e. some people have elevated cholesterol levels as a result of specific diseases or medical conditions. In these individuals, treating the underlying medical problem often results in an improvement in cholesterol levels, Thus, any patient whose cholesterol levels are elevated should be screened for one of the causes of secondary lipid
disorders. These causes are: diabetes, hypothyroidism, obstructive liver disease, chronic renal failure and drugs (anabolic steroids, progesterone drugs and corticosteroids) (Mayes 1988).

2.4.2.3 Relation of triglycerides and cholesterol with obesity

Obesity in most individuals is linked to an elevated level of both triglycerides and cholesterol. Since the elevated levels of cholesterol (hypercholesterolaemia) may have a health threatening effect due to the fact that it is a component of atherosclerotic plaques and that it elevates the individuals risk of coronary heart disease. In addition, it also increases the risk of formation of gallstones (Mendez-Sanchez et al 2002) (Figure 2.7). Excess triglycerides in plasma is called hypertriglyceridemia.It is linked

to the occurrence of coronary artery disease in some individuals. Elevated triglycerides may be a consequence of other diseases, such as untreated diabetes mellitus, by accumulating in pancreatic β-cells and causing it`s dysfunction (Shimabukuro et al 1997). Like cholesterol, increases in triglyceride levels can be detected by plasma measurements. Very high triglycerides can cause pancreatitis, an enlarged liver and spleen, and fatty deposits in the skin called xanthomas. It was reported that the levels of HDL-cholesterol decreases consistently with increasing BMI, while the levels of LDL-cholesterol and triglycerides and the ratio of total total cholesterol to HDL-cholesterol increaseds steadily (Reeder et el 1992).

Figure 2.7 Atherosclerotic plaque deposits in the artery



2.4.3 Insulin:

Insulin is a peptide hormone secreted by groups of cells within the pancreas called β-cells of the Islet of Langerhans. It plays an important role in maintaining normal glucose levels in the blood both after the intake of a high carbohydrate meal and during fasting state.

2.4.3.1 Structure of insulin:

Insulin consists of two polypeptide chains (A & B chains), linked covalently by disulphide bonds (Rodwell 1998). The A-chain has an N-terminal Gly and a C-terminal Asn , the B chain is made of amino acids and has Phe and Ala as the N-and C-terminal residues , respectively (Granner 1998).

2.4.3.2 Synthesis and Secretion of insulin:

Insulin is synthezised in the ß-cells of the islets of langerhans as a pre pro-insulin, which is stored as proinsulin. In this molecule the A and B chains are linked together by C-peptide. Prior to the secretion of insulin, the C-peptide is removed and insulin is secreted by the pancreas in the blood in response to an increase in blood sugar level and other factors (Granner 1988). These include hormonal factors like: growth hormone, cortisol, estrogens and non hormonal factors such as amino acids in blood. Most cells of the body have insulin receptors which bind the insulin and activate a signal transduction pathway. The insulin receptor is a cell surface receptor of the tyrosine kinase family.

- ***Relation of insulin with obesity***

Insulin resistance, even in the absence of diabetes, is associated with central obesity, hypertension, polycystic ovarian syndrome, dyslipidemia and atherosclerosis. insulin resistance is coupled with hypertriglyceridemia, low HDL-C and an enhanced secretion of VLDL, as well as disorders of coagulation and weight gain, and since obesity is a risk factor for diabetes, insulin resistance predisposes to obesity and the development of diabetes (Saltiel 2000). There is a syndrome which is characterized by obesity, hyperlipidemia, diabetes and hypertension as well as abnormal glucose metabolism. This syndrome is called the metabolic syndrome and it is associated with

atherosclerosis (Alexander et al 2003). It is caused by insulin resistance. This is seen mainly in diabetes mellitus Type 2 which is caused by a dual effect: insulin resistance and ß-cell failure. Insulin resistance is a state in which a given concentration of insulin is associated with a subnormal glucose response (Meigs 2002). Thus, the ß-cells of the pancreas secrete increased amounts of insulin to maintain euglycemia. However, over time, functional defects in insulin secretion prevent the ß-cells from maintaining high rates of insulin secretion, resulting in impaired glucose tolerance and eventually diabetes mellitus type 2, the dysregulation of carbohydrate and lipid metabolism that occurs as a consequence of insulin resistance exacerbates the progression of insulin resistance, this leads to the deterioration of glucose homeostasis and the development of glucose intolerance, which after a while leads to diabetes, and adipose cells produce more fatty acids, whereas the liver generates more glucose in an upregulated manner, and the ß-cells undergo complete failure resulting in the late stages of diabetes which needs high doses of exogeneous insulin (Saltiel 2000; Szapary et al 2002).

- ***Leptin receptor polymorphism, leptin, obesity, insulin and lipids in Saudis***

A few studies indicate an association between the pentanucleotide polymorphism in the leptin receptor gene and obesity. No studies have been carried out in the Saudi population. This is a pilot study to determine the frequency of the pentanucleotide insertion in the leptin receptor gene in obese and non-obese Saudi individuals. In addition, to investigate the levels of leptin in different leptin receptor polymorphic forms.

Since a close association exists between leptin and obesity, obesity and insulin, obesity and lipids and insulin and lipids, studies are essential to investigate the association between leptin, insulin and lipids. Furthermore, studies are also required to determine if different polymorphic forms of the leptin receptor influence not only the levels of leptin and hence obesity, but how do they affect insulin levels and plasma lipids.

This would show whether different leptin receptor polymorphic forms may be associated with an increase rate of atherosclerosis and hence, cardiovascular disease.

3.0 Objectives

This study was designed to fulfill the following objectives:

(i) To compare leptin levels in obese and non obese Saudi males and females.

(ii) To study leptin receptor polymorphism in obese and non obese Saudis.

(iii) To study the nature of correlation between leptin, cholesterol, triglycerides and insulin in obese and non obese Saudis.

(iv) To compare the levels of leptin, cholesterol, triglycerides and insulin in obese and non obese Saudis with the different leptin receptor genotypes.

4.0 Method & Material

4.1 Materials

The following chemicals and kits were used during the course of this study.

4.1.1 Chemicals used for DNA extraction:

1) 2x lysis buffer: The 10x lysis buffer was prepared by dissolving 41.45 gm of ammonium chloride (770mM), 4.6gm of potassium hydrogen carbonate (46mM) and 20ml of 0.5M EDTA solution pH 7.5 (10mM) in 1 litre of distilled water. Approppriate dilutions were made to prepare 1x & 2x buffers.
2) 0.5 M EDTA solution of pH 7.5: This solution was prepared by dissolving 186.1 gm of disodium ethylene diamine tetra acetate.2H2O in 800 ml of distilled H2O. pH was adjusted to 7.5 using NaOH and the volume was made up to 1 litre by distilled water.
3) Salt/EDTA buffer. Salt/EDTA buffer was prepared by dissolving 4.39 gm (75mM) sodium chloride , and 50 ml of the 0.5M EDTA solution pH 7.5 (25 mM) in 1litre of distilled water.
4) 10 % SDS: 10 % SDS was prepared by dissolving10 gm of sodium dodecyl sulfate (SDS) in 90 ml of distilled H_2O and heating to 68 ^{0}C to dissolve, pH was adjusted to 7.2 by the addition of a few drops of concentrated HCl and the volume was adjusted to 100 ml.
5) Proteinase K: 100 mg of proteinase K was dissolved in 5 ml distilled H_2O.
6) Chloroform/isoamyl alcohol (25:1): 96 ml chloroform, 4 ml isoamyl alcohol were mixed together.
7) 10 mM Tris , mM EDTA (PH 7.5): 1.2 gm of Tris and 0.3722 gm of EDTA were dissolved in 1 litre of distilled water, and the pH was adjusted to 7.5 by NaOH.
8) Redistilled phenol saturated with 100mM Tris-HCl (pH 8).

4.1.1.1 Chemicals used for redistilling the phenol.

* (0.5 M Tris pH 7.5): Tris buffer was prepared by dissolving 60.55 gm of Tris base in 800 ml of distilled H_2O. pH was adjusted by the addition of concentrated HCl.
* 1x TNE: The 1x TNE buffer was prepared by dissolving 1.2 gm of Tris, 3.7gm of EDTA , and 8.7 gm of NaCl in 1 litre of distilled water. the pH was adjusted to 7.5 with NaOH.

- ***Preparation of redistilled phenol:***

Phenol was prepared carefully and was redistilled. The phenol was equilibrated to a neutral pH, this phenol was kept frozen and was melted in a water bath at 60 ^{0}C. An

equal volume of 0.5M Tris pH 7.5 was added and the solution was shaken briefly using a separating funnel in the water bath . The lower layer was collected until it came out clear, after that an equal volume of 1x TNE was added, and the phases were allowed to separate again and the lower layer was collected again, then another volume of 1x TNE was added and the lower layer was collected again. The step was continued until the Ph of the lower layer was 7.6-7.9 and the phenol was stored in the freezer.

4.1.2 Chemicals used for agarose gel DNA electrophoresis

1) 100 x TE buffer: The 100 x TE buffer was prepared by dissolving121.1 gm of Tris, and 37.2 gm of EDTA in 800 ml distilled water, and the pH was adjusted to pH 7.5 by adding NaOH. The final volume was made up to 1 litre. 1 x TE buffer was prepared by diluting 10 ml of 100 x TE buffer to 1 litre using distilled water.
2) Ethidium bromide.

4.1.3 Chemicals used for polymerase chain reaction (PCR)

1) DNA Taq Polymerase kit: The kit contains the enzyme (Taq polymerase) and the buffer.
2) DNA sample: Each sample was diluted to obtain a final concentration of 20 ng /ml using the OD 260 readings. The concentration of DNA was calculated and diluted.
3) Primers: Two primers AW1 & AW2 were used, The nucleotide sequence of the

primers were as follows:

AW1 which is made up of 30 base pairs. 5`-ATATGGGTAATATAAAGTGTAATAGAGTA-3`

AW2 which is made up of 21 base pairs. 5`-AGAGAACAAACAGACAACATT-3`

The primers were dissolved in distilled water.

4) dNTPs: A mixture of 100 mM dNTPs was used.

4.1.4 Chemicals used for restriction endonuclease digestion of DNA with Rsa 1

The enzyme (Rsa1) was provided in the form of a kit , this kit contained:

1) The enzyme (5000 units/ml).

2) A buffer: (10 x buffer:10 Mm Tris HCl pH(8), 7mM $MgCl_2$ 50 mM NaCl, 7mM Mercaptoethanol, 0.01 % BSA).

4.1.5 Chemicals needed for DNA Acrylamide electrophoresis

1) 10 x TBE buffer: The 10 x TBE buffer was prepared by dissolving 121.1 gm of Tris, 51.35 gm of boric acid and 3.72 gm of EDTA in 1 litre distilled water. A specific dilution was performed to prepare the 1x TBE buffer needed.

2) 20 % Ammonium per sulfate: 0.2 gm ammonium persulfate were dissolved in 1ml distilled water.

3) 30 % Acrylamide stock solution: 73.5 gm of acrylamide , 1.5 gm of bisacrylamide were dissolved in 250ml distilled water.

4.1.6 Human Leptin Kit

Leptin levels were estimated by using kits from Linco Research, St Charles, MO,

USA.

i. Materials Provided in the Kit

1) Assay Buffer (40 ml/vial): 0.05 M Phosphosaline Ph 7.4 containing 0.025 M EDTA, 0.08 % Sodium Azide, 1 % RIA Grade BSA and 0.05 % Triton X-100.

2) Antiserum (26 ml/vial): Rabbit anti-Human Leptin serum in assay buffer.

3) ^{125} I-Human Leptin (27 ml/vial): ^{125} I-Human Leptin label, HPLC purified (specific activity 135 µCi, calibrated to the 1st Monday of each month.

4) Lable-Hydrating Buffer (27 ml/vial): Assay buffer containing normal Rabbit IgG as a carrier. Used to hydrate ^{125} I-Human Leptin.

5) Standards (1 ml/vial): Purified Recombinant Human Leptin in Assay Buffer at the following concentrations: 0.5, 1, 2, 5, 10, 20, 50, 100 ng/ml)

6) Quality Controls (QC) 1 and 2 (2 ml/vial): Purified Recombinant Human Leptin in Assay Buffer.

Expected range

QC 1 2.0-4.0 ng/ml

QC 2 15.5-23.1 ng/ml

7) Precipitating reagent (260 ml/vial): Goat anti-Rabbit IgG Serum, 3 % PEG and 0.05 % Triton X-100 on 0.05 M EDTA, 0.08 % Sodium Azide.
All reagents were ready to use except ^{125} I-Human Leptin, which was hydrated using Label Hydrating Buffer.

ii. Materials Required but not provided

1) Borosilicate glass tubes,12x75mm

2) Automated Pipette with disposable tips.

3) 100 µl & 1 ml repeating dispenser.

4) Refrigerated swing bucket centrifuge.

5) Absorbent paper.

6) Vortex mixer.

7) Refrigerator.

8) Gamma Counter.

iii. Reagent Preparation

^{125} I-Human Leptin: contents lyophilized. Hydrated with entire contents of Label Hydrating Buffer. The mixtures were allowed to set at room temperature for 30min, with occasional stirring.

4.1.7 Cholesterol estimation kit

For cholesterol estimation kits from Roche (Germany) were used.

i. Materials provided by kit

1. R1 (cholesterol reagent). Piperazine-1, 4-bis (2-ethane sulfonic acid) (PIPES) buffer: 75 mmol/l, Ph 6.8, Mg^{2+} : 10 mmol/l, sodium cholate: 0.2 mmol/l, 4-aminophenazone ≥ 0.15 mmol/l, phenol ≥ 4.2 mmol/l, fatty alcohol polyglycol ether: 1 %, cholesterol esterase (Pseudomonas spec.) ≥ 0.5 U/ml, cholesterol oxidase (E. coli) ≥ 0.15 U/ml, peroxidase (horseradish) ≥ 0.25 U/ml, stabilizers, preservative.

ii. Materials required but not provided

1. 0.9 % NaCl

2. Calibrators and controls

3. Clinical chemical analyzers:

The analyzer was calibrated and standardization was performed at 37 ^{0}C.

4.1.8 Triglyceride estimation kit

For triglyceride estimation, kits from Roche were used.

i. Materials provided by kit

1. R1 buffer (buffer/4-chlorophenol/enzymes) PIPES buffer: 50 mmol/l, pH 6.8, Mg^{2+} : 40 mmol/l, sodium cholate: 0.2 mmol/l, ATP ≥ 1.4 mmol/, 4-aminophenazone ≥ 0.13 mmol/l, 4- chlorophenol: 4.7 mmol/l, potassium hexacyanoferrate (Π): 1 µmol/l, fatty alcohol polyglycol ether: 0.65 %, lipoprotein lipase (pseudomonas spec.) ≥ 5 U/ml, glycerokinase (Bacillus stearotermophilus) ≥ 0.19 U/ml, glycerol phosphate oxidase (E. coli) ≥ 2.5 U/ml, peroxidase (horseradish) ≥ 0.1 U/ml, preservative.

ii. Materials required but not provided

1. .0.9 % NaCl

2. Calibrators and controls

3. Clinical chemical analyzer: the analyzer was programmed as follows:

Temperature 37 ^{0}C

Sample volume 3µl

R1 volume 250µl

Wavelength1 : 505 nm

Wavelength 2 : 700 nm.

4.1.9 Insulin estimation kit

For insulin estimation (INS-IRMA kits) from BIOSOURCE (Belgium) were used.

i. Materials provided by kit

1) Anti-INS tubes(monoclonal antibodies)

2) Anti-INS-^{125}I (monoclonal antibodies) in phosphate buffer with bovine serum

albumin

3) Azide and inert red dye.

4) Standards

5) Washing solution.

6) Controls 1 and 2 in human serum and thymol.

ii. Materials required but not provided

1) Automated Pipettes for delivery of: 50 µl, 500 µl and 2 ml.

2) 5ml automated syringe for washing.

3) Aspiration system.

4) Gamma counter set for ^{125}I counting and vortex mixer and magnetic stirrer.

iii. Reagent Preparation:

/

A) Standards: the zero standard was reconstituted with 2 ml distilled water and the other

standards with 0.5ml distilled water.

B) Controls: the controls were reconstituted with 0.5 ml distilled water.

C) Wash solution: the contents of the vial were diluted in 700 ml distilled water, a magnetic stirrer was used for homogenization. All the reagents were brought to room temperature prior to use, and they were mixed by swirling.

4.2 Subjects

The study included 168 females and 31 males attending the outpatient clinics of King Khalid University Hospital. The purpose of the study was explained and informed consent was obtained before recruitment. The history and the essential information were recorded on specially designed forms (Appendix 1).

4.2.1 Extraction of Blood

Blood was extracted in the morning from 8-12 am from each individual after 8-12 hours fast by venepunture in EDTA tubes. The plasma was separated by centrifugation at 4000 rpm for 5 minutes at room temperature. The cells were used for DNA extraction.

4.2.2 Storage of Plasma

The plasma samples were stored frozen at -20^0C until required for analysis.

The blood cells were stored frozen until required for DNA extraction.

4.3 Methods

4.3.1 Procedure of DNA extraction

DNA was extracted manually from all blood samples investigated during this study,

using the following steps:

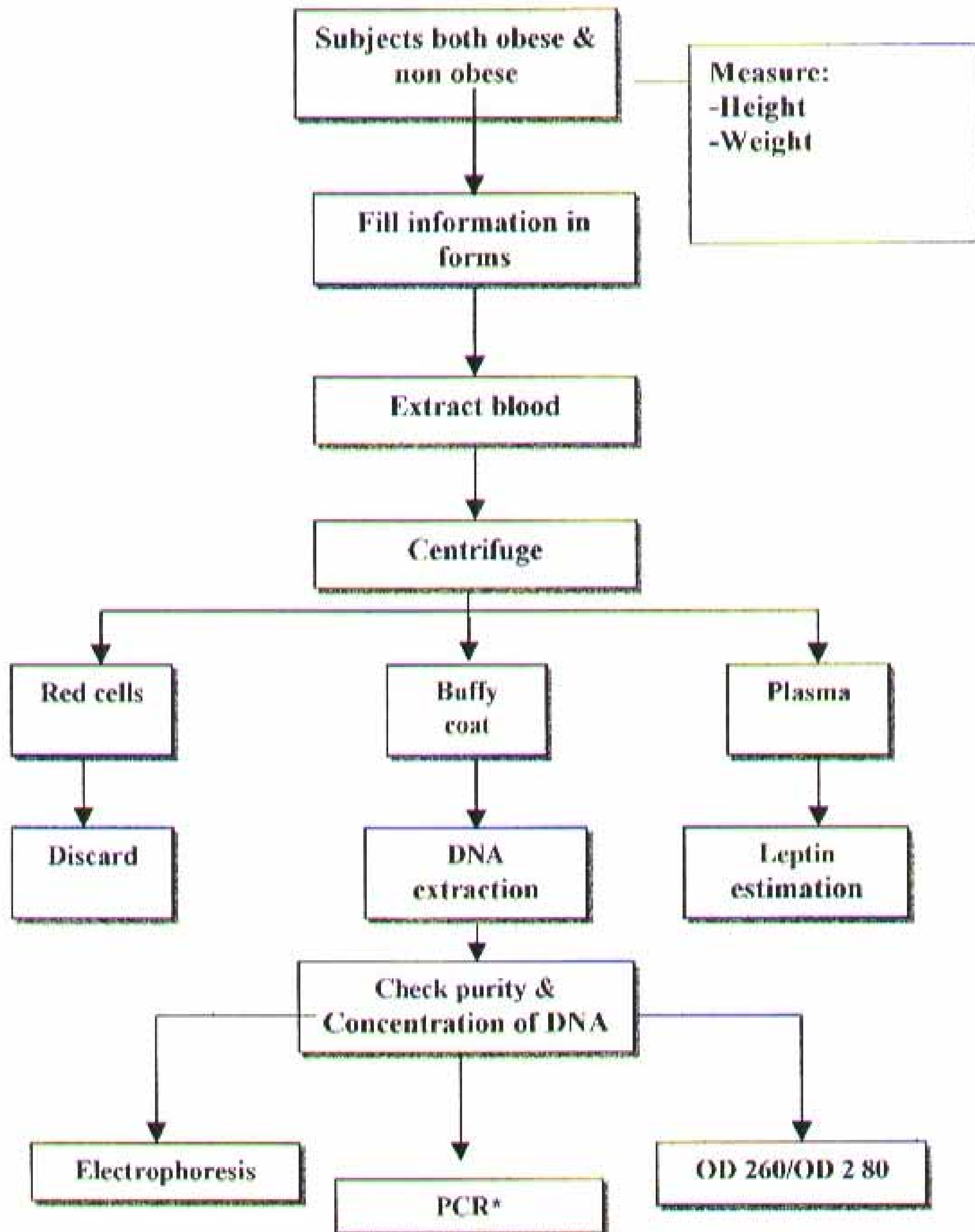
- ***Day :***

5 ml of the blood sample collected in EDTA tube was transferred to a 10 ml plastic centrifuge tube and was made up to the mark with 2 x lysis buffer. The tube was mixed on a tube rotator for 10 minutes. The nuclei were collected by centrifugation at 3000 rpm,
for 10 minutes at 4 ^{0}C. The supernatant was carefully poured out into a waste container, and was inverted to drain the pellets. The pellets were resuspended in 3 ml salt/EDTA buffer and mixed by vortexing. 0.3ml of 10 % SDS and 10 µl of 20 mg/ml Proteinase K were added and left overnight at 37 ^{0}C to digest the protein associated with chromatin.

- ***Day 2***

The next day 3 ml of phenol saturated with 100 mM Tris-HCl (pH 8) was added, mixed on the tube rotator for 5-10 minutes, and centrifuged at 2000 rpm for 5 minutes. The upper aqueous phase was removed and re-extracted with a further 3ml of phenol. The aqueous phase was extracted with 3ml of chloroform/isoamyl alcohol mixture 25:1 and the aqueous phase was transferred into a small glass vial with cover, 6ml of absolute ethanol were added to the vial, and it was shaken carefully to precipitate the DNA. The precipitate of DNA appeared as a thread –like structure was hooked out using a glass rod. The DNA was redissolved in 0.5ml of 10 mM Tris, 1mM EDTA (pH7.5) in a 2ml cryotube and stored at 4 ^{0}C. The sample was diluted with water (3 ml of distilled water and 25 µl of the sample) and optical density was measured at 260 nm and 280 nm. The ratio OD 260 nm /OD 280 nm was obtained to determine the purity of the DNA. The concentration of DNA was calculated from OD 260 values. The OD 260, OD 260/ OD 280 ratio and the Concentration of DNA in each sample are presented as appendix 2.

Work plan



*3` untranslated region

4.3.2 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed for each extracted DNA sample to determine the DNA status.

- **Principle:**

Electrophoresis is a technique used to separate and sometimes purify macromolecules that differ in size, charge or conformation. When charged molecules are placed in an electric field, they migrate toward either the positive (anode) or negative (cathode) pole according to their charge. Nucleic acids have a consistent negative charge imparted by their phosphate backbone, and migrate toward the anode. Small fragments of DNA can move more easily through the gel than larger ones and so all the different sizes spread out and separation occurs on the basis of size. Agarose is a galactose-based polymer extracted from seaweed, widely used in analytical and preparative electrophoretic separation of linear nucleic acids in the size range above 100 bp. For visualization of DNA, ethidium bromide is used, as DNA binds to ethidium bromide and gives a fluorescence, which can be seen under a UV light.

- **Procedure:**

One percent agarose gel was prepared by dissolving agarose in 1x TE buffer pH 7.5. The mixture was boiled with constant stirring using a magnetic stirrer to dissolve the agarose. 10 µl of ethidium bromide added and the gel was poured into the electrophoresis plate after the gel cooled down to about 50 ^{0}C, and a well comb (20 wells, 15 mm) was pressed down into the gel to make slots for sample application. Care was taken to ensure that there were no air bubbles in the gel. The gel was left to stand at room temperature for 15-20 minutes to polymerize. Each DNA sample (2 µl) was mixed in a well plate with 5 µl of 1 x TE buffer, and 1ul of the dye (stop

solution). After the gel settled, the comb was removed carefully and the plate was placed on the electrophoretic equipment connected to the electrical supply. The plate was covered with 1x TE buffer. The samples were applied into the wells and a voltage of 100v and a current of 40 Ampers was applied and electrophoresis was carried out for 1 hour (Figures 4.1 and 4.2). After electrophoresis, the plate was carefully removed, dried gently and visualized under a UV lamp. A photograph of the gel was taken using a polaroid camera (Figure 4.3).

Figure 4.1 Agarose gel electrophoresis showing sample amplication

Figure 4.2 Agarose gel electrophoresis apparatus

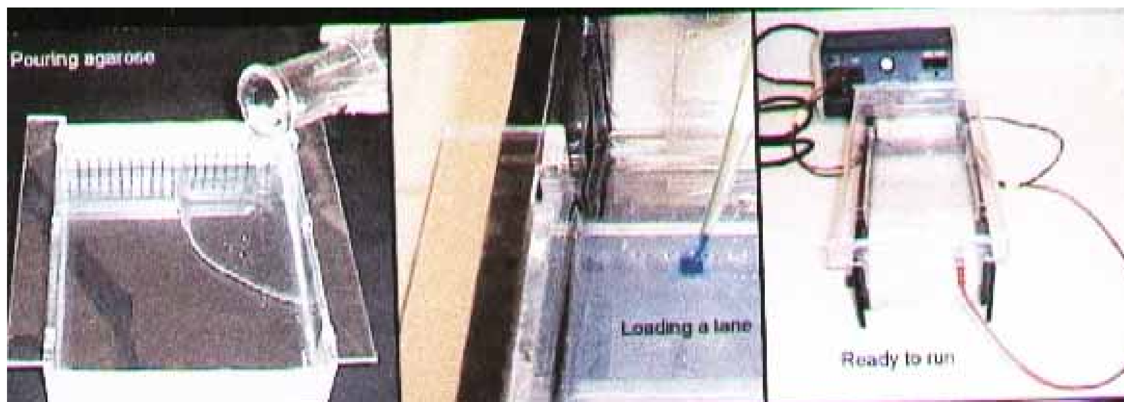


Figure 4.3 Polaroid picture of DNA bands visualized by ethidium bromide

4.3.3 Amplification of DNA by polymerase chain reaction

- **Principle:**

The PCR is an in vitro technique where a given DNA sequence is identically copied using specific primers that flank both sides of the DNA to be amplified and a DNA polymerase. The number of copies of the DNA fragment rises exponentially, since every newly synthesized DNA-sequence is also a matrix for the next copy.
For PCR, the following is needed:
(i) A thermostable DNA-polymerase: The most used polymerase is the Taq-polymearase. It is isolated from the bacteria *Thermophilus aquaticus* which occurs in hot springs. This enzyme is used for the PCR reaction, where the DNA is being denatured at a temperature of 95 ^{0}C, whereas the DNA-polymerase remains stable.
(ii) The template-DNA: The template-DNA can consist of very different DNA`s. For example: genomic DNA libraries and cDNA libraries. In this study genomic DNA extracted from Saudi Individuals was used (or genomic DNA).
(iii) Oligonucleotides (primers): Primers are short, single-stranded sequences of mostly 20-25 nucleotides length, which link with another single-stranded matrix by complimentary base pairing. They serve as the starting point for the synthesis of the complimentary strand of the DNA-polymerase.

(iv) Nucleotides (dNTPs): dNTPs are the units used for the synthesis of the new DNA-strand. With the help of the DNA-polymerase they are attached to the end of the primer complimentary to the matrix, thus creating the DNA-daughter strand.

In Principle the PCR-reaction is subdivided into three steps:

1) Denaturation: During denaturation, the template DNA is separated (denatured) into its two separate strands by heating up the temperature to 95 ^{0}C.

2) Annealing: This involves the annealing of the primer to the denatured DNA. The temperature is lowered to a degree specific for the primer, which generally lies between 55 ^{0}C and 70 ^{0}C. This guarantees, that the primer takes it place at the specific DNA-recognition-sequence (according to their complementary bases) of the single-stranded DNA-template-sequence.

3) Synthesis or extension: The third step, the synthesizing, takes place at a temperature of around 72 ^{0}C. This corresponds to the optimal temperature for the Taq-polymerase to work. The polymerase prolongues the paired short oligonucleotides (primers) according to the DNA-matrix, until the double-stranded DNA-molecule is complete again. The three steps are repeated in cycles, usually 30-35 times As an exponential increase occurs following the 30-35 cycles that, there is enough DNA to be detected and analyzed

- **Procedure:**

For PCR the <u>master mix</u> was prepared containing the following for

each sample:

Distilled water	7.9 µl
Buffer(10x)	2 µl
MgCl	1.5 µl
dNTPs	2 µl

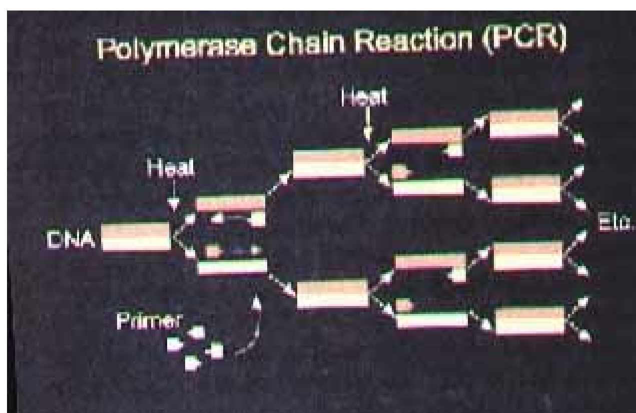
Primer 1	2 ul
Primer 2	2 μl
Taq Polymerase	0.1 μl (containing ½ a unit)
the Total volume:	**17.5 μl.**

From each diluted DNA sample 3 ul are taken and transferred to a PCR tube, and 17.5 ul of the master mix (mentioned above) was added and a drop of mineral oil was placed on top of each sample to protect against evaporation due to the high temperature in the cycler. The samples were centrifuged at 1000 rpm for 1minute and placed in a Perkin-Elmer thermo- cycler .

The PCR was performed as follows:

Initial denaturation for 5 minutes at 95 ^{0}C followed by 30 cycles of denaturation at 95^0 C for 1 minute, annealing at 55 ^{0}C for 1 minute and synthesis at 72 ^{0}C for 1 minute (Figure 4.4). After completion of the 30 cycles, the PCR product (1 μl) was subjected to electrophoresis in 2 % agarose gel (as above) and the remaining PCR product was stored at 4 ^{0} C until required for restriction endonuclease digestion.

Figure 4.4 PCR

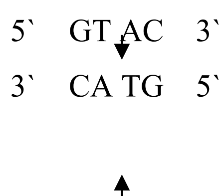


4.3.4 Rsa1 Digestion:

- **Principle:**

Restriction endonucleases (REs) are bacterial enzymes that cleave double-stranded DNA. There are two kinds of restriction endonucleases: Type 1 REs are important in bacterial function but do not cleave DNA at specific sequences. Type 2 REs require highly specific sites for DNA cleavage. The cleavage site specific for many REs have been defined. They cut DNA within or near to their particular recognition sequences, which typically are four to six nucleotides in length with a twofold axis of symmetry. i.e. palindromic sequence. Each restriction endonuclease has a specific sequence and number of nucleotides required to create the recognition site. The PCR product of the genomic DNA studied here, is either a band of 114 bp, or a band of 119 bp depending on the presence or absence of the pentanucleotide polymorphism in the leptin receptor gene. Since these two bands are difficult to differentiate from eachother, restriction of the PCR product is performed to visualize the difference between the two bands, since the 114 bp band is not cut by the restriction endonuclase, hence if the pentanucleotide insertion is present, the restriction product will be two bands, 1 band of 90 bp which can be seen and another of 29 bp which is too small to be visible. The restriction endonuclease used here is Rsa 1.

Rsa 1 is an endonuclease which cuts the DNA at the following sequence and generates blunt ends.



- **Procedure:**

For restriction endonuclease digestion with Rsa 1, 10 µl of the PCR product were pipetted into a tube and 0.2 µl of the enzyme Rsa1 and 1µ of the 10x buffer were added and the tube was left overnight at 37 ^{0}C. The following day Polyacrylamide electrophoresis was performed on the restriction endonuclease digestion product. The

product obtained was either a 114 bp or two bands of 90 and 29 bp depending on the absence or presence of the pentanucleotide polymorphism (insertion).

4.3.5 Acrylamide electrophoresis

- **Principle:**

The principle of Acrylamide electrophoresis is the same as previously mentioned for agarose, the only difference is the kind of gel used. The reason why another gel is used is due to the fact that after DNA is digested with the restriction endonuclease Rsa 1 the product is quite small in base pairs, so a gel with a high resolving power is needed for separating and characterizing the mixture of DNA strands present.

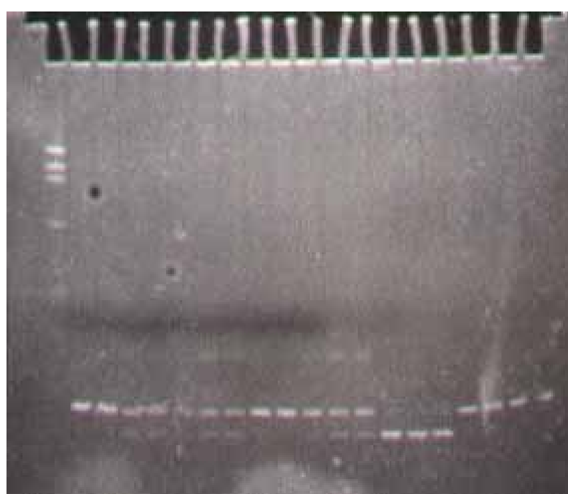
Polyacrylamide is a cross-linked polymer of acrylamide. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20 %. Polyacrylamide gels are significantly more difficult to prepare than the agarose gels, as oxygen inhibits the polymerization process, the gel must be poured between glass plates.

- **Procedure**

The glass plates used for the gel preparation were washed and then dried using a tissue, then the plate was prepared by fixing its 3 ends together using tape , while leaving the fourth end open. For more fixation, clips were added to the 3 sides. the plate was made to stand up in a gel holder. The 10% gel was prepared, by using10.6 ml of the 30 % acrylamide stock solution, 35µl of Temed and 175 µl of ammonium sulfate, and mixing with 4 ml of the 10 x TBE buffer and 25.4 ml of distilled water. The gel was then poured between the glass plates (after waiting a few minutes) using a syringe. Care has to be taken not to form any air bubbles. Pouring was stopped when the gel reached the edge of the glass plate. A previously washed and dried well comb was applied carefully at the top of the gel for making slots for applying the samples after the gel has polymerized 12 µl of the PCR product samples were mixed with dye (blue orange loading dye 7 ul). After the gel had polymerized [takes about 30 -40minutes], the well comb was removed carefully as well as the fixing clips and

the tape from the bottom of the plate were removed and any excess gel was rubbed away. The plate was moved to the electrophoresis equipment and carefully fixed in place using the knob on the 2 stands. The 1x TBE buffer was carefully added until it filled about 1/3 of the tank. Before performing the actual electrophoresis, the pump was switched on for the buffer to circulate, (about 30minutes). The sample for electrophoresis was prepared by mixing with the dyes. The samples and the marker (x 174 rf DNA , Hae 3 digest 500 µg/ml) (0.03 µl+5 µl dye) were applied. After all the samples were applied, the electrophoresis equipment was connected to the electrical supply, and electrophoresis was performed by applying a voltage of 25 v for 2 hours. After completion of electrophoresis, the plate was carefully removed from the holders by loosening the knobs of the electrophoresis equipment and was placed into a tray containing 1 litre of distilled water and 60 µl of ethidium bromide, a sheet of transparent paper was placed at the bottom of the tray. The gel was carefully removed from the plate and moved on to the transparent x-ray paper at the bottom of the tray and the gel was left in the stain for 20-30 minutes. Finally the gel was moved to a UV light source in order to visualize the bands of DNA.. (The x-ray paper is removed prior to adding the gel to the wet illuminated uv light plate). A picture of the gel was taken (Figure 4.5).

Figure 4.5 Polaroid picture of the PCR product after Rsa1 restriction



114 bp
90 bp

4.3.5 Leptin estimation (RIA)

Leptin levels in plasma were estimated using Radioimmunoassay technique.

- **Principle**

The basic principle of radioimmunoassay (RIA) is the use of radiolabelled Abs or Ags to detect Ag:Ab reactions. The Abs or Ags are labeled with ^{125}I isotope, and the presence of Ag:Ab reactions is detected using a gamma counter. The procedure depends on that a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited. If unlabelled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A calibration or standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive prepared labeled form of the antigen, a method whereby antibody –bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity. The Linco research inc. Human Leptin assay utilizes ^{125}I labeled Human Leptin and a Human Leptin antiserum to determine the level of leptin in serum, plasma or tissue culture media by the double antibody/PEG technique.

- **Assay procedure**

All reagents were brought to room temperature at least 30 min prior to starting assay. The reagents were dispensed in the following order and all reactions are carried out at room temperature: (A pipetting guide for all the solutions to be added for leptin estimated is presented as (Table 4.1).

- ***A) Day 1.***

1) 300 µl of Assay Buffer was pipetted to the non specific binding (NCB) tubes (3-4), 200 µl to reference (BO) tubes (5-6), and 100 µl to tubes 7 through the end of the assay.
2) 100 µl of standards and quality control was pipetted in duplicate.
3) 100 µl of each sample was pipetted in duplicate.
4) 100 µl of I Human Leptin was pipetted to all tubes.
5) 100 µl of ^{125} I-Human Leptin antibody was pipetted to all tubes except total count tubes (1-2) and NSB tubes (3-4).

6) The tubes were mixed on a vortex mixer, covered, and incubated overnight (20-24 hours) at 4^0 C.

- ***B) Day 2.***

7) 1 ml of cold (4^0C) Precipitating Reagent was added to all tubes (except Total Count tubes).

8) The tubes were mixed on a vortex mixer and incubated 20 minutes at 4 ^{0}C.

9) All tubes were centrifuged, (except Total Count tubes 1-2) for 20 minutes at 2000 rpm.

10) Immediately the supernatant of all tubes except Total Count tubes (1-2) was decanted, the tubes were drained for at least 15-60 seconds and the excess liquid was removed from lip of tubes by blotting.
11) All tubes were placed in a gamma counter and radioactivity was counted.

12) A standard curve was plotted by the counter computer, with count per minute x 10 (Bo/B %) on the y-axis and leptin concentration (ng/ml) for standards on the x-axis.The concentration of leptin for each sample was read directly and automatically from the standard curve by the computer. All tests were carried out in duplicates and the standard curve was prepared with each run separately. A typical standard curve is presented as Figure 4.1. The concentration of leptin in the samples was obtained from the standard curve, by the computer.

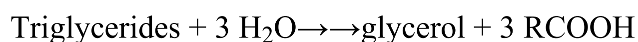
4.3.6 Estimation of triglycerides

Triglycerides were estimated using enzymatic colorimetric methods.

- **Principle of assay procedure**

The estimation of triglycerides is based on the method of Wahlefeld using an enzymatic colorimetric test. This method is based on using a lipoprotein lipase from microorganisms for the rapid and complete hydrolysis of triglycerides to glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff (Trinder endpoint reaction).
The reactions occur in the following sequence:

LPL



Triglycerides + 3 H_2O→→glycerol + 3 RCOOH

GK

Glycerol +ATP→→glycerol-3-phosphate +ADP

MG^{2+}

GPO

Glycerol-3-phosphate + O_2→→dihydroxyacetone phosphate +H_2O_2

peroxidase

H_2O_2 + 4-aminophenazone + 4-chlorophenol→→→→ 4-(p-benzoquinone-

monoimino)-Phenazone + $2H_2O$ + HCl.

- **Assay procedure:**

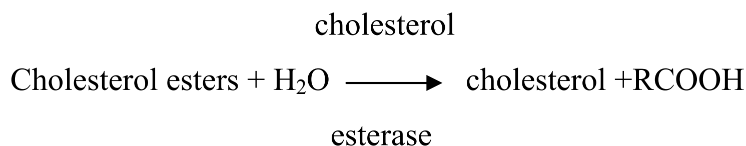
100 µl of each sample and of the controls were applied into the required cups and arranged in the specified places in the clinical chemical analyzer. The analyzer was calibrated and the set up of the assay was performed according to the instructions present in the analyzer. The temperature was set at 37 ^{0}C, and two wavelengths were used: 700 nm and 505nm.

4.3.7 Estimation of cholesterol

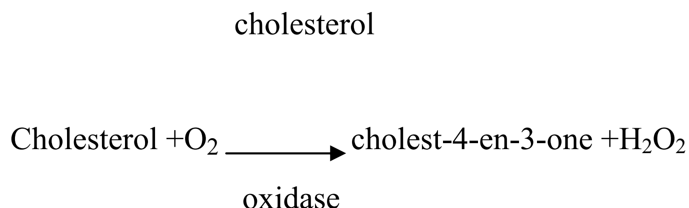
Cholesterol was estimated using enzymatic colorimetric methods.

- **Principles of assay Procedure:**

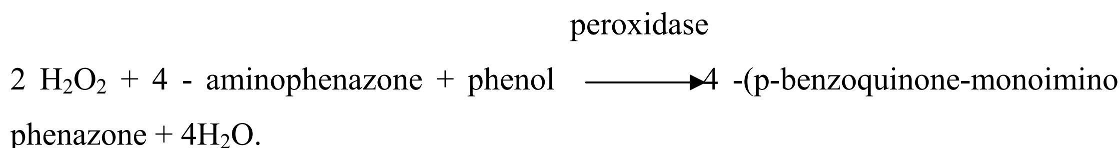
The method is based on the determination of Δ^4-cholestenone after enzymatic cleavage of the cholesterol ester bond by cholesterol esterase, conversion of cholesterol by cholesterol oxidase, and subsequent measurement by the Trinder reaction of the hydrogen
peroxide formed .
Cholesterol is determined enzymatically using cholesterol esterase & cholesterol oxidase.



Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids.



Cholesterol is converted by oxygen with the aid of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide.



Hydrogen peroxide produced forms a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The color intensity is directly proportional to the concentration of cholesterol and can be determined photometrically using clinical chemistry analyzers.

- **Assay procedure:**

100 µl of each sample and of the controls were applied into the required cups andarranged in the specified places in the chemical analyzer. The analyzer was calibrated and the assay was set up according to the instructions present in the analyzer for the estimation of cholesterol.

4.3.8 Estimation of insulin

Insulin was estimated using immunoradiometric methods (Irma).

- **Principles of insulin estimation**

In Irma, two different antibodies, capable of binding simultaneously to the analyte are used. One of these is immobilized on a solid support and serves to effectively extract the analyte from the sample. The second antibody is radiolabelled and serves to convert the extracted antigen into a radioactive product. As the complex is attached to the solid support, the" free" radiolabelled antibody in the solution can be easily separated out from that bound to the analyte. Amount of the radioactivity separated is compared with values of known analyte standards and the concentration of analyte present in the sample is calculated. The BIOSOURCE INS-IRMA is an

immunoradiometric assay based on coated tube separation. Mabs 1, the captured antibodies are attached to the lower and inner surface of the plastic tube. Standards or samples added to the tubes will at first show low affinity for Mabs 1. Addition of Mab2, the signal antibody labeled with ^{125}I will complete the system and trigger the immunological reaction. After washing, the remaining radioactivity bound to the tube reflects the antigen concentration.

- **Assay procedure**

1) The coated tubes were labeled in duplicate for each sample, standard and control. For determination of total counts, 2 normal tubes were labeled.
2) 50 µl of the samples, standard, controls were dispensed into the respective tubes and vortexed briefly.
3) 50 µl of tracer was added to each tube.
4) The rack containing the tubes was gently shaken.
5) The tubes were incubated 2 hours at room temperature.
6) The content of each tube was aspirated (except total counts). (It is important to make sure that the tip of the aspirator reaches the bottom of the coated tube in order to remove all liquid).
7) The tubes were then washed with 2 ml of the washing solution (except total counts). Foam formation due to the addition of the washing solution has to be avoided.
8) The tubes content was then aspirated (except total counts).
9)The tubes were washed again with 2 ml of the washing solution (except total counts) and then they were aspirated.
10) After the last washing the tubes were left standing upright for 2 minutes and the remaining drop of liquid was aspirated.
11) The tubes were then counted in the Gamma counter for 60 second

Table 4.1 Pipetting guide for Leptin estimation

	Step1	Step2-3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9-11
Tube #	Add assay buffer	Add stand/QC Sample	Add ^{125}I Leptin Tracer	Add Leptin Antibody	Vortex, cover and incubate 20-24 hrs at 4 ^{0}C	Add Precipitating Reagent	Vortex and Incubate 20 min at 4 ^{0}C	Centrifuge for 20 minutes, Decant and Count Pellets
1,2	…….	……….	100 µl	…………		……………..		
3,4	300 µl	………	100 µl	…………		1 ml		
5,6	200 µl	………	100 µl	100 µl		1 ml		
7,8	100 µl	100 µl of 0.5 ng/ml	100 µl	100 µl		1 ml		
9,10	100 µl	100 µl of 1 ng/ml	100 µl	100 µl		1 ml		
11.12	100 µl	100 µl of 2 ng/ml	100 µl	100 µl		1 ml		
13,14	100 µl	100 µl of 5 ng/ml	100 µl	100 µl		1 ml		
15,16	100 µl	100 µl of 10 ng/ml	100 µl	100 µl		1 ml		
17,18	100 µl	100 µl of 20 ng/ml	100 µl	100 µl		1 ml		
19,20	100 µl	100 µl of 50 ng/ml	100 µl	100 µl		1 ml		
21,22	100 µl	100 µl of 100 ng/ml	100 µl	100 µl		1 ml		
23,24	100 µl	100 µl ofQC1	100 µl	100 µl		1 ml		
25,26	100 µl	100 µl of QC2	100 µl	100µl		1 ml		
27,28	100 µl	100 µl of unknown	100 µl	100 µl		1 ml		
29,30	100 µl	100 µl of unknown	100 µl	100 µl		1 ml		

5.0 Results

This section presents the demographic data and the results of leptin receptor gene polymorphic studies, as well as the results for leptin, cholesterol, triglycerides and insulin in the total study population. The study group was divided according to the different leptin receptor genotypes and leptin, lipids and insulin were calculated for each group.
The results for these parameters in the groups with different genotypes and in the total study population are presented.

5.1 Demographic Data

The total study population was 199. Of these 168 were females while 31 were males. The mean, standard error of mean (SEM), median, mode, standard deviation (SD), skewness, standard error of skewness (SES), kurtosis, standard error of kurtosis (SEK), variance, 2.5th and 97.5th percentile, parametric range (mean ± 2 SD) and frequency distribution histogram for age, height, weight and BMI were obtained for the total study population as well as for the population divided into male and female. The results are present in the following sections:

5.1.1 Age in the total study population and in the male and female population

The minimum age in the total study population was 15 years and the maximum was 77 years (Table 5.1). The value of the mean, median and mode were 34.13, 32 and 28 years, respectively. As these values were not similar they indicate a non-Guassian

distribution which is seen in the frequency distribution histogram as a slightly positively skewed distribution (Figure 5.1) with skewness of 1.11 and kurtosis of 1.29. The parametric range was 9.51—58.75 years and the non-parametric range was 18-70 years. Whereas for the population divided according to sex, the minimum age for males was 15 years and the maximum was 77 years. The value of the mean, median and mode were 36.55, 33 and 40 years, respectively. The parametric range was 3.93-69.17 years and the non-parametric range was 15-77 years, respectively. For the females in the total study population the minimum age was 18 years while the maximum was 75 years. The mean, median and mode were 33.68, 32 and 29 years respectively. The parametric range was 10.82-56.54 years and the non-parametric range was 18-67.75 years. The age in the males and females were compared using 't' test and p value of 0.23. These results indicate that there was no significant difference in the age in the two sexes.

5.1.2 Body weight in the total study population and in the male and female population

The minimum body weight in the total study population was 44.1 Kg and the maximum was 126.8 Kg (Table 5.2). The mean, median and mode values were very close to each other (i.e. 67.22, 65 and 65 Kg, respectively) indicating a normal Guassian distribution. This was seen in the frequency distribution histogram (Figure 5.2), with skewness of 0.83 and kurtosis of 1.8. The parametric range of body weight in the total study population was 41.88-92.56 Kg, while the non-parametric range was 45.9-90.5 Kg. In males the minimum body weight was 50 Kg and the maximum was 91 kg. The mean, median and mode values were not very close 66.7, 65 and 58 Kg, respectively. The parametric range was 45.94-87.46 Kg and the non-parametric range was 50-91 Kg. In females the minimum weight was 44.1 Kg while the maximum weight was 126.8 Kg. The mean, median and mode for weight in females were different 67.31, 65 and 60 Kg, respectively. The parametric range was 41.15-93.47 Kg while the non-parametric range was 45.2-90.5 Kg. No significant difference in weight of the two sexes using students 't' test was seen ($p = 0.77$).

5.1.3 Height in the total study population and in the male and female population

The statistical data for height in the total study population is presented in Table 5.3. The values for mean, median and mode for height were close i.e. 1.59 m, 1.59 m and 1.6 m, respectively, indicating a normal Guassian distribution. This was seen in the frequency distribution histogram presented in (Figure 5.3) with skewness of 0.2 and kurtosis of -0.14. The minimum height was 1.37m while the maximum was 1.81m. The parametric range was 1.43-1.75 m and the non-parametric range was 1.45-1.75 m. When the results for male and female were compared, a significant difference in height was noticed. ($p = 0.001$). For male the minimum height was 1.51m and the maximum height was 1.81m.The values for mean, median and mode were 1.64, 1.63 and 1.54 m, respectively. The parametric range was 1.48-1.8 m and the non-parametric range was 1.51-1.81 m. For females the minimum height was 1.37m while the maximum height was 1.79 m. The mean, median and mode for height were 1.59, 1.58 and 1.6 m respectively. The parametric range was 1.43-1.75 m while the non-parametric range was 1.43-1.75 m.

5.1.4 Body mass index in the total study population and in the male and female population

In the total study population the BMI mean, median and mode were 26.59, 25.97 and 24.5 Kg/m^2, respectively (Table 5.4). The non-parametric range was 18.7-38.5 Kg/m^2 and the parametric range was 15.95-37.23 Kg/m^2. The lowest BMI was 15.05 Kg/m^2 while the highest was 51.4 Kg/m^2. Figure 5.4 presents the frequency distribution histogram of BMI in the total study population. It shows a slightly positively skewed distribution with skewness of 0.84 and kurtosis of 1.68.

On the basis of BMI, the population was grouped into normal weight (BMI 20-24.9 Kg/m^2), [this group includes a few individuals who were underweight (BMI < 20 Kg/m^2), they were included in the normal weight group due to the little number only very few], overweight (BMI 25-29.9 Kg/m^2) and obese (BMI ≥ 30 Kg/m^2). The

prevalence of normal weight, overweight and obese individuals in the total study population were 42.21%, 29.14% and 28.64 % respectively.

The results for both male and female were analyzed separately and a significant difference in the body mass index (BMI) between Saudi male and female was noticed. In males the BMI mean, median and mode values (25.07, 24.91 and 20.48 Kg/m^2) were lower than in females (26.87, 26.16 and 24.5 Kg/m^2). The minimum BMI in males was 16.33 Kg/m^2 and the maximum BMI was 34.36 Kg/m^2 , whereas the minimum and maximum BMI in females were 15.05 Kg/m^2 and 51.4 Kg/m^2 respectively. The parametric range in males and females was 16.05-34.09 Kg/m^2 and 16.03-37.71 Kg/m^2 respectively. Whereas, the non-parametric range in male and female was 16.33-34.36 Kg/m^2 and 18.79-39.27 Kg/m^2 respectively. The difference in the BMI in the males and females was statistically significant. The prevalence of normal weight, overweight and obese males was 51.61 %, 22.58 % and 25.80 % respectively, and the prevalence of normal weight, overweight and obese females was 40.47 %, 30.35 % and 29.16 % respecti

Table 5.1 Age in the total study population and in the male and female population

	Total	Males	Females
Mean	34.13	36.55*	33.68*
Std. error of mean	0.87	2.93	0.88

Median	32	33	32
Mode	28	40	29
Std. Deviation	12.31	16.31	11.43
Variance	151.53	266.12	130.57
Skewness	1.11	0.7	1.19
Std. error of Skewness	0.17	0.42	0.19
Kurtosis	1.29	-0.3	1.78
Std. error of Kurtosis	0.34	0.82	0.37
Minimum	15	15	17
Maximum	77	77	75
Percentile 2.5#	18	15	18
Percentile 97.5#	70	77	67.75

*p = 0.23

Figure 5.1 Age distribution in the total study population

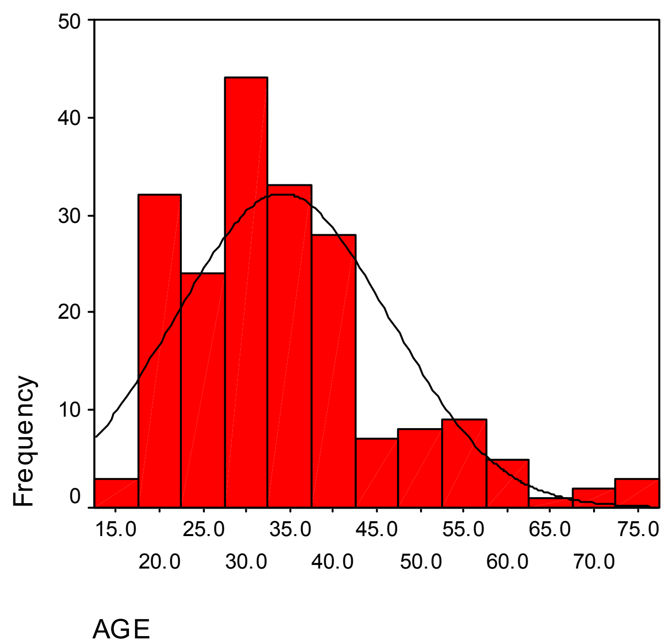


Table 5.2 Weight in the total study population and in the male and female population

	Total	Males	Females
Mean	67.22	66.7*	67.31*
Std. error of mean	0. 9	1.86	1
Median	65	65	65
Mode	65	58	60
Std. Deviation	12.67	10.38	13.08
Variance	160.7	107.82	171.1

Skewness	0.83	0.66	0.84
Std. error of Skewness	0.17	0.42	0.19
Kurtosis	1.8	0. 01	1.84
Std. error of Kurtosis	0.34	0.82	0.37
Minimum	44.1	50	44.1
Maximum	126.8	91	126.8
Percentile 2.5#	45.9	50	45.2
Percentile 97.5#	90.5	91	90.5

*p = 0.77

Figure 5.2 Weight distribution in the total population

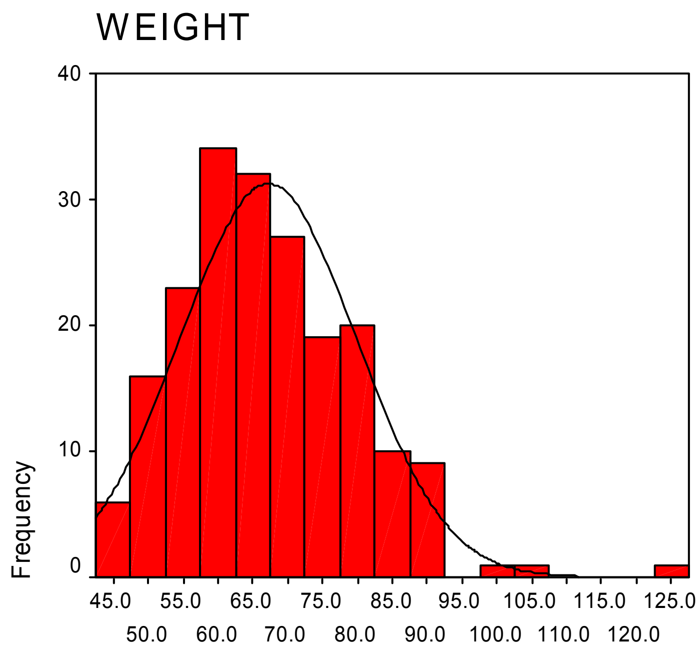


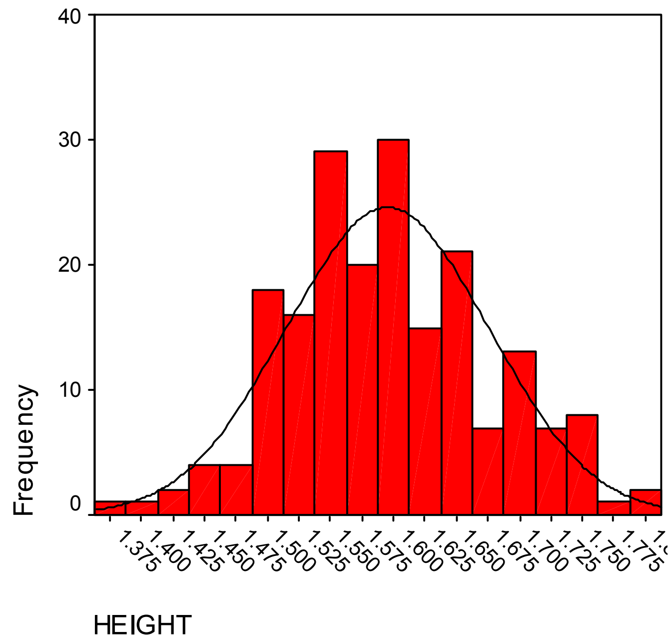
Table 5.3 Height in the total study population and in the male and female population

	Total	Males	Females
Mean	1.59	1.64*	1.59*
Std. error of mean	0.006	0.014	0.006
Median	1.59	1.63	1.58
Mode	1.6	1.54	1.6
Std. Deviation	0.08	0.079	0.08
Variance	0.006	0.006	0.006
Skewness	0.2	0.22	0.21
Std. error of Skewness	0.17	0.42	0.19

Kurtosis	-0.14	-0.07	-0.04
Std. error of Kurtosis	0.34	0.82	0.37
Minimum	1.37	1.51	1.37
Maximum	1.81	1.81	1.79
Percentile 2.5#	1.45	1.51	1.43
Percentile 97.5#	1.75	1.81	1.75

*p = 0.001

Figure 5.3 Height distribution in the total study population



40
30
20
10
0
Frequency
1.375
1.400
1.425
1.450
1.475
1.500
1.525
1.550
1.575
1.600
1.625
1.650
1.675
1.700
1.725
1.750
1.775
HEIGHT

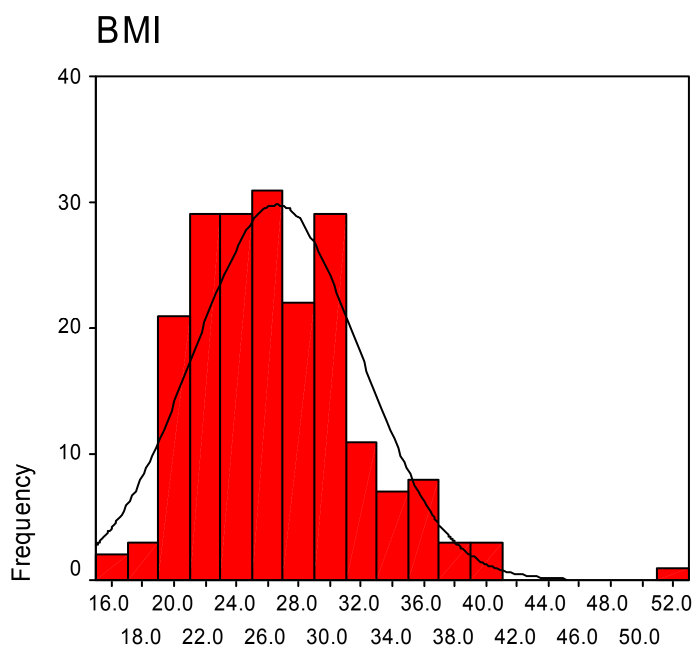
Table 5.4 Body mass index (BMI) in the total study population and in the male and female population

	Total	Males	Females
Mean	26.59	25.07*	26.87*
Std. error of mean	0.38	0.81	0.42
Median	25.97	24.91	26.16
Mode	24.5	20.48	24.5
Std. Deviation	5.32	4.51	5.42
Variance	28.29	20.33	29.38
Skewness	0.84	0.18	0.88
Std. error of Skewness	0.17	0.42	0.19
Kurtosis	1.68	-0.77	1.73
Std. error of Kurtosis	0.34	0.82	0.37

Minimum	15.05	16.33	15.05
Maximum	51.4	34.36	51.4
Percentile 2.5#	18.7	16.33	18.79
97.5#	38.5	34.36	39.27

*p = 0.05

Figure 5.4 Body mass index (BMI) distribution in the total study Population



5.2 Leptin receptor gene pentanucleotide polymorphism in the Saudi population

In this section the results of the studies on the leptin receptor gene pentanucleotide polymorphism are presented. The results obtained using PCR and Rsa 1 restriction endonuclease digestion are presented. These results were used to calculate the frequency of the different leptin receptor genotypes as well as allele frequency in the study population, and separately in the males and females. This section also includes a

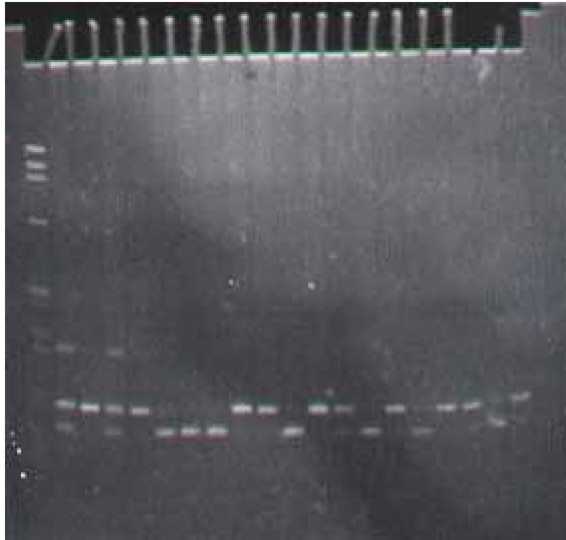
section on the frequency distribution of the different leptin receptor genotypes and the pentanucleotide insertion/deletion allele frequency in the study population and in the males and females divided according to BMI into different weight groups (normal weight, overweight and obese).

5.2.2 PCR and Rsa 1 restriction endonuclease digestion

Studies of leptin receptor polymorphism were carried out on 99 DNA samples, these were amplified using PCR and Figure 5.5 presents an electrophoretogram showing the pattern obtained in cases with and without the pentanucleotide polymorphism.
After the DNA was amplified, it was restricted using Rsa 1, to determine the presence or absence of the pentanucleotide polymorphism. In DNA, not cut by the restriction endonuclease (- -) only one band of 114 bp was obtained. If only one allele was cut (heterozygous) the result was (+ -) which is seen as two bands (see Figure 5.5) one of 114 bp and the other of 90 bp. If both alleles were cut (homozygous) the result was (+ +) this is seen as one band of 90 bp and a small undetectable band of 29 bp.

Figure 5.5 – Electrophoretic pattern obtained upon restriction of PCR product of the Leptin receptor gene with Rsa 1.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 1516 17 18 19 20



119 bp
90bp

This figure shows the different leptin receptor genotypes, lanes 3,5,9,10,12,13,15 and 17 show the (- -), lanes,6,7,8,11,14 and 16 show the (- +), while lanes 2,4,13, 17,18,19 and 20 showthe (+ +) genotype.

5.2.3 Frequency of the polymorphic forms of the leptin receptor pentanucleotide polymorphsim in the study population and in males and females

The frequency of the different polymorphic forms of the leptin receptor in the study population and in males and females of the population are presented in Table 5.5. The study population was 99, of who 20 were males and 79 where females. In the study population the frequency of the different genotypes was as follows: for the (- -) genotype there were 44 individuals which make up 44.44 % of the total, for the - +) genotype there were 27 individuals which are 27.27 % of the total and for the (+ +) genotype there were 28 individuals and the frequency was 28.28 %.
When the frequencies of the different genotypes in males and females were compared it was shown that the (- -) genotype had a higher occurrence in females than in males. The frequency of the (- -) genotype in females was 45.56 % while in male it was 40 %, whereas the frequency of the (+ +) genotype in males and females was 30 % and 27.84 % respectively. In comparison, the (- +) genotype had a slightly higher occurrence in males (30 %) compared to females (26.58 %). However, applying chi

square analysis ($\chi2$) to compare the frequencies in the males and females, no difference was observed in the genotype frequency in the males and females.

5.2.4 Frequency of the pentanucleotide polymorphic alleles in the study population and in males and females

The frequency of the (+) and (-) alleles in the study population and males and females of the population are presented in Table 5.6. The frequency of the (-) allele in the study population was 0.58 and for the (+) allele it was 0.42. The (-) allele frequency in the males and females was 0.55 and 0.589, while the frequency of the (+) allele was 0.45 and 0.411 in the males and females respectively. When the male and female results were compared, the $\chi2$ was 0.069 and the difference was not statistically significant. Fischer`s Exact Test was also applied and no difference was seen in the male and female results with a p = 0.721 (Odds Ratio 0.8542 and 95 % confidence interval = 0.4246-1.719).

Table 5.5 Genotype and allele frequency of the leptin receptor gene pentanucleotide polymorphism in the study population and in males and females

Genotype	Frequency in the study population (Total no =99)		Males (Total = 20)		Females (Total =79)	
	No	%	No	%	No	%
– –	44	44.44	8	40	36	45.56

- +	27	27.27	6	30	21	26.58
+ +	28	28.28	6	30	22	27.84

χ^2 = 0.2059; DF = 2; p = 0.9022 (NS)

Table 5.6 Allele frequency of the insertion/deletion pentanucleotide polymorphic forms of the leptin receptor gene in the study population and in males and females

Allele	Study population	Males	Females
-	0.58	0.5	0.589
+	0.42	0.45	0.411

- χ^2 = 0.069; DF = 1; p = 0.7978 (NS), Fischer`s Exact Test p = 0.7210 (NS)

5.2.5 Frequency of the leptin receptor pentanucleotide polymorphism in the different weight groups.

In an attempt to investigate if the leptin receptor gene pentanucleotide polymorphism occurred at a different frequency in the normal weight, overweight and obese

individuals, the frequency of the (+) and (-) genotypes and alleles was obtained in different BMI groups. The frequency of the different genotypes of the leptin receptor in the study population divided into different weight groups and in males and females in different weight groups is presented in Table 5.7. In the study population the frequency of the (- -) genotype in the normal weight group was 51.22 %, 39.28 % in the overweight group and 43.33 % in the obese group, whereas the frequency of the (- +) genotype in the different weight groups was 34.14 % in the normal group, 25 % in the overweight group and 20 % in the obese group. The (+ +) genotype occurred at a frequency of 14.63 % in the normal weight group, 35.71 % in the overweight group and 36.66 % in the obese group. Among the females, the frequency of the (- -) genotype in the normal, overweight and obese females was 51.61, 40 and 43.48 % respectively, the frequency of the (- +) in the normal, overweight and obese females was 29.03, 24 and 26.1 % respectively, whereas for the (+ +) genotype the frequency in the normal, overweight and obese females was 19.35, 36 and 30.43 % respectively.

In males, the frequency of the (- -) genotype in the normal, overweight and obese males was 40, 33.33 and 42.86 % respectively, for the (- +) genotype the frequency for the normal and overweight males was 50 and 33.33 % respectively, whereas the frequency of the (+ +) genotype in the normal, overweight and obese males was 20, 33.33 and 57.14 % respectively. Though the frequency of the (+ +) genotype was higher in the overweight and obese males and females, no statistically significant difference was seen applying the chi square Test for independence [$\chi^2 = 6.013$; DF = 4 and p = 0.1982].

5.2.6 Frequency of the pentanucleotide polymorphic alleles of the leptin receptor in the study population and in males and females divided into different weight groups

The frequency of the pentanucleotide polymorphic alleles of the leptin receptor gene in the study population and in males and females divided into different weight groups are presented in Table 5.8. The frequency of the (-) allele in the study population in

the normal, overweight and obese individuals was 0.68, 0.52 and 0.53 respectively, the frequency of the (+) allele in the normal, overweight and obese individuals was 0.32, 0.48 and 0.46 respectively. In the males the frequency of the (-) allele in normal, overweight and obese males was 0.65, 0.5 and 0.43 respectively, whereas the frequency of the (+) allele normal, overweight and obese males was 0.35, 0.5 and 0.57 respectively. For females, the frequency of the (-) allele in normal, overweight and obese females was 0.66, 0.52 and 0.43 respectively, whereas the frequency of the (+) allele in normal, overweight and obese females was 0.34, 0.48 and 0.43 respectively.
The (+) allele occurred at a higher frequency in the overweight and obese individuals, but the difference in the frequency of the (-) and (+) alleles, was not statistically significant in the different weight groups ($p > 0.05$).

Table 5.7 Frequency of the genotypes in the study population divided into different weight groups

Group	Weight groups	Study population (99)					
		_ _		- +		+ +	
		No	%	No	%	No	%
Study population	**Normal (41)**	21	51.22	14	34.14	6	14.63
	Overweight (28)	11	39.28	7	25	10	35.71
	Obese (30)	13	43.33	6	20	11	36.66
Males	**Normal (10)**	4	40	5	50	1	20
	Overweight (3)	1	33.3	1	33.33	1	33.33
	Obese (7)	3	42.86	N/A	N/A	4	57.14
Females	**Normal (31)**	16	51.61	9	29.9	6	19.35
	Overweight (25)	10	40	6	24	9	36
	Obese (23)	10	43.48	6	26.1	7	30.43

Table 5.8 Allele frequency of the pentanucleotide polymorphic alleles of the leptin receptor gene in the study population and in males and females divided into different weight groups

Group	**Allele**	
Study population	+	-
Normal	0.32	0.68
Overweight	0.48	0.52
Obese	0.46	0.53
Males		
Normal	0.35	0.65
Overweight	0.5	0.5
Obese	0.57	0.43
Females		

Normal	0.34	0.66
Overweight	0.48	0.52
Obese	0.43	0.56

$\chi^2 = 4.931$; DF = 2; p = 0.085

5.3 Biochemical analysis

The results of biochemical analysis in the total study population and in male and female are presented in this section. The parameters investigated included leptin, cholesterol, triglycerides and insulin.

5.3.2 Leptin in the total study population and in the male and female population

The results of leptin analysis in the total study population investigated during this study and in the population divided into male and female are presented in Table 5.9. In the total study population the mean, median and mode values for leptin were 15.7, 14.77 and 12.29 ng/ml respectively. Leptin showed a Guassian distribution as seen in Figure 5.6 with skewness of 0.63 and kurtosis of 0.71. The minimum value was 0.65 ng/ml and the maximum value was 46.98 ng/ml. The parametric range was 0.28-31.12 ng/ml and the non-parametric range was 2.99-31.71 ng/ml.

A significant difference was seen when the results in male and female were analyzed separately. The mean, median and mode values were higher in females (16.4, 15.5 and

12.29 ng/ml respectively) compared to males (11.95, 11.4 and 0.65 ng/ml respectively). The minimum and maximum values for leptin in males were 0.65 ng/ml and 26.75 ng/ml, whereas in females the minimum value was 2.99 ng/ml and the maximum was 46.98 ng/ml. In males the parametric range was 0-26.39 ng/ml and the non-parametric range was 0.65-26.75 ng/ml. For females, on the other hand the parametric range was 1.18-31.62 ng/ml and the non-parametric range was 4.1-32.79 ng/ml. The results in the males and females showed a statistically significant difference in the leptin levels, with significantly higher levels in the females compared to males (p= 0.003).

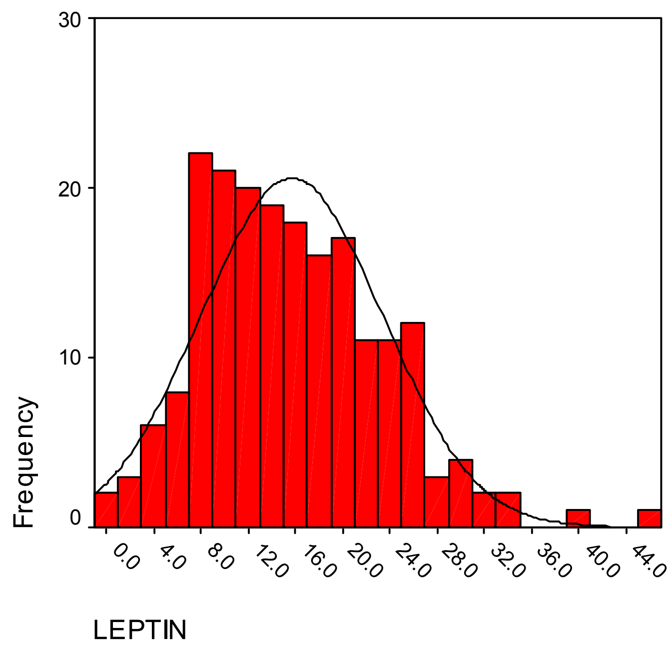
Table 5.9 Leptin levels in the total study population and in males and females

	Total	Males	Females
Mean	15.7	11.95*	16.4*
Std. error of mean	0.55	1.3	0.59
Median	14.77	11.4	15.5
Mode	12.29	0.65	12.29
Std. Deviation	7.71	7.22	7.61

Variance	59.44	52.2	58
Skewness	0.63	0.33	0.72
Std. error of Skewness	0.17	0.42	0.19
Kurtosis	0.71	-0.44	0.81
Std. error of Kurtosis	0.34	0.81	0.37
Minimum	0.65	0.65	2.99
Maximum	46.98	26.75	46.98
Percentile 2.5#	2.99	0.65	4.1
Percentile 97.5#	31.71	26.75	32.79

*p= 0.003

Figure 5.6 Leptin level distribution in the total study population


30
20
10
0
Frequency
0.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
32.0
36.0
40.0
44.0
LEPTIN

5.3.3 Lipids in the total study population and in the male and female population

The lipids investigated during this study included cholesterol and triglycerides. This section presents the results of these parameters in the total study population, and in the population divided into male and female.

5.3.3.1 Cholesterol levels in the total study population and in the male and female population

The levels of cholesterol in the total study population and in male and female are presented in Table 5.10. The mean value of cholesterol in the total study population was 4.76 mmol/l. The values of the median and mode were 4.69, 3.8 mmol/l, respectively. The cholesterol levels showed a Guassian distribution in the total study population. This was confirmed by the frequency distribution histogram (Figure 5.7) with skewness of 0.47 and kurtosis of 0.34. The parametric range was 2.5-7.02 mmol/l and the non-parametric range was 2.9-7.42 mmol/l. The minimum value was 2 mmol/l and the maximum was 8.73 mmol/l.
Based on the cut-off lipid values for the classification of hyperlipidaemias, published in the " Report of the National Cholesterol Education Program Expert Panel on Detection, evaluation and Treatment of High Blood Cholesterol in adults 1998" presented in Table 5.11, the population was grouped into those with normal cholesterol level and with borderline risk and high risk for development of coronary heart disease (CHD), as well as those with hypercholesterolaemia. Of the total population, 67 individuals (33.66 %) were classified as hypercholesterolaemic. Of these and 44 individuals (22.11 %) were borderline and 23 individuals (11.55 %) were at high risk for developing CHD.
When the results of males and females were analyzed separately, no significant difference was observed in cholesterol level between the two sexes. The mean, median and mode in males were 4.56, 4.5 and 3.5 mmol/l respectively, compared to

the mean, median and mode of females 4.8, 4.71 and 4.5 mmol/l respectively (Table 5.10). The parametric range for cholesterol in the males was 2.08-7.04 mmol/l and the non-parametric range was 2.9-7.64 mmol/l, while in females the parametric range for cholesterol was 2.58-7.02 mmol/l and the non-parametric range was 2.75-7.34 mmol/l. The minimum value of cholesterol observed in male and female were 2.9 mmol/l and 2 mmol/l and the maximum values were 7.64 mmol/l and 8.73 mmol/l respectively. The prevalence of hypercholesterolaemia in the two sexes was: 29.03% in the males and in the females it was 34.52. Almost (16.13 %) of the males were having borderline risk and 12.9 % were at high risk for developing coronary heart disease, while in the females, 23.21 %were at borderline risk and 11.31 % were at high risk for developing CHD.

Table 5.10 Cholesterol levels in total study population and in the

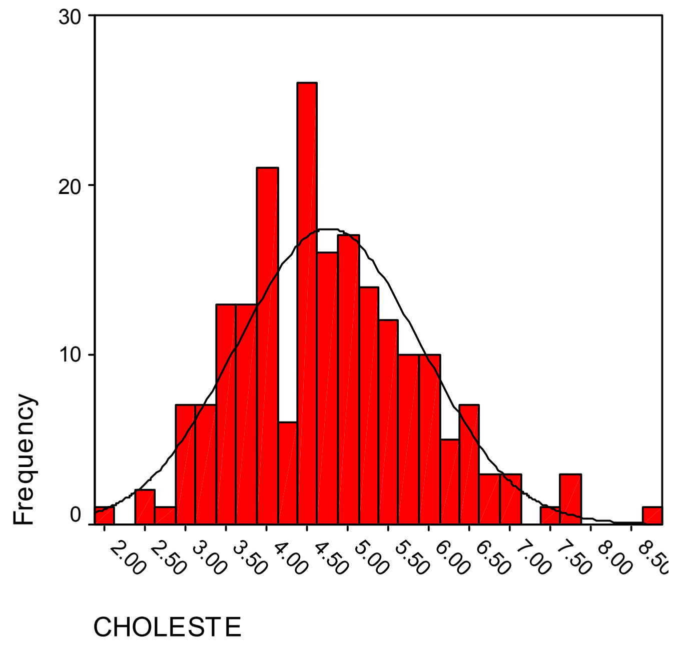
male and female population

	Total	Males	Females
Mean	4.76	4.56*	4.8*
Std.error of mean	0.08	0.22	0.08
Median	4.69	4.5	4.71
Mode	3.8	3.5	4.5
Std. Deviation	1.13	1.24	1.11
Variance	1.29	1.55	1.24
Skewness	0.47	0.75	0.45
Std. error of Skewness	1.72	0.42	0.19
Kurtosis	0.34	-0.14	0.55
Std. error of Kurtosis	0.34	0.81	0.37
Minimum	2	2.9	2
Maximum	8.73	7.64	8.73
2.5[#]	2.9	2.9	2.75

Percentile 97.5#	7.42	7.64	7.34

*p = 0.28

Figure 5.7 Cholesterol level distribution in the total population



5.11 Cut-off lipid values for classification of hyperlipidaemias

	Desirable	Borderline high risk	High risk CHD
Cholesterol: (mg/dl) (mmol/l)	<200 (5.18)	200-239 (5.18-6.19)	>240 (6.22)
Triglycerides: (mg/dl) (mmol/l)	<250 (2.83)	250-500 (2.83-5.65)	>500 (5.65)

From Ref. Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in adults. Arch Inter Med 1998; 148:36.

5.3.3.2 Triglycerides in the total study population and in the male and female population

Triglycerides were studied in the total study population and the results are presented in Table 5.12. The mean, median and mode for the total study population were 1.25, 1.1 and 0.61 mmol/l respectively. As these values were not similar to each other this indicated a skewed distribution. The frequency distribution histogram of triglycerides in the total study population is presented as Figure 5.8 with skewness of 2.86 and kurtosis of 14.96. The minimum value was 0.37 mmol/l and the maximum value was 6.69 mmol/l. The parametric range was 0-2.71 mmol/l and the non-parametric range was 0.46-3.12 mmol/l. Applying the cut-off values presented in Table 5.11, 5 individuals (2.5%) were classified as hypertriglyceridaemic.

When the results for male and female were analyzed separately, a difference was seen in the triglyceride levels in the two sexes. In the males, the triglyceride mean (1.55 mmol/l) was slightly higher when compared to the female triglyceride mean (1.23 mmol/l). The median and mode for male were 1.13 mmol/l and 0.68 mmol/l and in female they were 1.05 mmol/l and 0.61 mmol/l, respectively. In males the minimum triglyceride value was 0.65 mmol/l and the maximum value was 6.69 mmol/l, The parametric range was 0-3.87 mmol/l and the non-parametric range was 0.65-6.69 mmol/l. While in females the minimum value was 0.37mmol/l and the maximum value was 3.61mmol/l, The parametric range was 0-2.47 mmol/l and the non-parametric range was 0.45-3.06 mmol/l. Applying the cut-off values presented in

Table 5.11, 3 females (1.78%) and 2 males (6.45%) were classified as hypertriglyceridaemic.

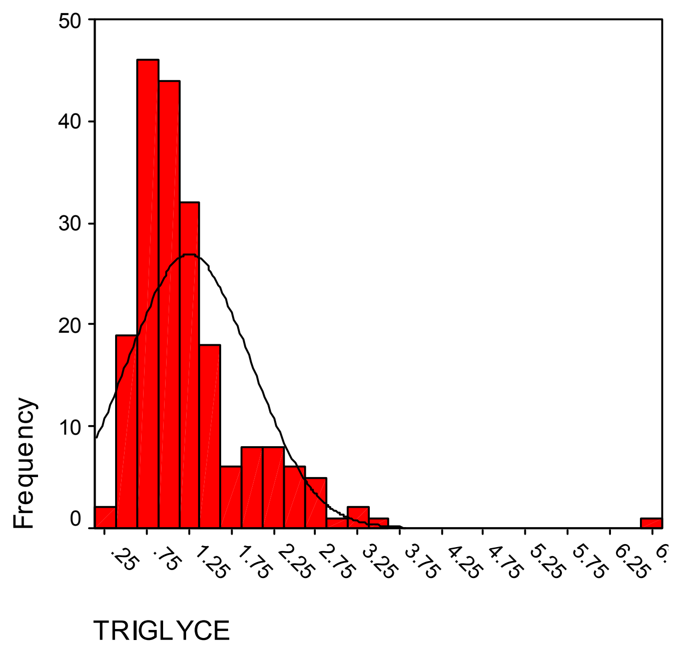
Table 5.12 Triglyceride levels in the total study population and in the male and female population

	Total	Males	Females
Mean	1.25	1.55*	1.23*
Std. error of mean	0.05	0.21	0.05
Median	1.1	1.13	1.05
Mode	0.61	0.68	0.61
Std. Deviation	0.73	1.16	0.62
Variance	0.54	1.3	0.41

Skewness	2.86	3.11	1.56
Std. error of Skewness	0.17	0.42	0.19
Kurtosis	14.96	12.68	2.6
Std. error of kurtosis	0.34	0.82	0.37
Minimum	0.37	0.65	0.37
Maximum	6.69	6.69	3.61
Percentile 2.5[#]	0.46	0.65	0.45
Percentile 97.5[#]	3.12	6.69	3.06

*p = 0.014

Figure 5.8 Triglyceride level distribution in the total study population



50
40
30
20
10
0
Frequency
.25
.75
1.25
1.75
2.25
2.75
3.25
3.75
4.25
4.75
5.25
5.75
6.25
6.
TRIGLYCE

5.3.4 Insulin in the total study population and in the male and female population

Insulin levels were measured in the total study population and in male and female and the results were presented in Table 5.13. In the total study population the values of insulin mean, median and mode were 13.6, 9.8 and 4.4 μU/ml respectively. As these values were not close to each other they indicate a non-Gaussian distribution, which is also seen in the frequency distribution histogram (Figure 5.9) as a positively skewed distribution with skewness 3.03 and kurtosis of 11.95. The parametric range for insulin was 0-39 μU/ml, whereas, the non-parametric range was 3.34-53.48 μU/ml. The lowest value of insulin was 1 μU/ml and the highest value was 88.8 μU/ml.

When the results insulin levels in the male and female individuals were analyzed separately, it was shown that insulin levels were slightly lower in males than in females. This can be seen in the values of the mean, median and mode. In the males the values were 13.13, 11.14 and 3.37 μU/ml, respectively whereas, in the females they were 13.68, 9.65 and 4.4 μU/ml, respectively. In males the lowest value of insulin was 3.37 μU/ml and the highest value was 44.74 μU/ml. While in females the lowest value was 1 μU/ml and the highest value was 88.8 μU/ml. The parametric range for insulin males was 0-32.73 μU/ml, while the non-parametric range was 3.37-44.74 μU/ml, while in females the parametric range was 0-40.04 μU/ml, while the non-parametric range was 3.32-59.38 μU/ml. The difference in the mean of insulin in the males and females was not statistically significant ($p > 0.05$).

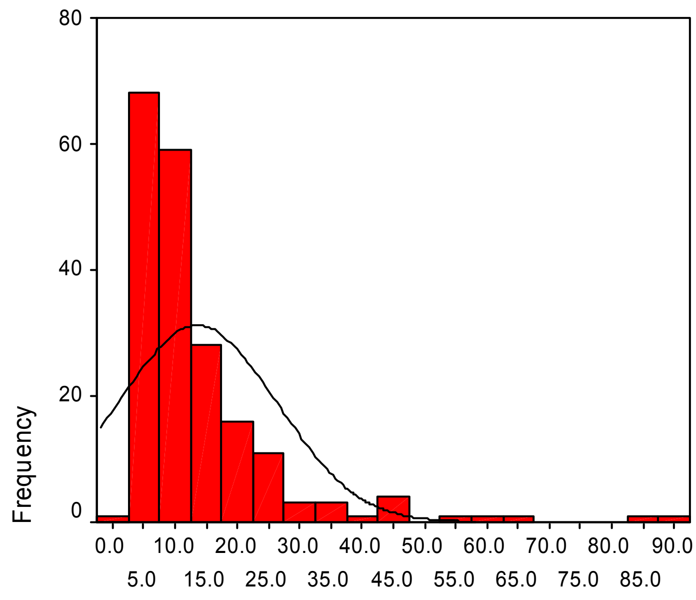
Table 5.13 Insulin levels in the total study population and in the male and female population

	Total	Males	Females
Mean	13.6	13.13*	13.68*
Std. error of mean	0.9	1.76	1.02
Median	9.8	11.14	9.65
Mode	4.4	3.37.	4.4
Std. Deviation	12.7	9.8	13.18
Variance	161.2	96.13	173.8
Skewness	3.03	2.13	3.06
Std. error of Skewness	0.172	0.42	0.19
Kurtosis	11.95	4.78	11.89
Std. error of Kurtosis	0.34	0.82	0.37
Minimum	1	3.37	1

Maximum	88.8	44.74	88.8
2.5# Percentile	3.34	3.37	3.32
97.5#	53.48	44.74	59.38

*p = 0.78

Figure 5.9 Insulin level distribution in the total study population


INSULIN
Frequency
80
60
40
20
0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
5.0
15.0
25.0
35.0
45.0
55.0
65.0
75.0
85.0

5.4 Value of Biochemical parameters in individuals with different leptin receptor Polymorphic forms in the study population and in males and females

In this section demographic data and biochemical parameters were analyzed separately in individuals with different leptin receptor pentanucleotide polymorphic forms in the study population and in males and females .The parameters compared included: age, height, weight, BMI, leptin, cholesterol triglyceride and insulin.

5.4.2 Age range in the study population and in males and females with different polymorphic forms of the leptin receptor

Age range of the individuals carrying the different leptin receptor polymorphic forms in the study population as well as in male and female is presented in Table 5.14. The mean for age ± SD and the significance of the difference in results of males and females are presented in Table 5.14. In the study population the mean of age for individuals with genotypes (- -), (- +) and (+ +) were 36.59, 30.67 and 35.54 years, respectively. In the males the mean age in the different leptin receptor genotypes was 39.25, 29.67 and 40.33 years respectively, while in the females the mean age in the different genotypes was 36, 30.95 and 34.23 years respectively. No significant difference was noticed in age between the different genotypes of the leptin receptor in male and female..

Table 5.14 Age range in individuals with different pentanucleotide leptin receptor genotypes

Genotype	Study gp Mean ± SD	Males Mean ± SD	Females Mean ± SD	p
– –	36.59 ± 14.55	39.25 ± 17.06	36 ± 14.14	0.57
- +	30.67 ± 8	29.67 ± 12.82	30.95 ± 6.45	0.74
+ +	35.54 ± 15.87	40.33 ± 23.56	34.23 ± 13.54	0.41

5.4.2 Weight range in the study population and in males and females with different pentanucleotide leptin receptor genotypes

The weight range of the study population and of male and female in the population with the different leptin receptor genotypes are presented in Table 5.15. For the study population the mean of weight in the different genotypes (- -), (- +) and (+ +) was 67.04, 67.58 and 69.76 Kg respectively and these values were not significantly different. In the male group the mean of weight in the different genotypes was 68.5, 61.17 and 78.67 Kg respectively, while in female the mean of weight in the different genotypes was 66.72, 69.42 and 67.33 Kg respectively. A significant difference was observed in weight between the males and females with the (+ +) genotype, but not

for the (- +) and (+ +) genotypes. Within the males, the highest weight was in the (+ +) genotype compared to the (- -) and (- +) genotypes. However, this was not observed in the females.

5.4.3 Height in the total study population and in males and females with different pentanucleotide leptin receptor genotypes

Height range in the study population as well as in males and females in the different leptin receptor genotypes are presented in Table 5.16. In the study population the mean of height for the different genotypes was 1.62, 1.6 and 1.58 m respectively and the difference between the different genotypes was not significant.

In the males the mean of height in the different leptin receptor genotypes was 1.64, 1.67 and 1.66 m respectively, While the mean for height in the different genotypes in females was 1.62, 1.59 and 1.56 m respectively. A statistically significant difference in height between males and females was noticed for the (+ +) genotype and for the (- +) genotype, but no difference was seen in the (- -) genotype. Within males and females there was a slight difference in the height in the different genotypes, but the difference was not statistically significant.

5.4.4 Body mass index (BMI) in the study population and in males and females with different pentanucleotide leptin receptor genotypes

The body mass index (BMI) range in the study population and in the males and females with the different leptin receptor genotypes (- -), (- +) and (+ +) are presented in Table 5.17. For the study population the mean of the BMI in the different leptin

receptor genotypes was 25.38, 26.43 and 27.78 Kg/m^2, respectively. These values were significantly different where the BMI was lowest in the (- -) genotype and highest in the (+ +) genotype.

In males the mean of BMI in the different genotypes was 25.75, 21.98 and 28.61 Kg/m^2 respectively, whereas in females the BMI mean in the different leptin receptor genotypes was 25.29, 27.7 and 27.55 Kg/m^2 , respectively. A significant difference was noticed for BMI for the (- +) genotype between males and females ($p < 0.05$), in the (- -) and (+ +) genotypes there was no significant difference ($p > 0.05$)

Table 5.15 Weight range in individuals with different pentanucleotide leptin receptor genotypes

Genotype	Study gp Mean ± SD	Males Mean ± SD	Females Mean ± SD	p
_ _	67.04 ± 10.94	68. 5 ± 9.65	66.72 ± 11.31	0.65
- +	67.58 ± 15.52	61.17 ± 7.78	69.42 ± 16.8	0.1
+ +	69.76 ± 12.91	78.67 ± 11.74	67.33 ± 12.36	0.05

Table 5.16 Height range in individuals with different leptin receptor

genotypes

Genotype	study gp Mean ± SD	Males Mean ± SD	Females Mean ± SD	p
_ _	1.62 ± 0.095	1.64 ± 0.1	1.62 ± 0.094	0.52
- +	1.6 ± 0.085	1.67 ± 0.068	1.59 ± 0.081	0.02
+ +	1.58 ± 0.088	1.66 ± 0.061	1.56 ± 0.082	0.007

Table 5.17 Body mass index (BMI) in individuals with different pentanucleotide leptin receptor genotypes

Genotype	Study gp Mean ± SD	Males Mean ± SD	Females Mean ± SD	p
– –	25.38 ± 5.9	25.75 ± 5.19	25.29 ± 6.1	0.83
- +	26.43 ± 6.77	21.98 ± 3.36	27.7 ± 7	0.01
+ +	27.78 ± 4.55	28.61 ± 4.96	27.55 ± 4.52	0.62

5.4.5 Leptin levels in the study population and in males and females with different pentanucleotide leptin receptor genotypes

The levels of leptin in the study population and in male and female with the different pentanucleotide leptin receptor genotypes (- -), (- +) and (+ +) are presented in Table 5.18. In the study population the mean for leptin in the different genotypes was 14.27, 15.02 and 17.04 ng/ml respectively. A significant increase was observed from the (- -) to the (+ +) genotypes ($p< 0.05$).

Among the males the mean of leptin in the different genotypes was 9.69, 6.03 and 17 ng/ml respectively, while among the females the mean of leptin in the different genotype was 15.29, 17.59 and 17.05 ng/ml respectively. A significant difference in leptin levels in the (- -) and (- +) genotypes between the two sexes was noticed. Interestingly, in genotype (+ +) both male and female had high leptin levels and the difference in the males and females was not significant.

5.4.6 Lipid levels in the study population and in male and female with different pentanucleotide leptin receptor genotypes

The levels of lipids (cholesterol and triglycerides) were investigated in the individuals with different pentanucleotide leptin receptor genotypes to determine if there was any effect. The results of these parameters in the study population and in males and females with different pentanucleotide leptin receptor genot

Table 5.18 Leptin levels in individuals with different pentanucleotide leptin receptor genotypes

Genotype	Study gp Mean ± SD	Males Mean ± SD	Females Mean ± SD	p
_ _	14.27 ± 7.81	9.69 ± 8.54	15.29 ± 7.39	0.06
- +	15.02 ± 8.61	6.03 ± 4.15	17.59 ± 7.8	0.00
+ +	17.04 ± 8.39	17 ± 7.42	17.05 ± 8.8	0.98

5.4.6.1 Cholesterol levels in the study population and in males and females with different pentanucleotide leptin receptor genotypes

The levels of cholesterol in the study population in males and females with the different pentanucleotide leptin receptor genotypes (- -), (- +) and (+ +) are presented in Table 5.19.The mean in the study population for cholesterol in the different genotypes was 4.69, 4.78 and 4.57 mmol/l respectively.

In the males, the mean of cholesterol in the different genotypes was 4.38, 3.95 and 4.84 mmol/l, respectively. While in females the mean for cholesterol in the different pentanucleotide leptin receptor genotypes was 4.76, 5.02 and 4.5 mmol/l, respectively. A statistically significant difference in cholesterol levels in the (- +) genotype was noticed between the two sexes, with significantly higher cholesterol in the females. The other genotypes (- -) and (+ +) did not show any difference between males and females.

5.4.6.2 Triglyceride levels in the study population and in males and females with different pentanucleotide leptin receptor genotypes

The triglyceride levels in the study population and in males and females with different pentanucleotide leptin receptor genotypes is presented in Table 5.20. The mean of triglycerides in the different pentanucleotide leptin receptor genotypes in the study population was 1.39, 1.29 and 1.24 mmol/l respectively.

In males the mean of triglycerides in the different genotypes was 1.92, 1.55 and 1.68 mmol/l respectively, whereas for females the mean of triglycerides in the different leptin receptor genotypes (- -), (- +) and (+ +) was 1.27, 1.22 and 1.12 mmol/l respectively. A slight difference in triglyceride levels in the (- -) and (+ +) genotypes was noticed between the two sexes. The (- +) genotype did not show any significant difference.

5.4.7 Insulin levels in the study population and in males and females with different pentanucleotide leptin receptor genotypes

The levels of insulin in the study population and in males and females with the different pentanucleotide leptin receptor genotypes are presented in Table 5.21. The mean of insulin in the different genotypes in the study population was 15.67, 12.27 and 15.28 μU/ml respectively, In males, the mean of insulin in the different leptin receptor genotypes was 13.24, 15.14 and 13.33 μU/ml respectively, on the other hand, in females the mean of insulin in the different genotypes was 16.21, 11.45 and 15.82 μU/ml respectively. No significant difference in insulin levels was noticed in all genotypes between the two sexes.

Table 5.19 Cholesterol levels in individuals with different

pentanucleotide pentanucleotideleptin receptor genotypes

Genotype	Study gp Mean ± SD	Males Mean ± SD	Females Mean ± SD	p
_ _	4.69 ± 1.2	4.38 ± 0.74	4.76 ± 1.27	0.12

- +	4.78 ± 1.03	3.95 ± 0.72	5.02 ± 1	0.01
+ +	4.57 ± 1.16	4.84± 1.59	4.5 ± 1.04	0.53

Table 5.22 Triglyceride levels in individuals with different pentanucleotide leptin receptor genotypes

Genotype	study group Mean ± SD	Males Mean ± SD	Females Mean ± SD	p
_ _	1.39 ± 1	1.92 ± 1.99	1.27 ± 0.6	0.099
- +	1.29 ± 0.7	1.55 ± 0.87	1.22 ± 0.65	0.32
+ +	1.24± 0.7	1.68 ± 0.78	1.12 ± 0.64	0.08

Table 5.21 Insulin levels in individuals with different leptin receptor genotypes

Genotype	Study group Mean ± SD	Males	Females	P
– –	15.67 ± 11.59	13.24 ± 12.87	16.21 ± 11.41	0.52

- +	12.27 ± 9.14	15.14 ± 14.68	11.45 ± 7.18	0.39
+ +	15.28 ± 14.75	13.33 ± 6.72	15.82 ±16.36	0.58

5.5 Leptin, lipids and insulin levels in the total study population and

in males and females divided into different weight groups

The levels of leptin, lipids and insulin were analyzed in the total study population and in males and females divided into weight groups on the basis of the BMI (normal weight 20-24.9 Kg/m^2, overweight 25-29.9 Kg/m^2 and obese $\geq$ 30 Kg/m^2). These results are presented in this section.

5.4.8 Leptin levels in the total study population and in males and females divided into different weight groups

The levels of leptin were compared in the total study population and in males and females divided into normal, overweight and obese individuals and the results are presented in Table 5.22. In the total study population the mean of leptin in the different weight groups was 10.92, 16.87 and 21.56 ng/ml respectively. There was a significant increase in the leptin level and the difference in the levels in the normal weight, overweight and obese was statistically significant.

In males the mean of leptin in the different weight groups was 7.89, 17.29 and 15.37 ng/ml respectively. Whereas for females the mean of leptin in the different weight groups was 11.63, 16.81 and 22.57 ng/ml respectively. A statistically significant difference in leptin levels in the normal and obese weight groups was noticed between males and females ($p < 0.05$).

Table 5.22 Leptin levels in the total study population and in males and females divided into different weight groups

Weight gp	**Total study gp Mean ± SD**	**Males Mean ± SD**	**Females Mean ± SD**	**p**
Normal weight (1)	10.92 ± 5.12	7.89 ± 5.32	11.63 ± 4.84	0.008
Overweigth (2)	16.87 ± 5.75	17.29 ± 6.63	16.81 ± 5.69	0.84
Obese (3)	21.56 ± 8.18	15.37 ± 6.77	22.57 ± 8	0.02
P				

5.4.9 Lipids in the total study population and in males and females divided into different weight groups

This section presents the levels of lipids in the total study population and in males and females divided into different weight groups.

5.4.9.1 Cholesterol levels in the study population and in males and females divided into different weight groups

The levels of cholesterol in the total study population and in males and females divided into different weight groups (normal weight, overweight and obese) are presented in Table 5.23 . In the total study population the mean of cholesterol in the different weight groups was 4.45, 5.03 and 4.95 mmol/l, respectively.

In males, the mean of cholesterol in the different weight groups was 4.9, 5.28 and 4.68 mmol/l, respectively. While for females the mean of cholesterol in the different weight groups was 4.51, 4.99 and 5 mmol/l, respectively. No significant difference was noticed in the cholesterol levels in the different weight groups between males and females.

5.4.9.2 Triglyceride levels in the total study population and in males and females divided into different weight groups

Triglyceride levels were compared between different weight groups in the total study population and in males and females and are presented in Table 5.24. The mean of triglycerides in the total study population in the different weight groups was 1.17, 1.19 and1.42 mmol/l, respectively.

In males, the mean of triglycerides in the different weight groups was 1.66, 1.28 and 1.56 mmol/l respectively. On the other hand for females the mean of triglycerides in females in the different weight groups was 1.06, 1.18 and 1.4 mmol/l respectively. A significant difference in the triglyceride levels was observed in the males and females in the normal weight groups ($p = 0.01$), but not in the other weight groups.

5.4.10 Insulin levels in the total study population and in males and females divided into different weight groups

The levels of insulin in the total study population and in males and females divided into different weight groups are presented in Table 5.25. The mean of insulin in the total study population in the different weight groups was 12.22, 12.32 and 16.94 μU/ml, respectively. In males the mean of insulin in the different weight groups was 10.95, 15.43 and 15.46 μU/ml respectively, while in females the mean of insulin in the different weight groups was 12.51, 11.9 and 17.18 μU/ml, respectively. No significant difference in insulin levels was observed between the two sexes in the different weight groups.

Table 5.23 Cholesterol levels in the total study population and in males and females divided into different weight groups

Weight gps	Total study gp Mean ± SD	Males Mean ± SD	Females Mean ± SD	p
Normal weight (1)	4.45 ± 0.93	4.19 ± 0.92	4.51 ± 0.93	0.21
Overweight. (2)	5.03 ± 1.12	5.28 ± 1.55	4.99 ± 1.06	0.84
Obese (3)	4.95 ± 1.32	4.68 ± 1.38	5 ± 1.31	0.53

P				

Table 5.24 Triglyceride levels in the total study population and in males and females divided into different weight groups

Weight gp	Total study gp	Males Mean ± SD	Females Mean ± SD	P

	Mean ± SD			
Normal (1)	1.17 ± 0.85	1.66 ± 1.49	1.06 ± 0.57	0.01
Overweight. (2)	1.19 ± 0.64	1.28 ± 0.51	1.18 ± 0.63	0.64
Obese (3)	1.42 ± 0.64	1.56 ± 0.83	1.4 ± 0.62	0.52
P				

Table 5.25 Insulin levels in the total study population and in males and females divided into different weight groups

Weight gp	Total study gp Mean ± SD	Males Mean ± SD	Females Mean ± SD	p
Normal weight (1)	12.22 ± 11.65	10.95 ± 10.05	12.51 ± 12.04	0.59
Overweight. (2)	12.32 ± 12.2	15.43 ± 6.82	11.9 ± 12. 74	0.28
Obese (3)	16.94 ±14.19	15.46 ± 11.55	17.18 ± 14.67	0.71
P				

5.5 Correlations between leptin, demographic data and biochemical parameters

In this section, the nature of correlations between beteen, demographic data and biochemical parameters (age, weight, height, BMI, leptin, lipids and insulin) for the males and females are presented in Table 5. 26 and Table 5.27, respectively. These correlations were performed to study how the parameters relate with leptin.

Leptin levels correlated with several parameters and the correlations among the males and females were different. In the males a positive correlation was observed with BMI and cholesterol and a negative correlation with height, while in the females, leptin correlated positively with age, weight, BMI, triglycerides and insulin, but not with cholesterol.

Table 5.26 Correlations between the leptin, demographic data and biochemical parameters in males

		Age	Weight	Height	BMI	Leptin	CHO	TG	Insulin
Age									
	R	1.000	0.38*	-0.36*	0.5**	0.22	0.14	-0.27	-0.26
	P		0.03	0.04	0.004	0.23	0.47	0.15	0.15
Weight	**R**	0.38*	1.000	-0.02	0.86**	0.34	0.19	0.14	0.01
	P	0.03		0.91	0.000	0.06	0.29	0.46	0.96
Height	**R**	-0.36*	-0.02	1.000	-0.52**	-0.51**	-0.4*	0.47**	-0.08
	P	0.04	0.91		0.002	0.004	0.02	0.007	0.67
BMI	**R**	0.5**	0.86**	-0.52**	1.000	0.56**	0.4*	-0.1	0.08
	P	0.004	0.000	0.002		0.001	0.03	0. 6	0.65
Leptin	**R**	0.22	0.34	-0.51**	0.56**	1.000	0.49**	-0.28	0.11
	P	0.23	0.06	0.004	0.001		0.005	0.12	0.55

CHO	**R**	0.14	0.19	-0.4*	0.4*	0.49**	1.000	0.1	0.17
	P	.467	.293	.024	.026	0.005		0.58	0.35
TG	**R**	-0.27	0.14	0.47**	-0.1	-0.28	0.1	1.000	0.16
	P	0.15	0.46	0.007	0. 6	0.12	0.58		0.38
Insulin	**R**	-0.26	0.01	-0.08	0.08	0.11	0.17	0.16	1.000
	P	0.150	0.96	0.67	0.65	0.55	0.35	0.39	

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Table 5.27 Correlations between leptin, demographic data and biochemical parameters in females

		Age	Weight	Height	BMI	Leptin	CHO	TG	Insulin
Age	**R**	1	0.31**	-0.14	0.36**	0.23**	0.38**	0.43**	0.06
	P		0.000	0.07	0	0.002	0.000	0.000	0.86
Weight	**R**	0.31**	1	0.16*	0.87**	0.61*	0.18*	0.29**	0.22*

	P	0.000		0.04	0.000	0.000	0.02	0.000	0.003
Height	**R**	-0.14	0.161*	1.000	-0.33**	-0.1	-0.05	0.1	0.13
	P	0.07	0.04		0.000	0.21	0.49	0.18	0.09
BMI	**R**	0.36**	0.87**	-0.33**	1.000	0.63**	0.18	0.22**	0.175*
	P	0.000	0.000	0.000		00.000	0.02	0.004	0.02
Leptin	**R**	0.23**	0.61**	-0.1	0.63**	1.000	0.15	0.21**	0.32**
	P	0.002	0.000	0.21	0.000		0.05	0.008	0.000
CHO	**R**	0.38**	0.18*	-0.05	0.18*	0.15	1.000	0.5**	-0.16*
	P	0.000	0.02	0.49	0.02	0.05		0.000	0.03
TG	**R**	0.43**	0.29**	0.1	0.22**	0.21**	0.5**	1.000	0.22*
	P	0.000	0.000	0.18	0.004	0.008	0.000		0.004
Insulin	**R**	0.06	0.22**	0.13	0.175*	0.32**	-0.16*	0.22**	1.000
	P	O.45	0.003	0.09	0.02	0.000	0.03	0.004	

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

6.0 Discussion:

Obesity is a complex disorder, where both polygenic and monogenic phenotypes are known. The common form of obesity is maltifactorial and it`s development depends on the contribution of multiple genes (polygenic) which produce a susceptible genotype and on the presence of precipitating environmental factors obesity develops. An individual may carry the genetic factors and may not develop obesity if the suitable environmental factors for developing obesity were not present (like eating habits and low physical activity) (Bougneres 2002).

It has been suggested by many workers, that 'markers' of obesity can be identified, then it will be possible to predict presymtomatically who may develop obesity and by avoiding the environmental factors it may be possible to avoid the development of obesity and hence, the number of serious life threatening conditions which are a consequence of obesity could be controlled.

Since the completion of the human genome project, the entire human DNA sequence is now available, and this makes it easier to study the obesity susceptibility genes. In addition, there is a growing understanding of the human genome due to the high degree of homology between humans and common laboratory animals and the availability of tools needed for studying DNA in the laboratory (Bouchard 1997).

The obesity gene map of 2002 shows that the number of gene loci contributing to the development of obesity are more than 300 genes, markers and chromosomal regions (Chagnon et al 2003).

The monogenic form of obesity results from mutations in a certain gene which then causes obesity. Several genes including the melanocortin-4-receptor (MC4P), peroxisome- proliferator-activated receptor (PPAR) γ2 genes as well as the leptin gene and the leptin receptor gene have been implicated in the development of obesity (Chen et al 1999). About five single obesity genes in rodents have been identified.

These genes suggest the crucial role of leptin and melanocortin signaling systems in the pathogenesis of obesity. One of the reported leptin gene mutations in humans was a single nucleotide deletion at position 398, which resulted in a frame shift of the leptin coding region after Gly 123 and a premature termination of peptide synthesis. The affected subjects had hyperinsulinimia and low leptin levels (Chen et al1999).

Leptin is the product of the obesity gene and is mainly produced by adipose tissue and

is involved in the control of both food intake and energy expenditure (Mantzoros et all 1998). Leptin mediates its action by interaction with a receptor which is mainly expressed in the hypothalamus (Burguera et al 2000).

Leptin levels are an indication of body fat stores. These levels are also affected by gender where women have higher leptin levels than men, this may be due to the fact that women have a higher amount of body fat and a different fat distribution than men. It is known that subcutaneous fat produces more leptin mRNA than intra-abdominal fat (Mantzoros et al 1998).

Other factors affecting leptin production include hormones like insulin which increases leptin secretion and cathecholamines which decrease leptin secretion (Kim et al 2000).

It was believed by several investigations that obesity was a leptin deficient state, but recent studies have indicated that obese subjects have either normal or even elevated leptin levels indicating that the leptin production in these individuals was normal if not higher than in normal weight individuals. This finding has all lead to the suggestion that leptin resistance occurs in of obesity. Thus that the defect was possibly at the level of the leptin receptor. Recent evidence has shown that SOCS-3 (suppressor of cytokine signaling 3) is a leptin inducible inhibitor of leptin signaling and a potential mediator of leptin resistance in obesity (Mantzoros et all 1998).

Leptin resistance can also be caused by glucocorticoids which may interfere with the interaction of leptin with its receptor (Mantzoros et all 1998).

Another cause of leptin resistance is abnormal serum leptin binding or abnormal leptin catabolism, and leptin transport across the blood-brain barrier where the leptin receptor isoform responsible for the transportation of leptin across the BBB is Ob-a.

In obesity, it was observed that there was a decreased leptin transport across the BBB, this is because leptin is believed to cross the BBB by a saturable transport mechanism, and so in obesity there is reduced leptin uptake due to the increased competition from

the higher concentrations of endogenous leptin. This explains why the significant increase in leptin levels correspond with smaller changes of leptin levels in the CSF.

Leptin gene mutations which lead to obesity are reported in a few rare cases like for example in the two severely obese children who were members of a highly consanguineous Pakistani pedigree, which had a mutation in the leptin gene which lead to a deletion of a single guanine nucleotide in codon 133 (Montague et al 1997) as well as a single nucleotide substitution (A to G) was found at nucleotide 26 of the untranslated first exon (Hager et al 1997).

Since obesity is characterized by leptin resistance, the leptin receptor gene is a favourable candidate in the pathophysiological mechanisms leading to obesity (Chen et al 1999). The leptin receptor is a member of the class 1 cytokine receptor family and it`s gene has been mapped to chromosome 1p31. There are six isoforms of the receptor which are formed by alternative RNA splicing at the most C-terminal coding exon. The short forms are thought to be responsible for the transport of leptin across the blood-brain barrier, as well as a source of clearance, while the long form is the one which brings about leptin`s actions (Tartaglia 1997).

Any mutation or polymorphism of this receptor would affect the signal transduction and so it is of great importance to identify these mutations and their effect on human adiposity. Even though it is unlikely that a significant proportion of the variation in adiposity in humans will be explained by homozygosity for gross alterations in LepR. However, more subtle and common allelic variants or heterozygosity for loss of function of leptin may determine predisposition to increased adiposity within a permissive environment (Chung et al 1998).

Up to date, a total of 19 polymorphisms have been discovered in the human LepR gene. These include a pentanucleotide insertion/deletion polymorphism in the 3`-untranslated region of the LepR in obese subjects, especially females (Chagnon et al 2000). Two recent studies on obese subjects have reported that heterozygous carriers of the insertion allele showed lower insulin values than the homozygous carriers of the more common deletion allele (Lakka et al 2000). One of these studies was performed on around 122 finnish males, out of the total number of subjects, one was homozygous for the 3`-UTR insertion and 22 were heterozygous. The carriers of the insertion allele had a 79 % reduced risk of diabetes compared with non-carriers. The 3`-UTR insertion seems to be common in the healthy population since the carrier frequency of this allele was as high as 23.5 % amongst the control subjects. In addition, the carriers of the insertion allele had 28.7% lower fasting insulin levels than non-carriers (Lakka et al 2000). These results can be seen in Table 6.1

Table 6.1 Results of a Finnish study performed on the leptin receptor gene pentanucleotide polymorphism

Genotype	ins/ins	ins/del	del/del	

Total no. of subjects (122)	1 (0.89 %)	22 (18.03 %)	99 (81.14 %)	
Controls (81)	0	19 (23.5 %)		
Subjects with diabetes (41)	1	4 (9.8 %)		

The pentanucleotide insertion (CTTTA) in the 3`-untranslated region of the leptin receptor gene is located 60 nucleotides after the stop signal. This insertion causes the formation of a stem loop motif with eight complementary nucleotides forming the stem, and ten nucleotides forming the loop in the mRNA molecule. Stem loop sequences have been found to affect the stability of the mRNA by binding to regulatory proteins which in turn could affect the rate of degradation and/or translation of the mRNA (Oksanen et al 1998).

Table 6.2 shows genotype and allele frequency of the 3`-UTR insertion/deletion polymorphism in obese and lean Finnish subjects.

Table 6.2 Genotype and allele frequency of the pentanucleotide insertion/deletion polymorphism in lean and obese Finnish subjcts

Class	**Morbidly obese (n=249)**	**Lean (138)**	**P**
	Genotype		
del/del (- -)	0.767	0.761	
del/ins (- +)	0.217	0.239	
ins/ins (+ +)	0.16	0.00	0.3
	Alleles		
del (-)	0.876	0.88	
ins (+)	0.124	0.12	0.84

As seen from the Table 6.2, the frequencies of the insertion allele did not differ between obese or lean subjects. Four obese, but none of the lean subjects were homozygous for the insertion allele.

The mean leptin values for the Finnsih females with the different genotypes (- -), (- +) and (+ +) were 47.3, 44.2 and 47.3 ng/ml respectively, while in the males, the corresponding mean values were 22.2, 27.8 and 25 ng/ml respectively. The presence of the 3`-UTR pentanucleotide insertion doesn't imply an increases risk for morbid obesity in Finns. (Oksanen et al 1998).

Other polymorphisms discovered include: four amino acid variants of the coding region, four silent mutations and 9 intronic variants of the leptin receptor gene, as well as a CTTT repeat at intron 16 (Oksanen et al 1998; Chagnon et al 2000). Three of the amino acid variants are quite common, they include (Lys 109 → Arg) in exon 4, (Gln 223 → Arg) in exon 6 and (Lys 656→ Asn) in exon 14. It was observed from several performed studies, that out of these polymorphisms, the Gln 223 → Arg polymorphism had a strong linkage with fat mass and fat free mass, as well as BMI. The carriers of this allele had 4 Kg less fat free mass and higher BMI values when compared to non-carriers. The subjects carrying this polymorphism had increased triglyceride levels as well as low HDL- cholesterol after overfeeding as well as induced hyperinsulinaemia which is associated with alterations in lipoprotein lipase. This polymorphism results in a single amino acid change observed and may eventually change the binding capacity of LepR to leptin due to a change in charge from neutral to positive (Chagnon et al 1999; Chagnon et al 2000). This polymorphism had a recessive mode of inheritance (Yiannakouris et al 2001). Table 6.3, shows the results of a study performed on 118 Greek males and females, 89 were normal weight, while 29 were either overweight or obese. The (Gln 223→ Arg) polymorphism had a higher prevalence in overweight and obese subjects when compared to normal weight subjects (20.7 %, 4.5 %, p = 0.01). Such studies have not been performed in Saudis and may be suggested as a se to this pilot study.

Table 6.3 Genotype and allele frequency of some common polymorphisms in the Greek population

	Females (n=62)		Males (n=56)	
Variables	Mean	SD	Mean	SD
Age (yr)	17.5	1.6	17.8	1.8
BMI (Kg/m^2)	21.3	3.1	23.4	3.8
Leptin (ng/ml)	9.7	6	3.2	2.6
Polymorphisms				
	Lys109Arg	Gln 223 Arg	Lys 656 Asn	
Genotypes				
1/1	0.763	0.44	0.593	
1/2	0.229	0.475	0.339	
1/3	0.008	0.085	0.068	
Alleles				
1	0.877	0.678	0.763	
2	0.123	0.322	0.237	

Some examples of the intronic variants are the variants at position 20 (G→A) and 31 (T→C) of intron 7 and at position 37 (G→A) of intron 17. For the silent mutations there is a G→A mutation at position 2145 (Leu 715) of exon 13 and a G→A mutation at position 3057 (Pro 1090) of exon 18 (Oksanen et al 1998).

Recently, a splice mutation producing a skipping of exon 18, resulting in a truncated receptor lacking both the transmembrane and the intracellular domains of the LepR has been reported in a consanguinous family of Kabilian origin. In this family of nine children, three sisters were homozygous for the mutation which produced an early-onset morbid obesity developing in the first months of life with hyperphagia and hypogonadotropic hypogonadism (Chagnon et al 1999). A similar polymorphism

which occurs due to the skipping of exon 16 was discovered in a French population (Clement et al 1998).

The CTTT repeat together with the (Lys 656 Asn) polymorphism, could be related to a change in the cytoplasmic domain properties of the human LepR (Chagnon et al 1999).

No significant differences in allele frequencies or genotype distributions for the pentanucleotide polymorphism of the leptin receptor gene were observed between lean and obese subjects in the Quebec family studies as well as in other studies of British, Danish, French, American or Japanese populations (Chagnon et al 1999). It was obvious from these studies that different combinations of allelic variants from different genes may predispose to obesity, and the studies also made it clear that the allelic variants show a significant race and/or population component. Listed below, is a table which gives a picture about the frequency of the three most common previously mentioned polymorphisms in different populations (Chung et al 1997).

Table 6.4 Allele frequencies of variant alleles of LepR producing amino acid variations

Allelic	Asian	Black	Caucasian	Hispanic	Lean	Obese

position						
Percentage of subjects	1 %	11.3 %	67.5 %	20.1 %	13.9 %	86.1 %
Lys 109 Arg	25	21.9	21	22.9	26.3	20.2
Gln 223 Arg	50	37.3	33.6	36.6	42.7	32.7
Lys 656 Asn	0	11.4	16.8	14.8	19.5	14.6

Cardiovascular disease is a major cause of morbidity and mortality in both the diabetic and the normal population, so the development of strategies to recognize individuals who are at risk of CVD is valuable for prevention (Haffner 1998).

In humans, the production of leptin has been linked to adiposity, insulin and insulin sensitivity, this was considered when the results of a study Leva et al (1998) were analyzed. Metabolic risk factors for CHD (like elevated blood glucose), hypertension and diabetes mellitus were correlated, Hyperinsulinemia was shown to be an independent predictor of CHD (Leyva et al 1998; Haffner 1998). Leptin was considered a classic risk factor for a future coronary event in a study performed on hypercholesterolemic men (Wallace et al 2001). Another study provided evidence that there is a direct link between leptin and the risk for thrombotic complications, by modulating platelet function (Konstantinides et al 2001).

An interesting finding was that a reduction in leptin levels due to a restriction in calories and exercise in obese men suffering from type 2 diabetes mellitus was directly associated with reductions in serum triglycerides and cholesterol, which suggests a connection between leptin and lipoprotein metabolism that is not only due to weight reduction (Halle et al 1999).

Obesity is associated with supersaturation of bile with cholesterol because of an increased hepatic secretion of this sterol, this increases the risk of the formation of gall stones in obese subjects, and in a study by Mendez-Sanchez et al (2002), a negative correlation between leptin levels and HDL-C was noticed. These lower levels predispose the individual to gall-stone formation due to the accumulation of

cholesterol. The low levels of HDL-C are accompanied by hypertriglyceridemia, and insulin resistance.

The mean of leptin in the normal weight, overweight and obese Saudis was 10.92, 16.87 and 21.56 ng/ml, respectively, (the mean age being 34.13 years, while the mean height has 1.59 m and the mean weight 67.22 Kg in the Saudi population). There was a significant difference in leptin levels between the three weight groups. Leptin levels increased moving from the normal weight group to the obese weight group, and a positive significant correlation was obtained between the leptin levels and BMI in both males and females. This indicates that overall leptin levels increase with an increase in BMI and that most obese persons are insensitive to endogenous leptin production (Considine et al 1996). Comparison of these results with those reported in other populations shows several interesting differences and some similarities. The results of this study are different from the result obtained by Considine et al (1996), when the leptin mean of 136 lean and 139 obese subjects was measured. The leptin mean for the lean and the obese subjects was 3.13 and 7.5 ng/ml respectively. This difference may be due to the difference in the number of subjects studied.

Within the Saudies, Leptin levels were significantly different between lean and overweight Saudi males and females where the females had a significantly higher leptin levels compared to the male counterpart in the same BMI group. These results are in line with results reported in a study by Lucantoni et al (2000).

The leptin mean in the total study population and in males and females was 15.7, 11.95 and 16.4 ng/ml respectively, and the mean of BMI in the total study population and in males and females was 26.59, 25.07 and 26.87 Kg/m^2 respectively. Leptin correlated positively with BMI as seen in another study (Mendez-Sanchez et al 2002). The leptin and BMI means were slightly lower in males compared to females which is explained by the fact that that females tend to have more body fat than males and have a different fat distribution as males, and it is known that hormonal factors and gender differences in adipose tissue distribution play a role and this is because adipose tissue is more active in synthezising leptin than visceral tissue, and adipose tissue is represented more in females than in males. However, adiposity accounts for about 40 % of variability of leptin levels in obese individuals, leaving a considerable proportion of this variability unexplained. (Lucantoni et al 2000). Insulin has been study when a considered as a regulator of the production of leptin. This was reported in a study

when a by the slight elevation in leptin levels due to prolonged insulin infusions was observed (Leyva et al1998).

The insulin mean in the total study population and in the males and females was 14.27, 13.13 and 14.48 μU/ml respectively. The insulin mean was slightly higher in females than males in the Saudi population, this is caused by the fact that some of the females included in this study suffer from type 2 DM, and so were suffering from insulin resistance, where insulin is secreted normally or with even higher levels than in normal individuals but there is a defect in the insulin receptor (Saltiel 2000). In the Saudi population, insulin levels were the highest in obese individuals, which proves the point that obesity is associated with hyperinsulinemia and insulin resistance. Data from several populations suggest strong positive correlations between leptin and insulin concentrations, where insulin resistant subjects had higher leptin concentrations when compared to insulin-sensitive subjects independent of body fat mass, as well as the fact that elevated leptin levels predict subsequent development of obesity or type 2 diabetes in Japanese-Americans (Wallace et al 2001). Since these subjects were obese, they had elevated leptin levels as well. A study reported by O`Rourke et al (2001) on obese subjects showed elevated levels of both leptin and insulin and were found to be with an increased risk of atherosclerosis. The key step in the development of atherosclerosis is the deposition of cholesterol-ester filled macrophage foam cells, which contribute to the formation of the atherosclerotic plaque (O`Rourke et al 2001).

In this study, the cholesterol mean of the total study population and in males and females were measured and about 67 individuals of the total polulation (33.66%) were classified as hypercholesterolaemic and 44 individuals (22.11 %) were borderline and 23 individuals (11.55 %) were at high risk for developing CHD, whereas the prevalence of hypercholesterolaemia in the two sexes was: (29.03%) in the males and (34.52) in the females, about (16.13 %) of the males were borderline and (12.9 %) were at high risk for developing coronary heart disease, while in the females (23.21 %) were borderline and (11.31 %) were at high risk for developing CHD.

There was no significant difference in cholesterol levels between males and females in the Saudi population. The mean of cholesterol was slightly higher in females than in males, this is quite interesting and it can be explained due to the fact that the study population included a few very obese females with quite high cholesterol levels.

Obese Saudi females had higher triglyceride and cholesterol levels than both lean and overweight females, whereas overweight men had higher cholesterol levels than both obese and normal weight men. These results can be explained by the small number of overweight males in this study. Cholesterol and triglyceride levels were higher in the overweight and obese Saudis, which points to a high stimulation of cholesterol biosynthesis in obese individuals (Toplak et al 2000).

Since obesity is often associated with pancreatic β-cell dysfunction caused by accumulating triglycerides in excessive amounts and diabetes, a study (Shimabukuro et al 1997) has revealed that leptin depletes triglycerides in cells which express the OB-R receptor, through a direct mechanism that involves both an increase in FFA oxidation and a decrease in esterification

The triglyceride mean in the total study population as well as in males and females was 1.25, 1.55 and 1.23 mmol/l.

There was a significant difference in triglyceride levels between males and females in the Saudi population. This is an interesting finding, males had a much higher mean than females. So this indicates that these males are most probably obese and have elevated leptin due to leptin resistance.

This is the first report on leptin receptor polymorphism in the Saudi population. The polymorphism studied here is a pentanucleotide (CTTTA) insertion/deletion polymorphism which has been discovered in the 3`-untranslated region of the leptin receptor gene. A previous study performed on Finnish subjects showed that there was no significant difference in the frequency of the insertion allele (+) between obese (0.124) and lean subjects (0.12). No association was noticed between serum leptin or lipid levels with the pentanucleotide genotype in the obese individuals, on the other hand, serum insulin levels were lower in the heterozygous carriers of the insertion allele (- +) than in subjects who were homozygous for the deletion allele (- -). No lean subjects in the Finnish study were homozygous for the insertion allele, (Oksanen et al 1998), other than in this study on obese and lean Saudi individuals, were homozygous lean subjects for the insertion allele were found, this indicates that the pentanucleotide polymorphism (like in Finns) does not imply an increased risk for obesity in the Saudi population.

The mean age in individuals with the different genotypes (- -), (- +) and (+ +) in the Saudi population was 36.59, 30.67 and 35.54 years respectively, whereas, the mean height in individuals with the different genotypes was 67.04, 67.58 and 69.76 Kg

respectively, the mean height of individuals with the different leptin receptor genotypes was 1.62, 1.6 and 1.58 m respectively, while the mean BMI was 25.38,

26.43 and 27.78 Kg/m^2. These means are lower than the one mentioned by (Oksanen et al 1998) and by (Francke et al 1996) and by (Lakka et al 2000). The mean age and BMI for females in the Saudi population were similar to those mentioned in a study on Finnish females with POCS by (Oksanen et al 2000)

In the study on morbidly obese Fins, the leptin mean in males for the different genotypes (- -), (- +) and (+ +) was 22.2, 27.8 and 2 25 ng/ml respectively, in females, the corresponding mean values were 47.3, 44.2 and 47.3 ng/nl respectively. The frequency of the different genotypes and of the alleles in this study on individuals of the Saudi population showed a significant difference between lean and obese Saudis. The frequency of the (+ +) genotype in the obese group (36.66 %) was higher than in the lean group (14.63 %), as well as the frequency of the insertion allele which was higher (0.46) in obese Saudis when compared to the frequency in lean Saudis (0.32). The same difference in the frequencies of the different genotypes and of the insertion allele was noticed between lean and obese males and females of the Saudi population. These results indicate that this polymorphism is not a major marker of obesity in Saudis, for it was found in lean subjects as well, in the Saudi population.

The results obtained from this study indicated that in the Saudi population, the BMI mean seems to increase starting from the (- -) and reaching its highest level at the (+ +) genotype.

The leptin mean values obtained for the different genotypes in males (9.69, 6.03 and 17 ng/ml respectively) and females (15.29, 17.59 and 17.05 ng/ml) of the Saudi population, were a lot lower than the ones obtained in the Finnish study, this difference could be referred to the large number of morbidly obese individuals included in the Finnish study and who`s BMI >40 Kg/m^2.

As in the Finnish study, no significant association was found between lipid levels and the insertion allele in the Saudi population. Females who were heterozygous for the insertion allele had significantly higher cholesterol levels than heterozygous males, whereas males who were homozygous for the deletion allele (- -), had significantly higher triglyceride levels than homozygous females.

In the Saudi population, findings of lower serum insulin levels were noticed in heterozygous carriers of the insertion allele same as in the Finnish study were made.

The insulin mean of subjects who were heterozygous for the insertion allele (- +), were lower when compared to insulin mean of subjects who were homozygous for the deletion allele (- -). This result is noticed for the Saudi population, especially in Saudi females, but interestingly, not in Saudi males, where heterozygous males for the insertion allele (- +) had higher insulin mean when compared to males who were homozygous either for the deletion allele (- -) or the insertion allele (+ +).

The results of a serum insulin-to-glucose ratio test was performed on Finnish subjects who were not taking medication affecting glucose, insulin or lipids indicated that carrier of the insertion had lower values suggesting a lesser degree of insulin resistance (Oksanen et al 1998). A study published by (Francke et al 1997) reported the existence of the pentanucleotide 3`-UTR polymorphism in a French Caucasian obese individuals population, and that females who were heterozygous carriers of the insertion allele showed slightly lower insulin levels 30 minutes after an oral glucose load when compared to those females which were homozygous for the deletion allele. The results obtained from this study also ruled out the presence of a major role of this polymorphism in the development of obesity in the French population (Francke et al 1997). Another report by (Lakka et al 2000) confirms that alterations in the leptin signaling system could contribute to serum insulin levels and the development of type 2 diabetes. A study performed on 65 Japanese NIDDM patients showed that the frequency of the insertion allele (+) was lower than reported in the study by Oksanen et al (1998) and no significant difference were noticed in other measurements between the (- -) and (- +) genotypes (Okazawa et al 1999).

A study performed by (Oksanen et al 2000) on 38 females who had polycystic ovarian syndrome and on 122 controls, supported that variations in the LepR gene locus has an effect on insulin regulation. Lower insulin levels were noted in females who were carriers of the insertion allele when compared to non-carrier females No difference was noticed in the allele frequencies of the different pentanucleotide del/ins genotyped between the affected and the control females..

7.0 Future studies

From the results of this study, it has become clear that several areas of research still require further exploration and clarification.

1) More studies are needed on the relationship between leptin levels and future weight gain.

2) Research is needed to uncover mutations in the promoter and/or intron sequences of the leptin receptor gene, or in other regions flanking the gene.

3) The intracellular molecules that mediate leptin receptor signal transduction are of tremendous interest, and need further studies.

4) Further investigations are required for the identification of genes which are induced by leptin in relevant cell types and their role in body weight regulation .

5) More studies on other polymorphisms in the leptin receptor gene are needed to find out if there is a connection with obesity in the Saudi population.

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9.0 Appendix

Patient registration form

King Saud University

College Of Science

Department Of Biochemistry

Medical and Clinical Information Form

1) General Information About Patient:

Patient No: ____________ Name: _________________________

Age: ____ (years) Nationality: _____________________

Sex : [M] [F]

Married : [Y] [N] [D] [W] No. Of Children: ________

Weight (Kg): ______ Height (m): ________ BMI (Kg/m^2): ________

2) Medical History:

Diabetes: [Y] [N]　　　　Type [IDDM] [NIDDM] [GDM]

Obesity: [Y] [N]　　　　CHD: [Y] [N]

Hypertension: [Y] [N]

Others:

3) Laboratory Analysis:

Parameter	**Value**
- Leptin	
- Cholesterol	
-Triglycerides	
- Insulin	
- Leptin Receptor Gene	