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Complementary Genetic and Functional Analyses of Genes involved in Adiposity

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A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University
for the degree of Doctor of Philosophy

August 2003

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Abstract

Leptin is a 16-kDa protein that is primarily secreted by adipose tissue. It affects body mass regulation by constituting part of the adipostat, that acts to alert the brain of the body's stored energy levels. Additional roles in immune function, reproduction and inflammation are known.

Genetic studies of single nucleotide polymorphisms (SNPs), that exist in the extracellular domain of the leptin receptor gene, were undertaken in a population of European Caucasian postmenopausal women to investigate associations with indicators of adiposity. Homozygosity of the G allele of the LYS109ARG SNP was associated with lower mean fat mass levels and BMI. Furthermore, linkage disequilibrium was detected between this SNP and GLN223ARG, which in previous studies was also associated with indicators of adiposity. No associations were found between the LYS656ASN SNP and the tested phenotypes.

To complement genetic studies of the leptin receptor, cDNA constructs representing different combinations of the alleles for SNPs in the leptin receptor were generated and subsequent expression of protein variants was conducted in COS-7 cells. Using a radioactive ligand-binding assay, labelled leptin was shown to specifically bind to the LYS109ARG223 and GLN223ARG protein variants, thereby testing the effect of the GLN223ARG mutation on LBA. Preliminary data, suggest that the ARG allele appeared to bind less leptin than the GLN.

Genetic studies were carried out on polymorphisms in related candidate genes. A promoter polymorphism (G -2548 A) in the leptin gene was associated with lower mean BMI and leptin levels in a cohort of European Caucasian postmenopausal women.

Individuals who lacked the 2 repeat allele of the variable number tandem repeat (VNTR) polymorphism present in intron two of the interleukin 1 receptor antagonist gene had an association with lower leptin levels but not BMI or fat mass. This suggests a potential feedback and / or cross-talk mechanism between leptin and members of the IL-1 family of cytokines in processes other than adiposity. Immunity and inflammation are processes where the interleukin one receptor antagonist protein has a prominent role and in which the function of leptin is increasingly being investigated, therefore an interaction between the two cytokines may be specific for these conditions.

The TNF alpha (G –308 A) SNP was also investigated but no associations were observed between this SNP and the phenotypes in the postmenopausal cohort.

To investigate the influence of the leptin receptor gene in conditions at the opposite end of the body weight spectrum to obesity, a case-control association study was undertaken to compare allele frequencies of the LYS109ARG, GLN223ARG and LYS656ASN leptin receptor SNPs between anorexic women and controls. No significant differences were observed in allele or haplotype frequencies.

Acknowledgements

I would like to thank Dr. Alex Blakemore for her supervision and guidance throughout my PhD studies.

I would also like to thank Professor Anna Kessling for supporting my studies during my time at the department of Medical and Community Genetics, Imperial College London.

This PhD was funded by Knoll Pharmaceuticals and Sheffield Hallam University.

I would like to thank the colleagues and friends who I have encountered in my 'tale of two cities'. From my time in Sheffield thanks to Bev, Emma, Naomi, Hafid, Mahmoud, Mike, Arun, Rachel and David. From London, Emma (again), Hafid (again), Pippa, Soji, Kieron, Michelle, Diane, Simon, Howard and Una. It has been a pleasure to meet so many nice people during different stages of my PhD.

Thanks too, to Jen and Will for keeping me entertained, fed and watered at opportune moments.

And finally thanks to my parents for all their support (both moral and financial!).

I am indebted for their constant enthusiasm and assistance that have sustained me during the good and trying times of my studies.

Abbreviations

A	Adenine
AGRP	Agouti-related protein
AN	Anorexia nervosa
AP	Alkaline phosphatase
ARG	Arginine
ASN	Asparagine
BMI	Body mass index
bp	base pair
C2	Ca^{2+} binding fold domain
C/EBP	CCAAT/enhancer binding protein
C	Cytosine
cDNA	complementary DNA
CHO	Chinese hamster ovary cells
CK	Cytokine receptor domain
CMV	Cytomegalovirus
CRF	Corticotrophin releasing factor
CPM	Counts per minute
D'	Coefficient of linkage disequilibrium
db	*diabetes*
DEXA	Dual energy x-ray absorptiometry
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Dideoxynucleotide triphosphate
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ERK	Extracellular factor regulated kinases
F3	Fibronectin type III domain
fa	*fatty*
FBS	Fetal bovine serum
G	Guanine
G-CSF	Granulocyte-colony stimulating factor
GLN	Glutamine
HCL	Hydrochloric acid
HEK293	Human embryonic kidney 293 cell line
HUGO	Human genome organisation
HRP	Horse-radish peroxidase
I	Iodine
IL	Interleukin
IL-1ra	Interleukin one receptor antagonist protein
IL1RN	Interleukin one receptor antagonist gene

JAK	Janus kinase
kb	kilobase
kDa	kilodalton
LBA	Leptin binding assay
LEP	human leptin gene
Lep	mouse leptin gene
LEPR	human leptin receptor gene
Lepr	mouse leptin receptor gene
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
LYS	Lysine
MAPK	Mitogen activated protein kinase
$MgCl_2$	Magnesium chloride
mRNA	messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NIH	National Institutes of Health
nm	Nanometre
NPY	Neuropeptide Y
ob	*obese*
Ob-R	Leptin receptor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PRO	Proline
PTP1B	Protein tyrosine phosphatase 1B
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SOCS	Suppresor of cytokine signalling
Sp1	Stimulatory protein 1
SPSS	Statistical package of the social sciences
STAT	Signal transducer and activator of transcription
T	Thymine
Taq	*Thermus Aquaticus*
TBE	Tris-borate EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNF-alpha	Tumour necrosis factor alpha
μCi	MicroCuries
VNTR	Variable number of tandem repeats
YAC	Yeast artificial chromosome

Publications relevant to this thesis

Paper:

SNPs in the leptin receptor gene: studies in anorexia nervosa
Meechan DW, Quinton ND, Brown K, Eastwood H and Blakemore AIF
Pyschiatric Genetics (In press).

Abstracts and poster presentations:

European Congress on obesity, Antwerp, 2000
Meechan DW, Quinton ND, Laird SM, Li TC and Blakemore AIF. 2000, Serum leptin and leptin binding activity in women who suffer recurrent miscarriage during early pregnancy. *International Journal of Obesity* **24**: P198.

American Society of Human Genetics, San Diego, 2001
Meechan DW, Quinton ND, Pieri LF, Brown KMO, Eastwood HE and Blakemore AIF (2001) Analysis of the leptin receptor SNP GLN223ARG in anorexia nervosa. *American Journal of Human Genetics* **69** (4): A2363.

British Society of Human Genetics, York, 2003
Meechan DW, Quinton ND, Eastell R and Blakemore AIF (2003)
Molecular and functional studies of SNPs in the human leptin receptor gene. *Journal of Medical Genetics* (In press).

American Society of Human Genetics, Los Angeles, 2003
Meechan DW, Quinton ND, Eastell R and Blakemore AIF (2003)
Complementary genetic and functional analyses of SNPs in the *LEPR* gene.
American Journal of Human Genetics (In press).

Table of Contents

1.1 Adiposity

Adiposity is the level of fat present in the body. The opposing processes of energy intake and energy expenditure set this level. Precise and definite mechanisms are employed to measure and hence regulate levels of adiposity. Any elements which lead to a dysregulation of this system will affect body composition and health status.

1.1.1 Obesity

Obesity is one of the most common and serious health problems faced by industrialised societies. In essence, it becomes manifest when energy intake (via food) exceeds energy output over a period of time. The epidemic of obesity is most striking in the USA with 63% of men and 55% of women being classified as overweight (Must *et al.*, 1999) and figures are rising.

It has been associated with many diseases such as coronary heart disease, type 2 diabetes mellitus, and osteoarthritis. Although the exact mechanism for the association with these conditions has not been established, the associated life expectancy, quality of life and health cost issues are a relevant and major cause for concern.

Obesity is accompanied by complications such as hypertension and atherosclerosis, which can lead to heart failure. An analysis of the relationship between adiposity and incidence of heart failure was undertaken by Kenchaiah *et al.* (2002) in the Framingham Heart study. In this study, a graded increase in the risk of heart failure with increasing obesity was highlighted among the 5881 participants.

A study of more than 11,000 women carried out in the US showed that measurements of increasing obesity were the dominant predictor of type 2 diabetes (Colditz *et al.*,

1995) although unequivocal medical evidence explaining the relationship is not yet known.

Skeletal systems may be affected by increased weight bearing. Hip and knee osteoarthritis is most common in white Europeans compared to other groups with approximately 7-25% of Caucasian individuals over 55 years of age suffering from the disorder in the hip. Felson and colleagues (1998) identified strong epidemiological evidence that increasing levels of obesity lead to susceptibility of knee osteoarthritis and a more moderate influence on osteoarthritis of the hip.

Fat distribution has different characteristics associated with gender. This is anecdotally observable with men tending to have more abdominal fat ('apple-shape') whereas women tend to have more fat on their hips and buttocks ('pear-shape'). Furthermore, changes in body fat distribution occur in women through reproductive cycling and childbearing (For review see, Legato, 1997). The different distribution of adipose tissue between sexes may have unique health implications.

It is difficult to associate many benefits with obesity. Yet it has been hypothesised that natural selection favoured a thrifty genotype in human history where a lower rate of energy expenditure and / or hyperphagia would lead to increased fat stores during times of food surplus. Body fat reserves would then be advantageous during times of food shortage (Heel, 1962). Unfortunately, in modern western societies where food is plentiful and physical exercise in the general population is on the decline this adaptation would no longer be beneficial resulting in marked obesity.

1.1.2 Underweight

At the other end of the body composition spectrum is the underweight phenotype. Underweight is associated with a variety of health issues including loss of reproductive function, depressed immunological responses and starvation (Hsu, 1980)

One of the most distressing manifestations of underweight is that of anorexia nervosa (AN). Classification criteria include maintenance of bodyweight at 85% or less of normal weight adjusted for age and height, amenorrhoea in female sufferers and an intense fear of weight gain. Indeed, behavioural patterns encompassing perfectionism and obsessionality are common amongst AN sufferers (Bulik *et al.*, 2003). In addition, AN can be further classified into restricted eating and binge/purge sub-types. As being underweight is a criteria for AN, individuals suffer the physiological consequences of starvation, and mortality figures approaching 16% have been recorded after a 20 year follow up study (Crisp *et al.*, 1992).

1.1.3 Measurements of adiposity

There are several methods of measuring indicators of adiposity, which are used in studies, with each method having associated drawbacks and benefits. A frequently used indicator is body mass index (BMI), where the weight of an individual in kilograms is divided by the square of their height in metres. This is a simple measurement to carry out but does not differentiate between fat mass and lean mass (Table 1.1 highlights World Health Organisation weight classifications taken from epidemiological data).

BMI kg/m^2	WHO classification	Popular description
<18.5	Underweight	Thin
18.5-24.9		Healthy, normal
25-29.9	Grade 1 overweight	Overweight
30-39.9	Grade 2 overweight	Obese
>40	Grade 3 overweight	Morbidly obese

Table 1.1: Classification of weight based upon BMI measurements

Another frequently used measurement is skinfold thickness, which can be carried out using callipers at multiple surface sites on the body. Specifically the SF8 value measures the sum of skinfold thickness at eight different points of the body but is unable to measure abdominal or intramuscular fat.

Bio-impedance measurements determine conductance of an electrical impulse through the body based on the principle that lean mass conducts better than fat mass. This is a simple and effective device, but is no more accurate than the previous tests. Variation in measurements can result from the point during the day at which readings are taken and also hydration levels at time of measurement.

Dual energy x-ray absorptiometry (DEXA) measurements afford accurate determination of fat and lean mass but require specialised equipment and handling making them impractical in field measurements.

1.1.4 Heritability of body composition

The role of heritability in the susceptibility to obesity and AN has been examined. Twin studies of adults from industrialised nations have resulted in heritability estimates varying between 50 and 80% using BMI as an indicator of obesity (Stunkard *et al.*, 1986, Allison *et al.*, 1994, and Borecki *et al.*, 1998), with lower values for family studies (For review, Comuzzie and Allison, 1998). Such variation is thought to come from age-specific effects of genes on BMI, as well as gender specific differences (Korkeila *et al.*, 1991). Twin studies in children show the highest heritability, with about 80% for BMI (Pietilainen *et al.*, 1999).

Results of twin studies of eating disorders, including AN, have been less consistent than studies of obesity. Observations of higher concordance rates for AN amongst monozygotic twins versus dizygotic twins (differentiating between shared genes and environment versus shared environment) was not observed by Walters and Kendler, (1995) using a general twin registry. However, Treasure and Holland (1994) found a higher level of AN amongst monozygotic twins versus dizygotic in a more targeted study using twin registries, advertisements and clinical populations. As such, the genetic and environmental contribution to eating disorders requires clarification and new twin studies are in progress.

1.2 The Adipostat

Located in the brain, the hypothalamus is the central mediator of regulation of body composition. To fulfil this role, it is able to detect the level of adiposity via a factor secreted by adipose tissue into the blood stream. After which, this factor is able to interact with the hypothalamus and activate specific downstream signals which moderate food intake and energy expenditure. This concept was historically termed as the adipostat (Kennedy, 1953).

1.2.1 Main players of the Adipostat

There are three distinct pathways that contribute to the adipostat as a whole. Neural mechanisms, that are activated as a result of gut distension following food intake, are undoubtedly involved in restricting further food intake by stimulating the appropriate areas of the brain (Tack *et al.*, 2002). Also gut hormones (for example PYY 3-36), which are released postprandially from the gastrointestinal tract in proportion to the calorie content of the meal, reduce food intake (Batterham *et al.*, 2002).

Thirdly, cytokines that are secreted from adipocytes (fat cells) into the blood stream in direct relation to the body's fat reserves and level of food intake.

Leptin is a 16 kilodalton (kDa) cytokine, which is primarily expressed by adipocytes, which secrete the protein into the blood circulation. When food is scarce, the adipocytes secrete less leptin into the bloodstream. It is also released from the adipocytes (above basal levels) a period of time after food intake.

Leptin is able to cross the blood brain barrier and specifically bind to leptin receptors within the arcuate nucleus region of the hypothalamus of the brain. The hypothalamus

is crucial in maintaining body weight control and the main area where leptin exerts its actions by informing the brain about the status of energy reserves. Hence, it is the blood borne factor, first postulated by Kennedy (1953). There are two specific cell types within the arcuate nucleus region of the hypothalamus that have a high concentration of leptin receptor and to which leptin binds. These cell types are able to express the pro-opiomelanocortin (POMC), agouti-related protein (AGRP) and neuropeptide Y (NPY) hormones. Cells that release NPY (a 36 amino acid peptide) stimulate food intake via the hormone binding to a sub-set of cells called the paraventricular nucleus activating specific downstream signals (Stephens *et al.*, 1995). Stephens and colleagues (1995) demonstrated that administration of recombinant leptin to lean and *ob/ob* mice suppressed food intake by having an inhibitory effect on the amount of NPY released. This was experimentally determined because the area of the arcuate nucleus, that stained positive for an antisense ribonucleotide probe specific for NPY messenger RNA, was decreased after leptin delivery.

Various POMC-derived melanocortin peptides including α-melanocortin stimulating hormone (α-MSH) are able to act on a cell sub-set within the lateral hypothalamic area, inhibiting food intake (Fan *et al.*, 1997, Elias *et al.*, 1999).

At the molecular level, the binding site for α-MSH is the melanocortin 4 receptor (Mc4R). α-MSH behaves as a high affinity agonist, initiating downstream anorexigenic responses to this receptor. Using similar techniques to those that were used on the NPY pathway, leptin binding was shown to have a stimulatory effect on this pathway in rats (Schwartz *et al.*, 1997).

The agouti related protein (AGRP) is a potent antagonist of Mc3R and Mc4R (Ollmann *et al.*, 1997). Levels of this protein are reduced subsequent to leptin

administration and ubiquitous expression of human AGRP in transgenic mice results in obesity. By competing with the same receptor sites as POMC derived hormones, AGRP inhibits the satiety effects of leptin.

Evidence suggests that pathways involving NPY, POMC and AGRP participate in an integrated system within the hypothalamus, contributing to the adipostat mechanism. Considering the combined affects of leptin on this pathway, leptin can be described as a satiety factor in that it reduces the desire to keep eating after food intake (Gibbs *et al.*, 1973) and so plays a key role in regulating the bodies' energy balance. Table 1.2 lists several neurotransmitters that affect feeding behaviour.

Stimulatory	**Inhibitory**
Neuropeptide Y	5-hydroxytryptamine
Orexin	Corticotrophin releasing factor (CRF)
Melanin concentrating hormone	CART
Agouti-related protein	α-melanocyte stimulating hormone
Galanin	Glucagon-like peptide 1

Table 1.2 Neuropeptides that affect feeding behaviours

1.3 Leptin and the Leptin Receptor

1.3.1 Identification and organisation of the *ob* (leptin) gene

There are at least five strains of mice that show an obese phenotype. The autosomal recessive, obese strain of mice (*ob*) identified in the 1950s is associated with obesity and type II diabetes (Ingalls *et al.*, 1950) (see figure 1.1).

Parabiosis is a technique that can be used to investigate such phenotypes by employing surgical techniques to cross-circulate blood between mutant and wild type mice. This procedure demonstrated a deficiency in a blood-borne factor that regulated food intake and metabolism by virtue of the fact that cross-circulation ameliorated the *ob* mouse phenotype (Coleman *et al.*, 1978). However, the blood-borne factor was not actually identified until 1994, when a positional cloning approach to locate and identify the mouse obese gene was undertaken. To achieve this, a genetic and physical map of the region was obtained followed by isolation of the gene and detection of the causative mutation (Zhang *et al.*, 1994). For the positional cloning, a yeast artificial chromosome (YAC) contig was generated spanning a region on mouse chromosome 6 known to contain the gene due to preliminary localisation by RFLP analysis. The critical region containing the gene was further refined, and DNA from the critical region cloned into exon-trapping vectors. Putative exons were screened against corresponding RNA in northern blotting experiments and one particular exon hybridised to a 4.5kb RNA species from white adipose tissue only. This transcript was absent in *ob/ob* mice. Subsequent isolation of cDNA clones (mouse leptin cDNA sequence is available at http://www.ncbi.nlm.nih.gov via the GenBank database, accession number NM008493) revealed a 167 amino acid open reading frame (ORF)

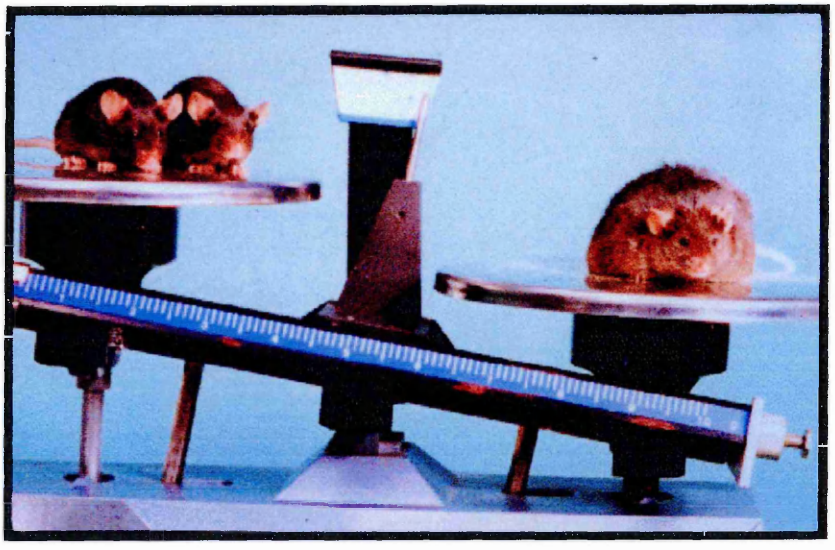


Figure 1.1: Picture of *ob* mouse compared to wild type mice (Picture from front cover of *Nature*, volume 372, issue 6505)

which in *ob/ob* mice contained a C to T base change at codon 105 coding for a premature stop codon. The human genome organisation (HUGO) subsequently named the mouse gene *Lep*. They proceeded to hybridise the mouse *Lep* cDNA to a human adipose tissue cDNA library, isolating homologous clones with 84% amino acid identity in the ORF between the two species (human leptin cDNA accession number NM000230). Leptin protein delivered intraperitoneally to *ob/ob* mice reduced feeding and body weight (Weigle *et al.*, 1995), proving that the gene caused the *ob* phenotype. Following the breakthrough of locating the gene, He and colleagues (1995) determined its genomic organisation and promoter activity. The gene comprised a maximum of 4 exons (a minority of the *Lep* mRNA accommodated an extra untranslated exon) with the coding sequence present in exons 2 and 3 (see figure 1.2). Using successive deletion mutants of the promoter sequence (cloned into a luciferase reporter vector to analyse changes in promoter activity), the 762-bp promoter was shown to have a minimal promoter region of 161bp. The minimal promoter contained Sp1 and CCAAT/enhancer-binding protein (C/EBP) motifs.

The human homologue (named by HUGO as *LEP*) spanned 20kb, with 3 exons separated by 2 introns (10.6kb and 2.3kb respectively), and the promoter region contained a TATA box-like sequence and several *cis*-acting regulatory elements including C/EBP motifs but also an AP-2 binding site and GC boxes (Isse *et al.*, 1995). Moreover, Isse and colleagues (1995) assigned the chromosomal position of the human *LEP* gene by fluorescence in situ hybridisation using probes specific for the ob gene, these hybridised at position 7q31.3. This agreed with comparative mapping of mouse chromosome 6, containing the *LEP* gene, to a syntenic portion on human chromosome 7q that was later refined to position 7q31.3 by genetic mapping (Green *et al.*, 1995).

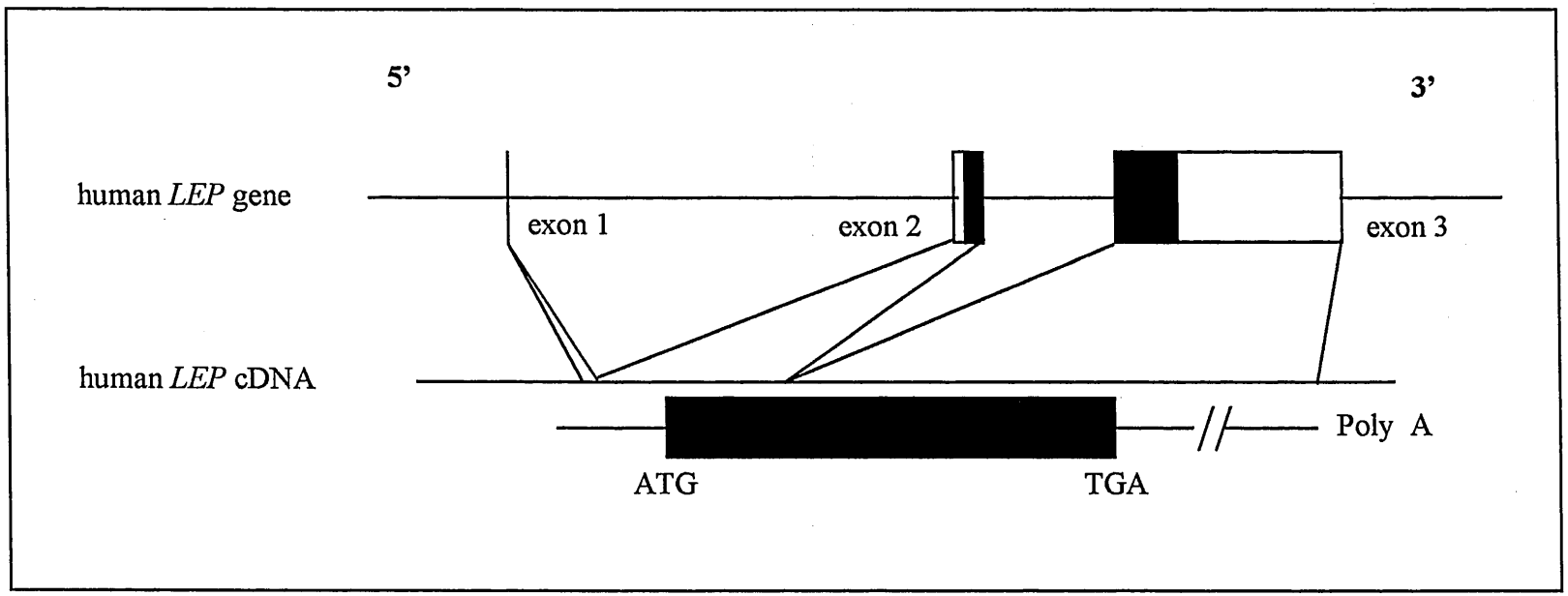


Figure 1.2: Schematic representation of the human *LEP* gene and cDNA. The coding region of the human *LEP* gene is indicated by shaded boxes. The translation start (ATG) and stop (TGA) codons are included (diagram adapted from Isse *et al.*, 1995).

1.3.2 Leptin protein structure

Leptin is a 16-kDa protein expressed and secreted by the adipose tissue. Using X-ray crystallography, Zhang *et al.*, (1997) determined the structure of the protein (see figure 1.3). For this, they took advantage of an introduced glutamine to tryptophan amino acid substitution at codon 100 that dramatically improved the ability of the protein to crystallise whilst still maintaining its biological activity. Cysteine residues at codons 96 and 146 were shown to form a disulphide bond and mutation of either rendered the protein biologically inactive. This indicates that disulphide formation is important for structure folding and receptor binding. An overall four-helix bundle structure was revealed that is shared with members of the long-chain helical cytokine family such as G-CSF, LIF and IL-6. These structures consist of four antiparallel alpha-helices arranged in a twisted helical bundle forming a hydrophobic cylindrical core. The similarity of these ligands and the sequence similarity of their receptors are suggestive of similar mechanisms of ligand receptor interaction.

1.3.3 Identification and organisation of the leptin receptor gene

Upon discovery of the leptin gene, the tools became available to locate its native receptor. Tartaglia and colleagues (1995) generated a series of leptin-alkaline phosphatase (AP) fusion proteins and initiated a binding survey of cell lines and tissues. Leptin binding sites were located to the mouse choroid plexus. Using this piece of information, a cDNA expression library was constructed from the tissue, screened with a leptin-AP fusion protein and a leptin receptor gene identified

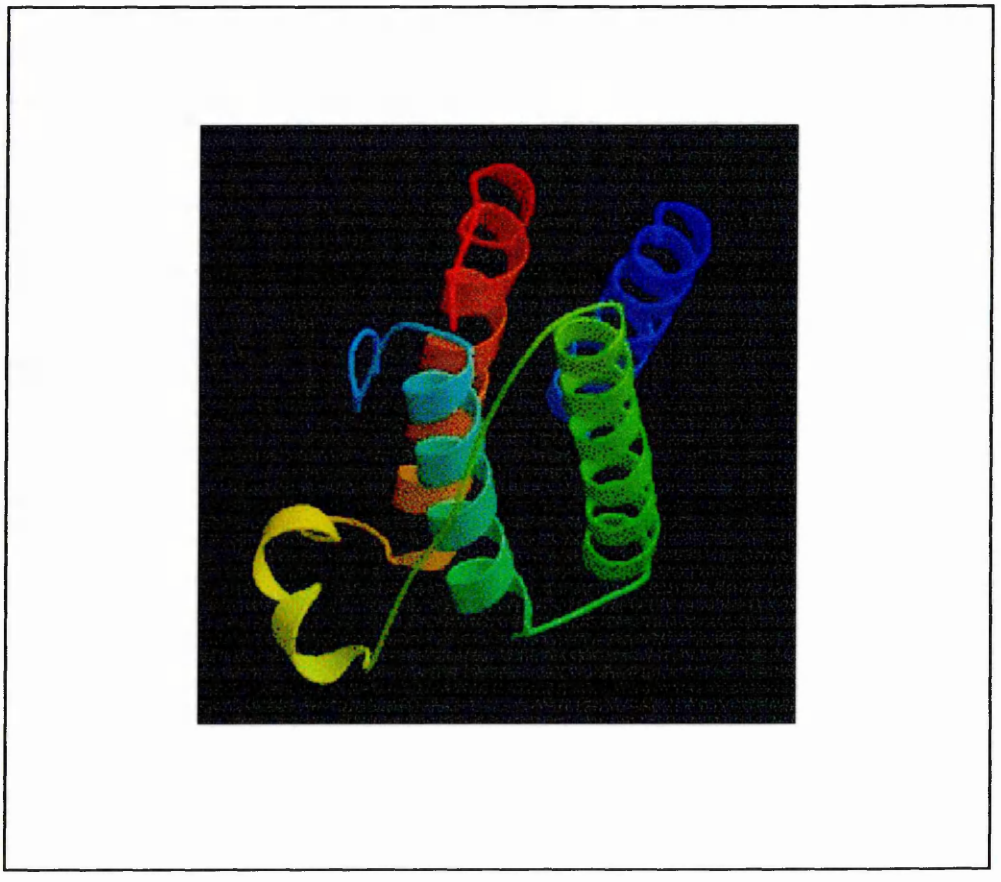


Figure 1.3: Three dimensional model of the human leptin protein structure containing four anti-parallel alpha helices (red, blue, green and turquoise) which form a barrel structure with a hydrophobic core (Picture derived from Zhang *et al.*, 1997).

(mouse leptin receptor accession number NM146146 and classified as *Lepr* by HUGO). By hybridising the mouse cDNA to a human cDNA library, a human homologue was found (accession number NM002303 and named *LEPR* by HUGO). It was syntenically mapped, in relation to the *db* locus in mice, to chromosome 1p.

In an effort to identify the gene responsible for the mouse *db/db* phenotype (which exhibits obesity, hyperphagia and insulin insensitivity), Lee and colleagues (1996) generated refined physical and genetic maps at the *db* locus.

Furthermore, they utilised exon trapping and cDNA selection from mouse hypothalamus to identify candidate genes from the *db* region. Several cDNA clones contained sequence identical to the extracellular domain *LEPR* sequence. Some of these clones varied in their C-terminal sequences beyond the point of the transmembrane domain suggestive of splice variants coding for varying length cytoplasmic tails and were designated Ob-Ra to Ob-Re. The study identified that RT-PCR products from hypothalamic RNA in *db/db* mice had a longer fragment length of Ob-Rb whilst sequencing of these products indicated a 106-bp insertion between a splice donor site at codon 889 and acceptor site at codon 890. A G→T mutation 108-bp downstream from codon 890 was shown to result in the creation of a splice donor site enabling incorporation of the 106-bp sequence. This inserted sequence coded for a termination codon five amino acids after the splice junction and so the mutant protein lacked most of the cytoplasmic region in *db/db* mice. This indicated a lack of downstream signalling following leptin binding and provides an explanation for the obese phenotype seen in *db/db* mice.

The Zucker (*fa*) rat is another rodent model of obesity that has a mutation located in the leptin receptor gene. Chua and colleagues determined that the gene responsible for

the *fa* rat phenotype was the same gene responsible for the *db* mouse by a combination of genetic mapping, and genomic Southern blot analysis of *fa* and *db* rodent DNA with a mouse extracellular domain leptin receptor cDNA probe.

The *fa* rat was subsequently shown to be homozygous for an A to C DNA base missense mutation that introduces a glutamine to proline amino acid substitution at codon 269 (Philips *et al.*, 1996, White *et al.*, 1997).

In humans, Chung and colleagues (1996) developed a refined genetic map of human chromosome 1p in the region of *LEPR* by radiation hybrid mapping. Using a panel of individuals from a family, 18 polymorphic markers were genetically mapped to chromosome 1p31 relative to *LEPR* with the aim of identifying new markers for the analysis of human pedigrees segregating with obesity.

1.3.4 Leptin receptor protein structure

The murine leptin receptor was classified as a single membrane-spanning receptor. A mature extracellular domain of 816 amino acids (minus a putative 22 amino acid signal sequence for peptide secretion) was determined from the cDNA sequence followed by a predicted 23 amino acid transmembrane domain (Tartaglia *et al.*, 1995).

The amino acid sequence is most closely related to the gp130 signal-transducing component of the IL-6, receptor with conservation of the characteristic cysteine and Trp-Ser-X-Trp-Ser motifs (Tartaglia *et al.*, 1995). Sequence comparison places the receptor within the class I cytokine receptor family together with the IL-6 receptor and growth hormone receptor (GH-R). The extracellular regions of these receptors are characterised by the presence of multiple domains including the cytokine receptor (CK), Ca^{2+} binding fold (C2) and fibronectin type III (F3) domains (see figure 1.4).

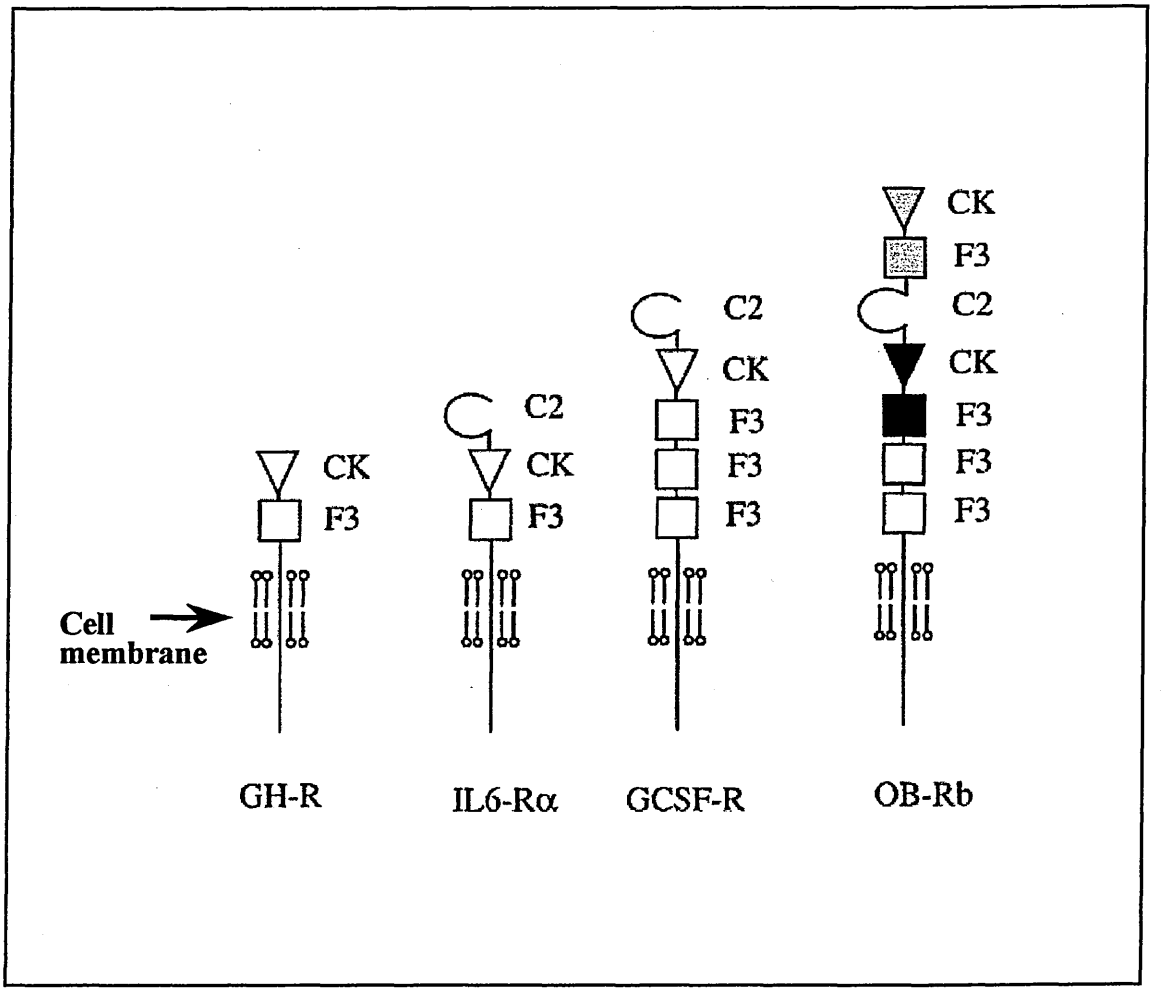


Figure 1.4: Comparison of extracellular domain of human leptin receptor protein with other members of the class I cytokine family (CK = cytokine homology domain, F3 = fibronectin type 3 domain and C2= ca^{2+} binding fold domain). Picture derived from Fong *et al.*, (1998).

High-resolution structures for GH-R provide good evidence for a ligand-binding region within a combined CK-F3 domain (Wells and de Vos, 1996). Although the crystal structure for the leptin receptor has not been determined, the extracellular domain contains two repeat CK-F3 domains highlighting potential ligand binding sites. Using COS-7 cells, Liu *et al.* (1997) expressed and characterised a soluble human leptin receptor containing the extracellular domain only. Immunoprecipitation of the receptor revealed a product with a molecular mass of approximately 130-150 kDa, which is higher than the 92-kDa protein predicted from the coding sequence alone. This suggests a high level of post-translational modification. Indeed, Haniu *et al,* (1998) purified a human leptin receptor from Chinese hamster cells and detected *N*-glycosylation approximating 36% of the total protein mass by digesting the receptor with the enzyme *N*-glycanase. Alternatively O-glycosylation, as detected by *O*-glycanase treatment was minimal.

In humans and mice, there are several isoforms of the leptin receptor with identical extracellular domains and varying length intracellular domains. In humans, differentially spliced transcripts have been observed with one 'long' and three variant isoforms of the receptor containing the complete extracellular domain and transmembrane domain, but varying sizes of the intracellular domain (Barr *et al.,* 1999). The different forms of the leptin receptor are termed as ObRa through to ObRe. The longest form (ObRb) contains a 303 amino acid tail, including two 12 amino acid JAK box domains that are required for JAK dependent signalling. The three variant isoforms have much shorter intracellular tails of 34 (ObRa), 44 (ObR**c**) and 96 (ObR**d**) amino acids each and only contain one JAK box domain. The fifth isoform is the soluble isoform, which is the extracellular domain alone (Lee *et al.,* 1996). Unlike mice, humans do not have mRNA coding for the soluble form, instead it is generated by proteolytic cleavage of

membrane-anchored leptin receptors by a metalloprotease (Maamra *et al.*, 2001). It is the only isoform, which is present systemically. In the blood it is able to bind to leptin and may play a role in modulating receptor signalling by competing for ligand binding and it may also reduce leptin degradation and clearance in serum (Huang *et al.*, 2001).

It has been stoichometrically demonstrated that leptin binds to its receptor in a 2:2 ratio (Devos *et al.*, 1997), i.e. two leptin molecules bind to two receptor molecules. This suggests a similar binding mode to that of granulocyte-colony stimulating factor (G-CSF) with its receptor (also a member of the class 1 cytokine receptor family). Hiroike and colleagues (2000) used the tertiary structure of G-CSF to build a computer model of the complexed leptin: leptin receptor structure in order to determine aspects of ligand binding. The model demonstrated a major and minor interface for both 1:1 leptin: leptin receptor complexes. The model also showed that site-directed mutations incorporated into the leptin protein (Verploegen *et al.*, 1997, Imagawa *et al.*, 1998, Boganet *et al.*, 1998) which were present within these interfaces led to reduced binding activity. These experimental and modelling results could therefore contribute to a rational drug design.

1.3.5 Leptin receptor signalling

Belonging to the class I cytokine receptor family, the leptin receptor lacks intrinsic tyrosine kinase activity. As such, it requires the recruitment and activation of receptor-associated kinases to initiate signal transduction pathways by phosphorylating tyrosine residues present in the receptors' cytoplasmic domain. Of the several isoforms, the long and short membrane-bound forms are the most predominant in tissues and are conserved between humans and mice, with the long form but not the short form having signalling capacity for most signalling pathways. To initiate specific down-stream

signalling, ligand binding to the extracellular domain is necessary. Yet it is interesting to note that several papers (Devos *et al*, 1997, White *et al.*, 1999) indicate a ligand-independent ability of the receptors to homo-oligomerise. Specifically, White and colleagues demonstrated that pre-formed leptin receptor complexes were formed following receptor immunoprecipitation from human embryonic kidney (HEK) 293 cells transfected with vectors encoding the long form of the protein. Ligand treatment of these cells minimally altered levels of oligomerisation. This would suggest that ligand binding is not essential for receptor oligomerisation but would presumably be required for alteration of signalling capacity. More recently, Couturier and colleagues (2003) used bioluminescence resonance energy transfer techniques to show that leptin receptors exist as constitutive dimers but that leptin induces conformational changes in the pre-existing receptor dimers.

A signalling pathway that the leptin receptor is able to activate is the JAK/STAT pathway. Leptin binding leads to a conformational change in the intracellular domain of the receptor enabling juxtaposition of JAK proteins, which phosphorylate each other and specific tyrosine residues of the receptor. Then, signal transducer and activator of transcription proteins (STATs) interact with the phosphorylated residues, are activated, forming hetero- or homodimers and translocate to the nucleus where they modulate transcription of target genes including *c-fos* and *c-jun*.

Both the *fa/fa* rat and *db/db* mouse have mutations that affect JAK/STAT signalling and analysis of these models stimulated investigation of the pathways involved. The mutation in *db/db* mice dramatically reduces expression of the long form of the receptor. Using electrophoretic mobility shift assays (EMSA), in which STAT proteins bind to specific DNA sites and exhibit reduced mobility on PAGE gels, Ghilardi and

colleagues (1996) showed that STAT 3, 5 and 6 were activated in normal mice but not in the case of *db/db* mice.

Expression vectors containing the leptin receptor with the *fa* point mutation in the extracellular domain, were able to constitutively activate transcription via STAT 1 and 3 independently of ligand binding (White *et al.*, 1997). STAT5B was not, however, constitutively activated and ligand-induced STAT5B activation was greatly reduced compared to the wild type receptor. Taken as a whole, these results suggested that the mutation induces a conformational change that partially mimics the ligand-bound state, enabling ligand-independent activation of STAT1 and 3 but not STAT5B.

The long form of the receptor contains the largest number of sequence elements that recruit JAKs. Having two conserved box motifs (which specifically recruit JAK proteins) compared to only one for the other isoforms (Baumann *et al.*, 1996, Ghilardi *et al.*, 1997).

Using site-directed and deletion mutagenesis strategies towards different isoforms of the murine leptin receptor, both Kloek and colleagues (2002) and Bahrenberg and colleagues (2002) reported that loss of the box 1 domain and immediate surrounding residues terminated JAK2 phosphorylation. Although the shorter forms of the receptor contain the box 1 domain, they lack these critical surrounding residues and fail to signal via the JAK2 kinase family.

In vitro studies have shown that activated JAKs, phosphorylate tyrosine residue -1138 on the long form of the murine receptor enabling recruitment of STAT3, and tyrosine residue 985 which recruits the SH2 domain containing protein, SHP-2 (Li *et al.*, 1999). Banks *et al* (2000) presented data suggesting that SHP-2 controls activation of extracellular factor-regulated kinases (ERKs), a set of serine/threonine kinases involved in regulation of gene transcription and cellular physiology. The methods used

to measure this relied upon detection of phosphorylated proteins, in this instance from the ERK pathway, whilst specific accumulation of mRNA transcribed from the c-*fos* gene was detected as well. Interestingly, in the same study mRNA accumulation of suppressor of cytokine signalling 3 (SOCS3) mRNA was detected after STAT3 activation suggesting that at least one function of STAT3 activation is to inhibit signalling by increasing levels of SOCS3. SOCS proteins have been shown to inhibit cytokine signalling. They contain a central SH2 domain that may inhibit signalling by binding to phosphorylated JAK proteins and/or by occupying phosphorylated residues on the receptor and indeed SOCS3 is recruited by the tyrosine residue at position 985 (Banks *et al.*, 2000, Bjorbaek *et al.*, 2000).

In vivo and *in vitro* studies in mice have shown that following leptin treatment, the tyrosine phosphatase, protein tyrosine phosphatase 1B (PTP1B) attenuates signalling by dephosphorylating JAK2. This subsequently blocks STAT3 phosphorylation and STAT3 induction of gene transcription (Cheng *et al.*, 2002, Kaszubska *et al.*, 2002, Zabolotny *et al.*, 2002). By reducing the expression of genes that are up regulated by leptin indicates that PTP1B negatively regulates leptin signalling. Inhibitors of PTP1B may therefore reduce leptin resistance, making it a potential target for drug therapies against obesity.

Chinese hamster ovary (CHO) cell lines expressing the short or long form of the rat leptin receptor were shown to have enhanced levels of tyrosine phosphorylation of STAT3, in long form expressing cells only, and mitogen-activated protein kinase (MAPK), in long and short form expressing cells. The data suggests that the short form is actually able to transmit signals via the MAPK pathway (Yamashita *et al.*, 1998). MAPKs are known to be stimulated by several growth factors and in turn

phosphorylate many transcription factors and cytoplasmic proteins involved in a number of cell functions.

The involvement of leptin signalling in biological processes other than weight regulation has become apparent. Recent studies in mice by Bates and colleagues (2003) demonstrated that substitution of an intracellular tyrosine residue, which is required for STAT3 signalling led to targeted disruption of STAT3 signalling in the long form of the receptor. The mice were obese but fertility was maintained. This result provided evidence for different biological effects resulting from different signalling pathways.

Such insights into the regulation and types of leptin receptor signalling will undoubtedly be explored further as studies continue and could highlight any potential dysregulation involved in various conditions (see figure 1.5 for overview of leptin receptor signalling).

1.3.6 Leptin and leptin receptor levels in humans

Leptin is expressed and secreted by adipocytes into the blood stream. A circadian rhythm (Sinha *et al.*, 1996) for leptin has been observed with circulating levels peaking at midnight and the early hours that could help to suppress appetite during the night.

Gender is another factor which affects leptin levels, with women having higher levels for a given fat mass compared to men (Schwartz *et al.*, 1996). This difference is likely to be influenced by sex hormone variation between the sexes and indeed a reduction in leptin levels is observed after the menopause (Rosenbaum *et al.*, 1996) possibly reflecting the reduction of female sex hormones. Gender specific differences in leptin mRNA expression have also been observed, with females having a higher ratio of subcutaneous to intra-abdominal leptin mRNA expression (Montague *et al.*, 1997a).

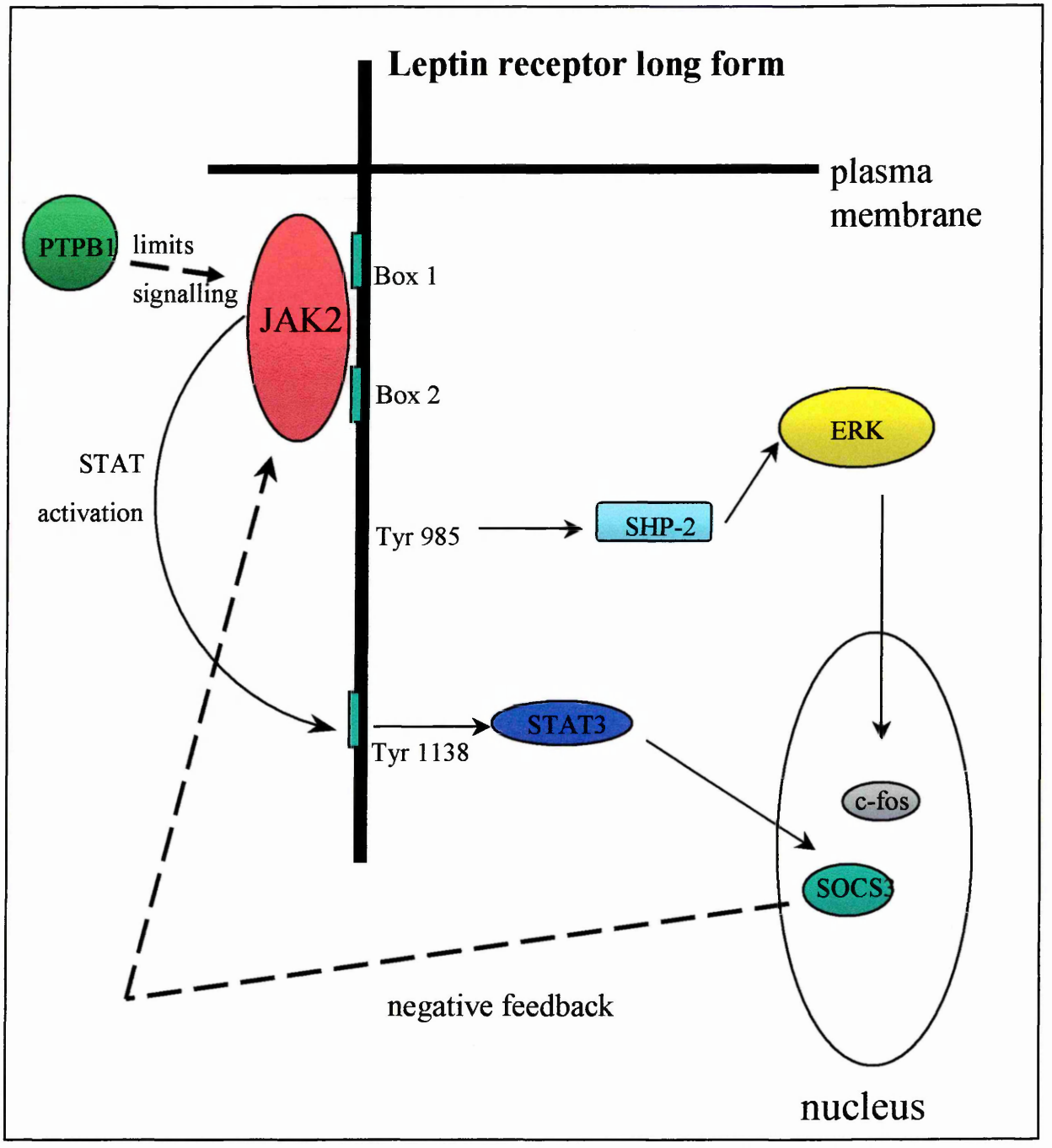


Figure 1.5: Schematic representation of model of signalling for the long form of the leptin receptor containing the two conserved box motifs required for interaction with JAK2. Upon ligand stimulation, JAK2 phosphorylates tyrosine residues on the receptor. These residues recruit STAT 3, which is tyrosine phosphorylated by JAK2, whereupon it translocates to the nucleus to mediate transcription of *socs3, c-jun* and other genes. SOCS3 protein ultimately feeds back by inhibiting JAK2 and binding to the phosphorylated tyrosine residue at position 985.

Leptin levels increase proportionally with increasing BMI and percentage body fat (Considine *et al.*, 1996). Basal levels of leptin are markedly increased in obese individuals. This state of 'leptin resistance' is characteristic of obesity and suggestive of a lack of signalling response despite the high levels of leptin. Brain perfusion studies in mice have established that leptin transport into the brain is a saturable mechanism (Banks *et al.*, 2000, Maness *et al.*, 2000). At high serum leptin levels, saturation of the transport mechanism may impart a rate limiting effect which may limit the cytokines satiety effects in the brain contributing to leptin resistance (Banks, 2003).

Soluble leptin receptor levels, as detected by enzyme linked immunosorbent assay (ELISA), are inversely correlated with percentage body fat (Ogier *et al.*, 2002, Van Dielen *et al.*, 2002) with soluble receptor levels being lower in obese and over-weight individuals. Individuals in Ogier's study undertook a three-month low calorie diet and an increase of soluble receptor levels with concurrent loss of fat mass were detected. One possible explanation of these results that requires investigation is that higher circulating levels of leptin down-regulate soluble leptin receptor release whilst high receptor levels may enhance leptin action in lean subjects.

Interestingly, in the same study an increased soluble receptor level was seen in men when compared to women, but not only in the obese state. On the other hand, Quinton *et al.* (1999) noticed no sex difference in leptin binding activity as determined by a radioactive leptin-binding assay of serum samples from a sample in which a range of BMI was represented. It is possible that the combination of lean and overweight individuals in this study design may explain the discrepancy with the results obtained by Ogier and colleagues.

1.3.7 Other activities of the leptin system

Leptin has been shown to be expressed from a range of tissues other than adipose tissue. These include the stomach (Breidert *et al.*, 1999), skeletal muscle (Wang *et al.*, 1998) and umbilical cord (Akerman *et al.*, 2002). As a result, research has opened up into the peripheral activities of leptin distinct from its role in the central regulation of appetite.

1.3.8 Reproduction

A major focus area of research aside from weight regulation is the involvement of leptin in reproductive function. An indication of this comes from the observation that leptin deficient *ob/ob* mice are infertile. Administration of exogenous leptin to the hypothalamus, causes maturation of the reproductive system and restoration of fertility (Weigle *et al.*, 1996). In humans, expression of leptin and the leptin receptor has been detected in the ovaries (Loffler *et al.*, 2001) and placenta (Hauguel-de Mouzon, 2003). It is suggested that leptin's ability to regulate the female's level of adiposity is important within the context of attaining long-term energy stores which are essential for reproduction. The availability of nutrients for fetal growth and development require substantial energy stores and indeed women who have BMI levels below a certain level will tend to be infertile. Furthermore, the onset of puberty is linked to fat storage, with pioneering studies by Kennedy and Mitra (1969) proposing that signals from fat were responsible for hypothalamic regulation of ovarian activity. In the *ob* mouse model, progression to puberty was rescued by administration of exogenous leptin. In humans with mutations in the leptin and leptin receptor, failure to progress through puberty was also observed (Strobel *et al.*, 1998, Clement *et al.*, 1998). Quinton and colleagues (1999) determined that leptin binding activity in serum was high in pre-pubertal years

in both boys and girls and fell during puberty. They postulated that the fall in leptin binding activity reflected a reduction in soluble leptin receptor (which is able to bind to leptin in the circulation) enabling more leptin to be available to the full-length leptin receptor causing the biological signal of leptin to be transmitted.

1.3.9 Leptin and the cytokine network

As has been demonstrated with many cytokines, leptin is able to interact within the cytokine network in several different situations in an overlapping and complementary manner.

For example, Santos-Alvarez *et al* (1999) reported that by increasing production of the cytokines, IL-2 and interferon gamma (IFN-γ), leptin enhances the activation and proliferation of circulating T lymphocytes in humans. Considering that the *db/db* mouse has reduced levels of T and B lymphocytes (Bennett *et al.,* 1996), these results suggest that by regulating other cytokines, leptin could have an important role in lymphopoiesis and is necessary for the proinflammatory immune response.

Another piece of evidence suggestive of a role for leptin in the immune response was recognised when administration of the endotoxin, lipopolysaccharide (LPS) in hamsters up- regulated leptin gene expression in adipose tissue and protein serum levels (Grunfeld *et al.,* 1996). These compounds can potently induce cytokine production *in vivo,* with leptin induction apparently mediated via release of IL-1 and TNF-α. (Sarraf *et al.,* 1997). Indeed, LPS fails to increase leptin levels in IL-1β knockout mice (Faggioni *et al.,* 1998) whilst *ob* mice exhibit attenuated levels of serum TNF-α and IL-6 in response to LPS.

The results of these studies suggest that leptin could interact with other cytokines to modulate responses to infection and inflammation which are associated with reduced food intake and weight loss.

Further evidence of the specific interaction of leptin with IL-1 has been seen in rats, Luheshi and colleagues (1999) observed that injecting leptin into the brains of normal rats increased IL-1β levels in the hypothalamus and, the central injection of IL-1 receptor antagonist (IL-1ra) inhibited the satiety action of leptin.

Leptin has been shown to induce the secretion of IL-1ra in human monocytes (Gabay *et al.*, 2001). Recently, the molecular mechanism of activation of the IL-1ra promoter via the long form of the leptin receptor was discovered (Dreyer *et al.*, 2003). It involves signalling through a MAPK pathway with binding of p42/44 MAPK protein and an uncharacterised complex of transcription factors to a nuclear factor κB/PU.1 site in the IL-1ra promoter region. Considering that MAPK pathways are usually proinflammatory, an overall inflammatory action of leptin has been postulated which is in keeping with the clinical observation of local inflammatory reactions after subcutaneous administration of leptin in humans (Heymsfield *et al.*, 1999).

1.4 Genetic Concepts and Methods

Although the genetic material is very alike in humans, throughout the human genome there are numerous variations at specific positions between individuals.

These sites of variation, or polymorphisms, are present in duplicate because the human genome is comprised of pairs of matched chromosomes. Each copy (allele) is inherited from an individual's parents and passed on to their offspring.

As a generalisation, DNA variants present in >1% of the population are termed polymorphisms. A particularly deleterious DNA variation is usually present in less than 1% of the population. However, as common polymorphisms can contribute to disease, the delineation between the two is becoming less clear. Genetic studies of diseases such as obesity/anorexia involve the analysis of such DNA variations, used as genetic markers.

1.4.1 Single Nucleotide Polymorphisms (SNPs): a type of genetic variation

SNPs are the most common form of DNA variation in the human genome occurring on average once every 0.3-1 kilobase of DNA (Schork *et al.*, 2000).

This form of DNA variation involves variation in a single base. Substitutions can occur within coding DNA sequence and, as such, may result in a substitution of one amino acid for another. If this is the case, the substitution may be conservative, whereby one amino acid is substituted for another amino acid with similar charge and size properties. Alternatively, a non-conservative change may take place involving the incorporation of an amino acid with different properties which may alter the protein's conformation and, perhaps, activity. A nonsense SNP substitution will code for one of the three stop codons resulting in the formation of a truncated protein. A 'silent' change may also occur whereby the substitution maintains the same amino acid. SNPs that are present in the third base of a triplet codon are an example of a silent polymorphism as any one amino acid can be coded for by different triplet codons, each one varying in the third base only. This degeneracy of the genetic code was described by Crick (Crick, 1966). SNPs can reside in non-coding sequences and therefore may have a minimal functional effect unless they are situated in conserved regions that influence gene

expression, such as the promoter sequences, or at splice recognition sites which are required for correct splicing of RNA transcription products.

The base change at any one SNP may generate or abolish a restriction site for a class II endonuclease enzyme. If so, then DNA amplified over this region can be conveniently digested using the appropriate enzyme, the products subjected to electrophoresis and individuals typed for the particular SNP. Recent advances in technology, including real-time PCR and denaturing high performance liquid chromatography have increased automation and sample throughput. The high frequency of SNPs, rapid screening characteristics and the potential to have a functional effect on gene expression or on the protein itself, makes SNPs desirable to study. This is because there are enough of them in and around genes to represent candidate polymorphisms (individually or in unison) some of which may influence a disease/disease trait. Such advantages coupled with the ease of typing for the SNP in individuals and the relative stability of SNPs over generations has led to international efforts to generate maps of SNPs spanning the human genome, one example being the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP).

1.4.2 Linkage disequilibrium

A phenomenon observed through studies of genetic variability is that of linkage disequilibrium. In a population, it has been described as the co-occurrence of specific alleles from more than one marker at a higher frequency than would be predicted by random chance (Genetics Home Reference database at the U.S. National Library of Medicine website, http://ghr.nlm.nih.gov/ghr//page/Home). This may be because the alleles are physically close so that the chance of being disrupted by recombination at meiosis is low and they are inherited together over successive generations. These

are inherited together over successive generations. These combinations of alleles are described as haplotypes, and changes in frequency at one allele may reflect / cause changes in the others. Therefore, the knowledge of whether a cluster of SNPs is in linkage disequilibrium is beneficial for two reasons, firstly, as analysis of any one SNP may represent the functional effects of a neighbouring SNP or secondly, a *combination* of SNPs represented by their alleles in a conserved haplotype may be contributing to a disease state.

1.4.3 Linkage analysis

Linkage studies have been classically used to analyse the co-segregation of DNA markers with a particular monogenic disease in family pedigrees. This methodology, in its simplest form involves studying pairs of affected siblings, but larger multiplex families are also used.

Unlike this qualitative classification which is based on the presence or absence of a disease, many diseases can be defined by an underlying quantitative trait, such as percentage body fat or BMI in obesity. This approach is commonly used when linkage studies are applied to multifactorial diseases, which may result from the combined effects of more than one gene and where patterns of hereditary are usually more complex than those of single-gene disorders. These traits provide a measure of disease severity and the trait itself may be affected by a smaller number of factors compared to the variety of genetic and environmental factors that lead to the presence or absence of the disease itself. Such features can enable the quantitative variable to have a stronger correlation with variants of one particular gene amongst the several genes which contribute to the disorder, improving the identification of causative susceptibility variants.

contribute to the disorder, improving the identification of causative susceptibility variants.

1.4.4 Association studies

The comparison of allele and genotype distribution between patients and controls is a strategy for the detection of disease susceptibility genes. Any observed difference in frequencies between the two groups may arise from a functional effect of the allele under investigation. Alternatively, the allele may be in linkage disequilibrium with a nearby susceptibility locus. The original SNP may therefore be a marker of susceptibility to a disease without having a causal role.

Such case control studies require careful matching. For example, maintaining the same ethnicity between cases and controls is important because different ethnic populations may have different frequencies for a particular allele, which could be mis-construed as a significant difference in an association study.

The confounding effect of population stratification on association studies is also a concern. In this scenario, a population is assumed to be homogenous with respect to allele frequencies for a particular marker but in reality is composed of several subpopulations each with different allele frequencies. If the subpopulations have different risks of disease then an association between the candidate gene and disease may be spuriously assigned.

Another consideration is cohort size as the greater its size, the more statistically significant the results will be. This may be of importance if the frequency of the variant allele, which is hypothesised to make individuals susceptible to a disease, is low (Schork, 2002).

1.4.5 Transmission disequilibrium test

This test detects association in the presence of linkage. It involves a statistical comparison between candidate susceptibility alleles transmitted or not transmitted to affected offspring from parents who are heterozygous for the DNA variant being studied. These studies circumvent the issue of needing to find carefully matched controls as analysis is based within a family.

1.5 Genetics of Leptin and the Leptin Receptor in Humans

Following the discovery of the leptin and leptin receptor genes and their involvement in obesity deduced from rodent models, these genes became obvious candidates for the condition in humans.

In obese humans, very few mutations have been discovered which affect obesity.

A rare example is described by Montague and colleagues (1997b) who demonstrated severe early-onset obesity in two children, from a highly consanguineous family of Pakistani origin, caused by a frameshift mutation at codon 133 in the *LEP* gene. This resulted in a mutant truncated leptin protein with an aberrant C-terminus, that was not secreted normally from cells (Rau *et al.*, 1999).

Another study by Clement and colleagues (1998) uncovered a homozygous mutation at a splice site, which causes deletion of exon 18 from the mature mRNA, forming a truncated leptin receptor lacking the transmembrane and intracellular domain. This results in morbid obesity for the affected individuals from a family of Kabilian origin.

Such null mutations that lead to morbid obesity have rarely been found in humans. This led researchers to believe that generally, obesity does not result from a single gene

defect and is probably a complex disorder with a combination of several genes and environmental factors likely to contribute or predispose to the disorder. Searches for common polymorphisms within key genes were mounted to determine whether they make a more modest contribution to regulation of obesity.

Sequencing of the *LEP* gene reported an A to G substitution in the untranslated exon one (Hager *et al.*, 1998) and a G to A base change at nucleotide position -2548 in the promoter region (Mammes *et al.*, 2000).

Location of these SNPs in the *LEP* gene enabled association studies to be conducted within normal populations and between obese and normal weight groups (as determined by obesity indicators such as BMI, percentage fat mass etc). This requires comparison of allele frequencies and distribution for each polymorphism separately and in combination (haplotype analysis).

Studies on the *LEP* gene have identified that the G-allele of the A to G base change in exon 1 is associated with lower leptin leptin levels (Hager *et al.*, 1998) in obese individuals. Examples of SNPs in the coding region of the gene are few (this may reflect the fact that the *LEP* gene is substantially smaller than the *LEPR* gene). Several SNPs were located in the 5' flanking region of the *LEP* gene, three of which were found to differ in allele frequencies between obese and non-obese women (W-D, Li *et al.*, 1999) The most significant difference was observed for the G to A -2548 base change SNP. In a separate study, Mammes and colleagues (2000) reported that the G allele was associated with lowered leptin levels and obesity in men only.

Studies by Considine and colleagues (1996), Gotoda and colleagues (1997) and Thompson and colleagues (1997) involved sequencing of the *LEPR* gene in obese individuals from Pima Indians, British and French populations. Pima Indians are a North American tribe who split several centuries ago into two geographical groups.

The Arizona Pimas who eat a high-fat, westernised diet have the highest incidence of obesity in the world, whilst their Mexican kindred are on average 26kg lighter, participate in extensive physical labour and live on a limited diet (Gibbs, 1996).

Several coding and non-coding polymorphisms were discovered. More than one SNP was identified in the sequence coding for the extracellular domain of the leptin receptor. An adenine (A) to guanine (G) base change generating the LYS109ARG amino acid substitution (both of which have a basic charge (+ve) at pH 6-7). Another A to G base change codes for the GLN223ARG substitution (resulting in a change from an uncharged amino acid to one with a basic (+ve) charge at pH 6-7 at that codon). A silent thymine (T) to cytosine (C) base change at codon 343 was located which maintains the presence of a serine residue (See figure 1.6 for location of leptin receptor SNPs).

Association studies of the *LEPR* gene by Gotoda and colleagues (1997) did not detect any difference in allele frequencies for any of the polymorphisms between obese and lean white British males. Thompson and colleagues (1997) observed that two polymorphisms in the non-coding region of the *LEPR* gene were present in obese Pima Indians but not normal weight Pima Indians. Due to the small sample size (twenty individuals) however, the significance of this result is unclear.

Since these initial investigations, many more studies have been undertaken in different ethnic groups with varying study designs. Some have reported an association whilst others have not. Positive associations of the GLN223ARG SNP with measures of adiposity have been detected in several studies (Chagnon *et al.*, 1999, Chagnon *et al.*, 2000, Yiannakouris *et al.*, 2001 and Quinton *et al.*, 2001) (see table 1.3) where the presence of the arginine residue is associated with an increase in such indicators of adiposity as BMI and fat mass. In contrast, other studies have failed to reveal an

association of any of the common SNPs with measures of adiposity (Echwald *et al.*, 1997, Matsuoka *et al.*, 1997, Silver *et al.*, 1997) (see table 1.3). These conflicting results may not be surprising due to the complex aetiology of obesity where different combinations of genes combined with environmental factors may contribute to the same phenotype (obesity) in different individuals. In such a condition, the effect of individual polymorphisms is harder to detect and also because different ethnic groups were studied, it is unsure whether a certain polymorphism is having a specific influence on a particular ethnic group. Gender and age differences are additional confounding factors that are known to influence leptin levels, with one example being that leptin levels fluctuate during the menstrual cycle (Shimizu *et al.*, 1997). Some studies have attempted to control for such problems. For example, Quinton *et al.* (2001) looked at a cohort of postmenopausal women only (Table 1.3). Heo *et al.* (2001) initiated a meta-analysis to increase numbers and, therefore, the power of the results by combining the data from several association studies of the leptin receptor gene. They concluded that there was no statistically compelling evidence that any of these alleles were associated with BMI or waist circumference in the general population. It is interesting to note, however, that the majority of their data was pooled from studies originally having a negative result and that they also concluded that certain genotypic effects could be population-specific.

Linkage analysis measuring co-segregation of the 24 hour respiratory quotient quantitative trait (a predictor of weight gain as determined by Ravussin and Swinburn, 1993) with obesity, found strong linkage at chromosome 1p31-p21.

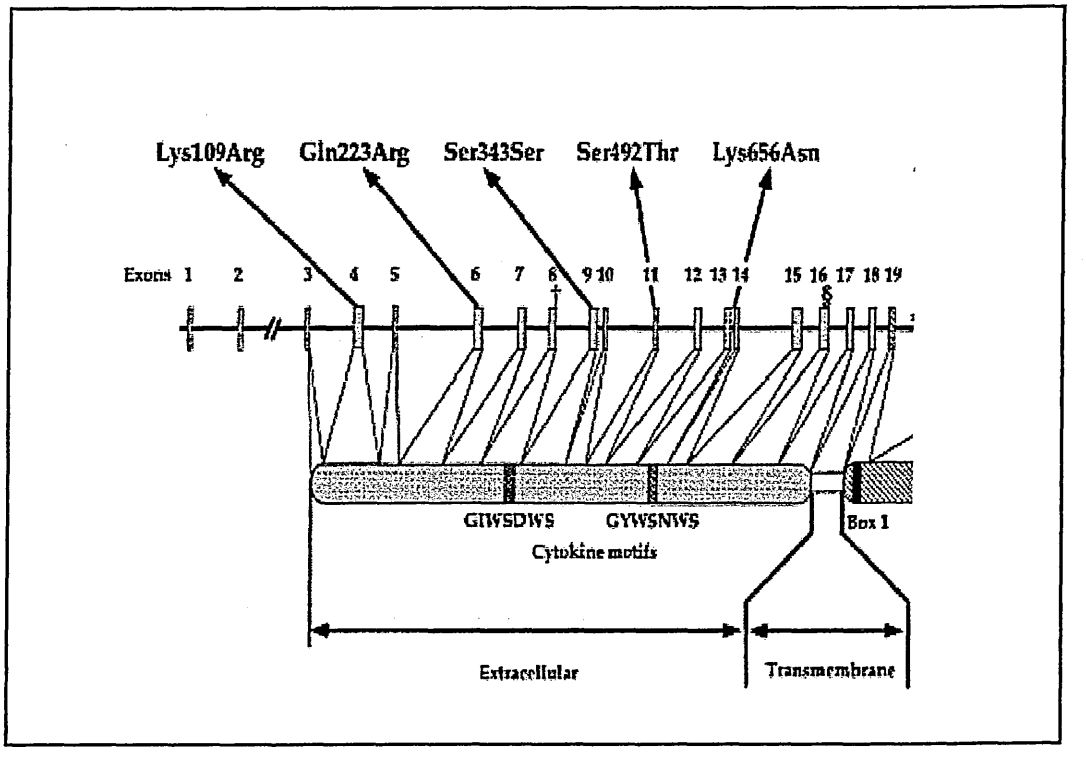


Figure 1.6: Schematic representation of the extracellular and transmembrane domains of the human leptin receptor protein and also the relative position of the *LEPR* gene exons and SNPs. Adapted from Matsuoka *et al.* (1997).

Author	Study type	Study size	Gender	Ethnic group	Results
Silver *et al.* (1997)	Case:control association	104 obese 107 lean	male / female	Caucasian	No association with BMI or waist to hip ratio detected for GLN223ARG or LYS656ASN
Thompson *et al.* (1997)	Case:control association	10 obese 10 lean	male / female	Pima Indian	Association of non-coding polymorphisms with high percentage fat
Gotoda *et al.* (1997)	Case:control association	190 obese 132 lean	male	British Caucasian	No association with BMI/skinfold thickness detected for LYS109ARG, GLN223ARG or LYS656ASN
Matsuoka *et al.* (1997)	Case:control association	47 obese 68 non-obese	male / female	Japanese	No association with BMI detected for LYS109ARG, GLN223ARG, or LYS656ASN
Chagnon *et al.* (1999)	Linkage and association	109 nuclear families 167 obese 141 lean	male / female	Black / Caucasian	GLN223ARG linkage with fat mass in Caucasians
Chagnon *et al.* (2000)	Linkage and association	115 black nuclear families 99 Caucasian nuclear families	male / female	Black / Caucasian	Association with GLN223ARG and fat mass, % fat, BMI and leptin levels in middle-aged Caucasian males. BMI and fat mass linked with K109R
Mammes *et al.* (2001)	Case:control association	179 overweight 289 lean	male/female	Caucasian	Fat mass associated with the SER343SER SNP
Quinton *et al.* (2001)	normal population	220	female (postmenopausal)	British Caucasian	Association of GLN223ARG with fat mass, BMI and leptin binding activity
Wauters *et al* -2001	Case:control association	198 overweight premenopausal 82 overweight postmenopausal	female	Caucasian	Postmenopausal Carriers of the ASN656 allele had increased hip circumference, total abdominal fat and subcutaneous fat
Yiannokouris *et al.* (2001)	Case:control association	89 lean 29 obese	male / female	Greek Caucasian	Association with GLN223ARG and fat mass/BMI

Table 1.3: Summary of selection of studies investigating associations/linkages of leptin receptor polymorphisms with measurements of adiposity.

1.6 Aims of the study

There were three main aims of the studies described in this thesis:

1. to identify and further characterise any associations between leptin receptor SNPs and measures of adiposity in:
   a) a postmenopausal female European Caucasian population;
   b) a female European Caucasian anorectic population

2. to identify associations between other relevant cytokine polymorphisms and measures of adiposity in a postmenopausal female European Caucasian population

3. to generate leptin receptor protein variants (representing the DNA polymorphisms), functionally characterise them, with the aim of comparing activity to determine if the SNPs have a functional effect.

These studies should enhance our understanding of the role of genetic variation in eating disorders and disease, whilst enabling functional analysis of these variations.

Chapter 2
Materials and Methods

2.1 Anthropometrical measurements

To determine height and weight, subjects were barefoot and lightly clothed. The BMI was calculated as the body weight (kilograms) divided by the square of the height (metres^2).

To determine fat and lean mass values, whole body scans were performed under a dual energy x-ray absorptiometry (DEXA) machine. By determining the varying levels of absorbance by the different anatomical structures of the body, a quantitative measurement (grams) of lean and fat mass was obtained.

2.2 Genotyping

2.2.1 Polymerase Chain Reaction (PCR) of SNPs in the human leptin receptor gene (*LEPR*)

The *LEPR* polymorphisms investigated were, LYS109ARG, GLN223ARG and LYS656ASN. PCR was carried out using either purified genomic DNA or DNA from dried blood spots as a template.

Primers used to amplify these regions of the leptin receptor are described by Echwald *et al.*, (1997) and Gotoda *et al.*, (1998).

PCR reactions were carried out in a 25µl volume. A negative control (whereby the DNA template was replaced with an equal volume of water) was included in each batch of PCR.

For all reactions, the *Taq* DNA polymerase (recombinant from *Thermus aquaticus*), deoxynucleotides (dNTPs), magnesium chloride (MgCl) and 10XNH_4 reaction buffer were supplied by Bioline. The primer oligonucleotides were supplied by Invitrogen.

The PCR reactions were conducted on an MJ research PT-225 thermal cycler.

Reaction components, conditions and primer sequences are presented in tables 2.1, 2.2 and 2.3.

Template DNA was supplied as either dried blood spotted onto standard neonatal blood cards (carried out by Professor Eastell's research group, Northern General Hospital, Sheffield) or purified DNA solutions extracted by Helen Eastwood at the Yorkshire Centre for Eating Disorders (Leeds).

	LYS109ARG	**GLN223ARG**	**LYS656ASN**
DNA solution[1]	2µl	2µl	2µl
$MgCl_2$ (Bioline)	2mM	2.5mM	1mM
10xNH_4 buffer	2.5µl	2.5µl	2.5µl
dNTPs (Bioline)	0.2mM	0.2mM	0.2mM
Forward primer	0.4µM	0.6µM	0.6µM
Reverse primer	0.4µM	0.6µM	0.6µM
Taq polymerase	2.5 units	2.5 units	2.5 units
Sterile H_20	To a final volume of 25µl	to volume of 25µl	To volume of 25µl

Table 2.1: Table of components for PCR mixture for LYS109ARG, GLN223ARG and LYS656ASN genotyping PCRs. Reaction volumes stated were the optimum reaction volumes observed following PCR optimisation.[1] Where the template is dried blood, DNA solution is replaced by 1mm^2 of card containing dried blood and the final reaction volume of 25µl is maintained by adjusting the sterile water volume.

PCR step[1,*]	LYS109ARG		GLN223ARG		LYS656ASN	
	Temperature (centigrade)	Time (seconds)	Temperature	Time	Temperature	Time
1. DNA denaturation	94	30	94	60	94	30
2. Primer annealing	54	30	49	60	54	30
3. DNA strand synthesis	72	30	72	150	72	30
4. Synthesis completion	72	300	72	300	72	300

Table 2.2: Table of PCR cycle conditions for LYS109ARG, GLN223ARG and LYS656ASN. [1] Steps 1 through 3 are repeated 35 times for all three PCRs. PCR cycle conditions were taken from Echwald *et al.*, (1997) and Gotoda *et al.*, (1998).
* For blood spot PCR, a pre-reaction denaturation step of 95°C for 10 minutes occurs, followed by addition of *Taq* polymerase and completion of the PCR reaction as above.

	Forward primer sequence	Reverse primer sequence
LYS109ARG	5'-TTTCCACTGTTGCTTTCGGA-3'	5'-AAACTAAAGAATTTACTGTTGAAACAAATGGC-3'
GLN223ARG	5'-ACCCTTTAAGCTGGGTGTCCCAAATAG-3'	5'-AGCTAGCAAATATTTTTGTAAGCAATT-3'
LYS656ASN	5'-TCCTATGAGAGGACCTGAAT -3'	5'-AGGGGCTTCCAAAGTAAAGTGACATTTTTCGC-3'

Table 2.3: Table of DNA primer sequences used for the LYS109ARG, GLN223ARG and LYS656ASN reactions.
PCR primer sequences were taken from Echwald *et al.*, (1997) and Gotoda *et al.*, (1998).

2.2.2 *LEPR* SNPs PCR product digestion

Digestion of the LYS109ARG, GLN223ARG and LYS656ASN polymorphisms were undertaken using the restriction enzymes *Hae* III (Promega), *Msp* I (Promega) and *Acc* II (Amersham Biotech) using the reaction components and conditions presented in table 2.4.

	LYS109ARG	**GLN223ARG**	**LYS656ASN**
PCR product	10µl	10µl	10µl
Enzyme	1µl (10 units)	1µl	1µl
Enzyme buffer	2µl	2µl	2µl
Sterile water	(to a final volume of) 20µl	20µl	20µl
Incubation time and temperature	16 hours at 37°C (water bath)	16 hours at 37°C	16 hours at 37°C

Table 2.4: Reaction components and conditions for LYS109ARG, GLN223ARG and LYS656ASN restriction digest genotype reactions.

After digestion, the samples were electrophoresed on 3% Nusieve agarose gels (BMA products) for the LYS109ARG and LYS656ASN polymorphisms and 2.5% agarose gels (Sigma) for the GLN223ARG polymorphism in order to visualise the different genotypes (Figure 6.1 in chapter 6).

2.2.3 PCR of SNPs in the *LEP*, TNF-α and IL1RN genes

PCR was carried out using DNA from dried blood spots as a template. The reaction conditions for the *LEP* G-2548A promoter SNP were carried out using the PCR conditions and primers reported by Mammes and colleagues (2000). The TNF- α G-308A SNP PCR conditions are essentially based upon those used by Fernandez-Real and colleagues (1997) and the ILRN variable number tandem repeat (VNTR) PCR used the PCR and primer conditions published by Tarlow *et al.*, (1993).

Reagents and equipment used were the same as described in section 2.2.1.

Reaction components, conditions and primer sequences are presented in tables 2.5, 2.6 and 2.7. The *LEP* G-2548A and TNF α G-308A SNPs were subsequently digested by restriction enzymes to visualise the different alleles for each SNP. Each allele present in individuals for the IL1RN VNTR was specifically amplified during the PCR reaction and detected by running the PCR products on a 2% agarose gel.

	LEP G -2548 A SNP	TNF-α -308 SNP	IL1RN VNTR
DNA bloodspot	1mm^2	1mm^2	1mm^2
$MgCl_2$ (Bioline)	2mM	1.5mM	1.5mM
10xNH_4 buffer	2.5µl	2.5µl	2.5µl
dNTPs (Bioline)	0.2mM	0.2mM	0.2mM
Forward primer	0.4µM	0.6µM	0.5µM
Reverse primer	0.4µM	0.6µM	0.5µM
Taq polymerase	2.5 units	2.5 units	2.5 units
Sterile H_20	To a final volume of 25µl	to volume of 25µl	To volume of 25µl

Table 2.5: Table of PCR components used for the *LEP* G-2548 A SNP, TNF-α -308 SNP and IL1RN VNTR PCRs. Reaction volumes stated were the optimum reaction volumes observed following PCR optimisation.

	LEP G-2548A SNP		TNF α -308 SNP		IL1RN VNTR	
PCR step[1,*]	Temperature (centigrade)	Time (seconds)	Temperature	Time	Temperature	Time
1. DNA denaturation	94	60	94	30	94	60
2. Primer annealing	5O	60	60	40	59	60
3. DNA strand synthesis	72	60	72	40	70	120
4. Synthesis completion	72	300	72	300	72	300

Table 2.6: Table of PCR cycle conditions for the *LEP* G-2548A SNP, TNF α -308 SNP and IL1RN VNTR.[1] Steps 1 through 3 are repeated 35 times for all three PCR reactions. * For blood spot PCR, a pre-reaction denaturation step of 95°C for 10 minutes occurs, followed by addition of the Taq polymerase and completion of the PCR reaction as above. The cycle conditions were taken from Mammes *et al.*, (2000), Fernandez-Real *et al.*, (1997) and Tarlow *et al.*, (1993) for the three polymorphisms respectively.

	Forward primer sequence	Reverse primer sequence
***LEP* G -2548A**	5'-TTTCCTGTAATTTTCCCGTGAG-3'	5'-AAAGCAAAGACAGGCATAAAAA -3'
TNF α -308	5'-AGGCAATAGGTTTTGAGGGCCAT -3'	5'-ACACTCCCCATCCTCCCGGCT -3'
IL1RN VNTR	5'-CTCAGCAACACTCCTAT -3'	5'-TCCTGGTCTGCAGGT -3'

Table 2.7: Table of DNA primer sequences used for the leptin G-2548A SNP, TNF α -308 SNP and IL1RN VNTR PCR reactions. The primer sequences were taken from Mammes *et al.*, (2000), Fernandez-Real *et al.*, (1997) and Tarlow *et al.*, (1993) for the three polymorphisms respectively.

2.2.4 *LEP* G -2548A and TNF-α G-308A SNP PCR product digestion

Digestion of the *LEP* G-2548A SNP and TNF α-308 SNP PCR products were undertaken using the restriction enzymes *Cfo* I (Promega) and *Nco* I (Promega) respectively. The reaction components and conditions presented in table 2.8.

	***LEP* G-2548A**	**TNF α -308**
PCR product	10μl	10μl
Enzyme	1μl (10 units)	1μl
Enzyme buffer	2μl	2μl
Sterile water	(to a final volume of) 20μl	20μl
Incubation time and temperature	16 hours at 37°C (water bath)	16 hours at 37°C

Table 2.8: Reaction components and conditions for the *LEP* G-2548A and TNF α -308 restriction digests.

After digestion, the *LEP* G-2548A samples were subjected to electrophoresis in 3% Nusieve agarose gels to visualise the different genotypes. The TNFα G-308A samples were subjected to electrophoresis on 9% polyacrylamide (PAGE) gels (Sigma) to visualise the different genotypes (See chapter 5, figures 5.1 and 5.2).

2.2.5 IL1RN VNTR genotyping

Following PCR, the IL1RN VNTR PCR products were applied directly to a 2% agarose gel. As each allele differs by 86bp, scoring of the different alleles can be determined directly from visualising size differences on an ethidium-stained gel (see chapter 5, figure 5.3).

2.3 Nucleic acid purification techniques

2.3.1 Plasmid purification from bacterial cultures

Inoculated bacterial cultures were incubated overnight in an orbital shaker (New Brunswick Scientific model G25) at 200 revolutions per minute (rpm) at a temperature of 37°C in 17x100mm tubes (Falcon) containing 3ml (LB) broth and 100µg/ml of the appropriate selective antibiotic. Subsequently, 1.5ml of culture was centrifuged in an Eppendorf tube for 5 minutes at 11,500 x g (Eppendorf 1615 centrifuge) to pellet the cells. The supernatant was removed and the pellet re-suspended in 100µl of solution 1 (50mM glucose, 10mM ethylenediamine-tetraacetic acid (EDTA) and 25mM Tris. hydrochloric acid (HCl) pH 8). To this was added 200µl solution 2 (0.2M sodium hydroxide (NaOH), 1% w/v sodium dodecyl sulphate (SDS) and the tube placed on ice for 5 minutes. Afterwards, 150µl ice-cold solution 3 (5M potassium acetate and glacial acetic acid) was added, the tube inverted slowly several times and then stored on ice for 5 minutes. The tube was centrifuged for 10 minutes at 11,500 x g at 4°C; the supernatant collected and transferred to a new tube. 400µl phenol:chloroform (24:1) was added to the supernatant, vortexed and centrifuged for 2 minutes at 11,500 x g. The supernatant was transferred to a new tube and 700µl ethanol added. The tube was incubated for 5 minutes on ice and then centrifuged for 5 minutes at 11,500 x g at 4°C. The supernatant was removed, and 1ml 70% ethanol added to the tube, which was then centrifuged for 5 minutes at 11,500 x g at 4°C. The supernatant was discarded and the DNA pellet air-dried, followed by resuspension in 50µl sterile water containing 5µg/ml RNAse A (Sigma).

2.3.2 DNA purification from agarose gel (Gene Clean protocol)

The DNA band of interest was excised from ethidium bromide-stained agarose gels over a UV transilluminator. The excised band was weighed to determine the approximate volume of the gel slice (0.1g approximately equals 100µl) and placed in a 1.5ml Eppendorf tube. Subsequently, the GeneClean DNA purification kit was used (Bio101). Four and a half volumes of a sodium iodide solution (Bio101) was added to the gel slice along with a half volume of TBE (Tris-Borate EDTA) modifier solution (Bio101). The mixture was incubated at 45-55°C in a water bath for 5 minutes to melt the gel. Five µl of EZ-glass milk silica suspension (Bio101) was added to the melted agarose and incubated for 5 minutes at room temperature with occasional mixing. The silica matrix was pelleted by spinning the Eppendorf tube for 5 seconds at 11,500 x g (Eppendorf 1615 microcentrifuge) and the supernatant discarded. The pellet was washed by resuspending the pellet in 400µl of New wash (Bio101), followed by centrifugation for five seconds at 11,500 x g and removal of supernatant. The wash step was carried out three times in total. To elute the DNA, the pellet was resuspended with 10µl of water and the suspension centrifuged for 30 seconds at 11,500 x g. The supernatant containing the DNA was transferred to a new tube.

2.3.3 Assessment of DNA concentration and purity

The concentration of DNA was estimated by measurement of absorbance at a wavelength of 260 nanometres (nm) (GeneQuant spectrophotometer). DNA samples were diluted 1:100 in sterile water and placed in a 1cm pathlength quartz cuvette (Amersham Biotech) and the absorbance measured at 260nm. Under such conditions, an absorbance unit of one is measured from a solution containing 50μg/ml DNA. Subsequently the concentration of the test sample can be calculated.

The purity of the DNA sample was assessed by measurement of the absorbance at 280nm as well as 260nm followed by calculation of the ratio between the two absorbance readings. A ratio of 1.8 was considered free of impurities. Samples typically gave ratios of 1.75 to 1.85.

2.3.4 RNA purification techniques

Extraction of total RNA from cultured cells was performed using a guanidinium thiocyanate-phenol-chloroform method first described by Chomczynski and Sacchi (1987). The presence of Tri reagent (Sigma) maintains the integrity of the RNA during and following disruption of cells.

2.3.5 Preparation of cultured cells for RNA extraction

Human embryonic kidney (HEK) 293 cells were provided as confluent monolayers.

The medium was removed and the cells washed twice in 10ml of 1x phosphate-buffered saline (PBS) (Sigma). Subsequently, 1ml of TRI reagent was added per $10cm^2$ ^{of} flask area. The liquid was pipetted vigorously to dislodge and lyse the cells after

which the samples were transferred to chilled 1.5ml Eppendorf tubes. Following incubation on ice for 5 minutes, 100µl of ice-cold chloroform (per ml of Tri reagent) was added to the tubes, mixed and incubated on ice for 10 minutes. The tubes were then centrifuged at 11,500 x g (Eppendorf 1615 microcentrifuge) for 15 minutes at 4° C. The top aqueous layer (approximately 500µl) was removed and placed into a fresh Eppendorf tube with an equal amount of ice-cold isopropanol. This mixture was incubated on ice for one hour. The centrifugation was then repeated at 11,500 x g for 15min at 4°C, the supernatant was discarded and the pellet resuspended in 250µl of 80% ethanol. The resuspended pellets were centrifuged at 11,500 x g for 5min at 4°C and the supernatant removed. The pellets were air-dried for 10 minutes and resuspended in 20-50µl of sterile H_2O.

2.3.6 Assessment of RNA concentration and purity

The RNA concentration for each sample was calculated by measuring the optical density of a diluted sample, at a wavelength of 260nm (GeneQuant spectrophotometer). In a 1cm-pathlength quartz cuvette, an absorbance reading of 1 unit at 260nm is equal to 40µg/ml single-stranded RNA. The original samples were then appropriately diluted to obtain an RNA concentration of approximately 1µg/µl.
The ratio of the absorbance at 260nm: 280nm was also calculated. This value reflected the purity of the RNA in the sample, with a value of 2.0 considered relatively free of impurities.

2.4 Reverse Transcriptase-PCR (RT-PCR)

2.4.1 First strand cDNA synthesis

Using the enzyme AMV (avian myeloblastosisvirus) reverse transcriptase (RT) (Gibco), first strand cDNA was synthesised using mRNA as a template.

For each RNA sample, a 36µl reaction mix was made containing 8µl of 5 x RT buffer, (500mM Tris-HCl, (pH 8.3), 50mM $MgCl_2$, 50mM DTT) (Gibco), 8µl of KCl (250mM), 16µl of dNTPs (containing equi-molar amounts of dGTP, dATP, dCTP and dTTP), 1.2µl of Oligo $(dT)_{12-18}$ primer (0.5mg/ml in diethyl pyrocarbonate treated water) (Gibco), 1.2µl (3units) reverse transcriptase and 1.6µl of sterile H_2O. This mixture was added to 4µl of RNA (1µg/µl). Two controls were run parallel to each sample. The first contained only sterile H_2O in replacement of RNA (negative control) and the second contained sterile H_2O in replacement of the RT enzyme (no RT control). The mixture was then overlaid with 40µl of mineral oil, to prevent evaporation, and placed in a MJ research PT-225 thermal cycler for the following reaction (table 2.9).

Step	Duration	Temperature
cDNA synthesis	1 hour	37.5°C
Enzyme denaturation	5 minutes	99°C
Holding temperature	∞	4°C

Table 2.9 First strand synthesis reaction conditions

The samples were then removed and stored at -20°C.

2.4.2 PCR of *LEPR* extracellular domain

PCR was used to amplify the cDNA for the extracellular domain of *LEPR*. Using the following primers designed specifically for this study:

Forward primer 5'-GGA TCC AAC TTG TCA TAT CCA ATT ACT CCT TG-3'

Reverse primer 5'- A CCT GCA TCA CTC TGG TGT TT-3'

The PCR reaction mix consisted of a 20µl master mix containing 15.5µl sterile water, 2.5µl 10 x NH_4 reaction buffer (Bioline), 0.5µl 50mM $MgCl_2$ (Bioline), 0.5µl of 20µM forward primer, 0.5µl of 20µM reverse primer (Invitrogen) and 2.5 units of *Taq* polymerase (Bioline). The reaction was conducted on an MJ research PT-225 thermal cycler using the following program (table 2.10).

PCR cycle step	Duration (seconds)	Temperature
DNA denaturation 1	180	96°C
DNA denaturation 2*	60	94°C
Primer annealing*	60	56.5°C
DNA strand synthesis*	150	72°C
Synthesis completion	480	72°C
Holding temperature	∞	4°C

Table 2.10: Conditions for extracellular *LEPR* PCR. * The steps, DNA denaturation 2 through to DNA strand synthesis were repeated for 35 cycles before proceeding to the synthesis completion step.

2.5 Recombinant DNA techniques

2.5.1 pGEM-T cloning

The extracellular *LEPR* cDNA obtained from the RT-PCR was cloned into the pGEM-T vector (Promega).

This plasmid is supplied pre-cut at a specific position, with thymine bases added at both ends of the cut site, forming single base overhangs. The termini of the RT-PCR products have adenine base overhangs added by *Taq* polymerase, facilitating complementary base pairing and ligation between the product and plasmid.

The insert and plasmid DNA were joined using T4 DNA ligase (Promega) in the following reaction mixture of 5µl 2x ligation buffer (Promega), 50ng of pGEM-T vector, 150ng of PCR product, 1µl of T4 DNA ligase (3 weiss units/µl) and sterile water to a final volume of 10µl. The reactions were incubated overnight at 4°C.

2.5.2 pCMV-Tag 2B cloning

The extracellular *LEPR* cDNA insert present in the pGEM-T vector was digested, agarose gel purified by the gene-clean method, and sub-cloned into the pCMV-Tag 2B expression vector (Stratagene). The insert and plasmid DNA were ligated together using T4 DNA ligase (Promega) in the following reaction mixture of 5µl 2x ligation buffer (Promega), 100ng of vector, 400ng of *LEPR* insert, 1µl of T4 DNA ligase (3 weiss units/µl) and sterile water to a final volume of 10µl. The reactions were incubated overnight at 4°C.

2.5.3 Site-directed mutagenesis

Site-directed mutagenesis was employed to engineer mutations in the *LEPR* cDNA insert in the pCMV vector at the sites corresponding to the LYS109ARG and GLN223ARG polymorphisms using the Stratagene quick-change mutagenesis kit.

For the first step of the process, DNA amplification occurred using the recombinant pCMV-Tag 2B vector as template and mismatched primers to incorporate the desired mutation. The site-directed mutagenesis amplification reactions were identical for both the LYS109ARG and GLN223ARG with the exception of the different primer sequences required for each. The reaction components comprised 5µl of 10x reaction buffer (Stratagene), 5ng of plasmid template, 125ng of oligonucleotide primer 1 and 2 (Invitrogen), 1µl of dNTP mix (Stratagene), 1µl of *PfuTurbo* (high fidelity) DNA polymerase (2.5 units pet µl, Stratagene) and sterile water to a final volume of 50µl. The reactions were conducted on an MJ research PT-225 thermal cycler using the following cycle parameters (table2.11) and primer sequences (table 2.12).

Amplification step	Duration (seconds)	Temperature
Plasmid denaturation 1*	30	95°C
Plasmid denaturation 2*	30	95°C
primer annealing *	60	55°C
DNA strand synthesis	960	68°C

Table 2.11: Conditions for amplification of mutant pCMV-Tag 2B plasmids. * The steps, plasmid denaturation 2 through to DNA strand synthesis were repeated for 16 cycles.

	Forward primer sequence	**Reverse primer sequence**
LYS→ARG (codon 109)	5'-GCAGACAACATTGAAGGAA**G***GA CATTTGTT -3'	5'-GAAACAAATGTC**C***TTCCTTCAA TGTTGTCTGC -3'
ARG→GLN (codon 223)	5'-GGTGGAGTAATTTTCC**A***GTCAC CTCTAATGTCAG -3'	5'-CTGACATTAGAGGTGAC**T***GGA AAATTACTCCACC -3'

Table 2.12: Primer sequences used to generate and amplify mutant pCMV-Tag 2B plasmids. *Bold case highlights the mis-matched base incorporated into the amplified products.

After plasmid amplification, the methylated parental plasmid DNA was digested with the enzyme *Dpn*I which targets methylated DNA only. Firstly, the samples were incubated on ice for 2 minutes and then 1µl (10U/µl) of *Dpn*I restriction enzyme (Stratagene) added. The samples were digested for 1 hour at 37°C in a water bath. The remaining, nicked, non-methylated daughter plasmids were subsequently available for transformation

2.5.4 Transformation

Following ligation reactions and/or site-directed mutagenesis reactions, bacterial cells were transformed with the relevant plasmid. The cells were supplied with growth media and an antibiotic selection agent for the recombinant plasmid, allowing replication and propagation of the cell colonies containing the recombinant plasmid on agar plates.

2.5.5 Cell transformation with the pGEM-T vector

Initially, 2µl of the ligation mixture was added to 50µl of JM109 competent bacterial cells (Promega) in an Eppendorf tube. The tube was placed in ice for 20 minutes and then placed in a water bath at 42°C for 45 seconds. The tubes were removed and returned to ice for 2 minutes after which 950ul of LB growth media added to them. The tubes were incubated at 37°C for 1.5 hours on an orbital shaker to enable the plasmid to sufficiently express its ampicillin resistance gene (New Brunswick Scientific model G25) at 200rpm and then plated out onto LB agar plates containing 0.5mM IPTG (Sigma), 80µg/ml X-Gal (Sigma) and 100µg/ml ampicillin (Sigma). The plates were incubated overnight at 37°C and colonies analysed the following day.

2.5.6 Cell transformation with the pCMV plasmid

5µl of the ligation mixture was added to 50µl of XL-10 gold competent bacterial cells (*recA*, *endA1* phenotype) (Stratagene) in an Eppendorf tube. The tube was placed on ice for 20 minutes and then placed in a waterbath at 42°C for 45 seconds. The tubes were removed and returned to ice for 2 minutes and then 950µl of LB growth media added. They were subsequently incubated at 37°C for 1.5 hours on an orbital shaker (New Brunswick Scientific model G25) at 200rpm so that the kanamycin resistant gene contained within the plasmid was adequately expressed. The media was subsequently plated onto LB agar plates containing 50µg/ml kanamycin antibiotic (Sigma). The plates were incubated overnight at 37°C. Colonies were analysed the following day.

2.5.7 Cell transformation with site-directed mutant pCMV plasmids

One µl of the D*pn* I-treated nicked plasmid DNA was added to a 17 x 120mm tube (Falcon) containing 50µl of XL1-Blue cells (Stratagene) and placed on ice for 30 minutes. After which, the reactions were incubated at 42°C for 45 seconds in a water bath. 0.5ml of NZY+ broth was added to each tube and the transformations incubated at 37°C for 1 hour in an orbital shaker (New Brunswick Scientific model G25) at 200rpm. 250µl of the transformation mixture was plated onto LB agar plates containing 100µg/ml kanamycin antibiotic (Sigma) and incubated overnight at 37°C. Colonies were analysed the following day.

2.5.8 DNA sequencing

For DNA sequence analysis of plasmid DNA, 1μg of plasmid DNA, at a concentration of 250ng/μl, was mixed with 12pmol of sequencing primer and delivered to the Advanced Biotechnology Centre (Imperial College London) for automated sequencing.

2.5.9 Plasmid digestion

DNA cloned into vectors was digested using appropriate type II restriction enzymes to cleave the insert DNA from the vector. The products were applied to 0.8% agarose gels, containing ethidium bromide, and subjected to electrophoresis. The products were visualised using a UV transilluminator. A typical digestion reaction included 500ng of plasmid DNA, 2μl of reaction buffer (Promega), 1μl of restriction enzyme 1 (10 units/μl) (Promega), 1μl of restriction enzyme 2 (10 units/μl) (Promega) and sterile water to a final volume of 20μl. The reactions were incubated at 37°C for 4 hours.

2.6 Electrophoretic Techniques applied to DNA samples

2.6.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise PCR, PCR digest, RT-PCR and restriction enzyme digestion products.

Agarose gels were prepared by adding the appropriate amount of agarose (Sigma) to 100ml of 1x Tris borate EDTA (TBE) buffer (e.g. 1 gram per 100ml gives a 1% gel). TBE was diluted in water from a 10x concentrate solution (Invitrogen). The agarose was dissolved by heating in a microwave. The agarose solution was allowed to cool and 2µl of ethidium bromide (Sigma) (stock solution 10mg/ml in water) was added. The agarose was left to set in the gel apparatus (Biometra). Samples were mixed with TBE loading dye (1% bromophenol blue in 10% sucrose in 50ml 1x TBE) in a ratio of 4:1 v/v. The appropriate molecular weight markers were loaded and gels electrophoresed for 1 hour at 80-100V. The DNA bands were visualised and images captured on a dual intensity ultraviolet gel analysis system (UVP Ltd).

2.6.2 Polyacrylamide gel electrophoresis (PAGE)

For detection of the TNF-α -308 digestion products, PAGE gels were utilised. Gels of a final acrylamide concentration of 9% were made by mixing 5.5ml of a 40% Acrylamide:Bisacrylamide solution (29:1 ratio) (Sigma) with 14.5ml of 1x TBE buffer. 25% ammonium persulphate (APS) (Sigma) was made up fresh each day a PAGE gel was required. 65µl APS and 35µl of TEMED (Sigma) were added last to the gel mixture in order to crosslink the acrylamide/bisacrylamide solution permitting polymerisation to occur. The PAGE gel system used to electrophorese the gels was the

Mini-PROTEAN III cell system (Bio-Rad). The apparatus was assembled according to manufacturers instructions and the gels allowed to polymerise for 40 minutes. Samples (mixed with TBE loading dye) were loaded onto the gel and electrophoresis carried out for 1 hour at 120 volts. Visualisation of bands was achieved by bathing the gels in 50ml of 1xTBE running buffer containing 2µl of ethidium bromide (10mg/ml stock solution) subsequent to electrophoresis. Gel images were captured using a dual intensity ultraviolet gel analysis system (UVP Ltd).

2.7Cell culture methods

2.7.1 COS-7 Cell culture methods

For recombinant protein expression studies, the mammalian COS-7 cell line (ECACC, Porton Down) was used. The cells originate from African green monkey kidney cells transformed with the SV40 virus.

2.7.2 Cell feeding

Adherent cells were grown in 6ml complete Dulbeccos modified Eagles medium (DMEM) media (10% foetal bovine serum (FBS) and 2mM glutamine) (Sigma) in a 37°C incubator at 5% CO_2 levels in 25cm^2 tissue culture flasks (Corning). Cells were fed 3-4 days by removal of the media and replacement with 5ml fresh complete media.

2.7.3 Cell counting

For accurate and reproducible cell densities, viable cell numbers were obtained by carrying out a trypan blue exclusion assay. Trypan blue is a dye which is taken up by dead cells but not viable cells. A haemocytometer was used to count the cells. This is a glass slide which contains 2 chambers of known volume. Each chamber contains a series of 1mm x 1mm squares. Viable cells were counted within each of these squares and the mean number of cells per square was calculated. Twenty microlitres of cells in complete media (Sigma) was mixed 1:5 with trypan blue (Sigma) and a small amount pipetted onto a haemocytometer, allowing a viable cell count to be made. The concentration of viable cells in the tissue culture flasks was subsequently calculated.

2.7.4 Sub-culturing

When the COS-7 cells reached 70-80% confluency in the tissue culture flasks, they were diluted 1:10 to obtain cell densities of approximately 10,000 cells / cm^2. Firstly, the cell media was discarded and the cells washed twice with 2ml PBS solution (Sigma). Subsequently, the cells were dislodged using 1ml of 1x trypsin / EDTA solution (Sigma) per flask. The cells were incubated at 37°C for 10 minutes and 5ml complete media added. The cells were resuspended by shaking and a small aliquot removed for cell counting. The appropriate amount of complete media was then added and the cells plated out into a suitable number of $25cm^2$ tissue culture flasks to obtain the correct cell density.

2.7.5 COS-7 cell transfection

Transfection of the COS-7 cell line with recombinant pCMV-TAG2b vector (and site-directed mutant derivatives) was required to facilitate production of the extracellular domain leptin receptor protein. Transfection was carried out using The TransIT COS transfection reagent (Mirus Corporation). This reagent consists of a mixture of 3:1 wt:wt ratio of cellular protein and cationic polyamine which forms a complex with the vector DNA enabling the DNA to enter the target cells by an endosomal pathway.

24 hours prior to transfection, COS-7 cells were split into the appropriate number of $25cm^2$ tissue flasks in complete media to obtain approximately 60-70% confluency the following day.

In a sterile 17 x120mm tube (Falcon), 8µl of TransIT- COS reagent (Mirus) was added dropwise to 200µl of serum-free DMEM media and mixed thoroughly by

vortexing. The mixtures were incubated at room temperature for 15 minutes. 4µl of COS Boss reagent (Mirus) was added to each mixture and subsequently incubated for 15 minutes at room temperature. 2µg of pCMVtag2b plasmid DNA, and each of the site-directed mutant plasmids, were each added separately to a TransIT -COS / COS Boss reagent mixture and incubated for 15 minutes at room temperature. Each TransIT -COS/COS Boss/DNA complex mixture was added dropwise into its own $25cm^2$ tissue culture flask and incubated for 72 hours at 37°C with a 5% CO_2 atmosphere.

2.8 Protein purification and analysis

2.8.1 Immunoprecipitation

The FLAG epitope tagged proteins produced after transfection of the COS-7 cells with recombinant pCMV vector can be affinity purified using highly specific antibodies raised against the epitope. This is the basis of the immunoprecipitation method carried out using the Sigma FLAG tagged protein immunoprecipitation kit containing monoclonal antibodies attached to an agarose resin. Immunoprecipitates were pooled from groups of tissue culture flasks, each group being transfected with a specific recombinant vector coding for one of the leptin receptor protein variants.

2.8.2 Cell lysis

The COS-7 cells were lysed to release the recombinant protein. The growth medium was removed and the cells washed twice with 5ml of 1x PBS buffer (Sigma). 1ml

lysis buffer (50 mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100) (Sigma) containing 10µl protease inhibitor cocktail (Sigma) (to inhibit protease degradation of the target protein) was added per 25cm^2 tissue culture flask. After which, the flask was incubated for 30 minutes on a shaker at 4°C. The cells were lifted using a sterile cell scraper (Nunc) and collected into a 1.5ml tube. The cells were centrifuged for 10 minutes at 12,000 x g (Eppendorf 1615 centrifuge) and the supernatant transferred to a fresh tube.

2.8.3 Flag fusion protein immunoprecipitation

40µl resuspended agarose resin (Sigma) was added to an empty 1.5ml tube. The resin was centrifuged for 5 seconds at 10,600 x g. The supernatant was subsequently removed. The packed gel was washed 4 times with 0.5ml of 1 x wash buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl) (Sigma) and after the last wash, 1ml of cell lysate was added. Samples were agitated overnight at 4°C allowing the antibody to bind to the FLAG epitope. The following day, the resin was centrifuged for 5 seconds at 10,600 x g and the supernatant discarded. The resin was washed three times with 0.5ml 1 x wash buffer.

2.8.4 Elution of the FLAG-fusion protein

Elution of the FLAG epitope tagged protein was carried out under native conditions by competition with a FLAG peptide. 150ng/µl FLAG peptide solution (Sigma) was added to the resin in each tube. The samples were incubated with gentle shaking for

30 minutes at 4°C to facilitate displacement of the target protein. Subsequently, the resin was centrifuged for 5 seconds at 10,600 x g. The purified, target protein was transferred to a fresh tube and stored at -20°C until further use.

2.8.5 Leptin receptor enzyme-linked immunosorbent assay (ELISA)

A double monoclonal sandwich enzyme immunoassay (Abcam) was used for the quantitative measurement of human leptin receptor to determine the concentration of the recombinant human leptin receptor protein variants. This was achieved by incubating leptin receptor standards and the samples in a microtitration plate coated with monoclonal human leptin receptor antibody. A second anti-human leptin receptor antibody, labelled with horseradish peroxidase (HRP), was added and incubated with the immobilised antibody-leptin receptor complex. Addition of the HRP substrate, comprising of tetramethylbenzidine containing H_2O_2, produced a colour reaction which was measured spectrophotometrically at a wavelength of 450nm. The absorbance is proportional to the concentration of the leptin receptor and by plotting the absorbance values versus the concentration of the leptin receptor standards; a standard curve was generated from which the concentration of the samples was determined.

50µl of each purified protein sample was diluted 1:3 with dilution buffer (Abcam).

100µl of standards and diluted samples were pipetted in duplicate into the microtitration plate wells. The plate was incubated at room temperature for one hour. Subsequently each well was washed 3 times in 350µl of wash solution (Abcam). 100µl of conjugate solution, containing the labelled antibody (concentration not provided in literature) (Abcam), was added and the plate incubated at room temperature for one

hour. Then the wells were washed four times in 350µl of wash solution after which 100µl of HRP substrate solution was added (concentration not provided in literature). The plate was incubated in the dark for 10 minutes at room temperature, after which the colour development was stopped by adding 100µl of stop solution (Abcam). Finally the absorbance was read at 450nm by a UV spectrophotometer (Dynatech MRX).

2.8.6 Leptin receptor protein deglycosylation

Purified leptin receptor products were deglycosylated with the amidase enzyme PNGaseF (New England Biolabs) which cleaves a wide range of oligosaccharides that are linked to asparagine residues in the protein sequence (N-linked oligosaccharides). The protein was denatured in 1x glycoprotein denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) (New England Biolabs) for 10 minutes at 100°C. Subsequently, the sample was incubated in 1x G7 reaction buffer (50mM sodium phosphate) (New England Biolabs), supplemented with 1% NP-40 (New England Biolabs), for 2 hours at 37°C.

2.8.7 Measurement of leptin binding activity

This procedure is based upon the method of Quinton and colleagues (1999) and is used to determine the specific binding of the recombinant human leptin receptor protein variants towards a radioactive ligand (leptin).

50µl of serum (control sample) and 0.5 units (1 unit equals 2ng of human leptin receptor) of purified recombinant human leptin receptor samples were each incubated

with 150µl of assay buffer (0.01M phosphate buffer pH 7.4, 0.18M $MgCl_2$, 1% BSA) and 100µl of ^{125} iodinated leptin (135µCi/µg) (Linco research Co) in the absence of excess cold leptin (in order to measure total binding of leptin to all possible binding sites within the sample) or presence of excess cold leptin (1µg) (R&D systems). The cold leptin displaced the radioactive leptin from specific sites leaving the radioactive leptin bound to non-specific sites, providing a non-specific binding measurement. Duplicate reaction samples were prepared for samples containing the cold leptin and also for those lacking the cold leptin. The samples were then incubated for 16 hours at 4°C. The bound and unbound fractions were separated by adding a charcoal slurry (2% charcoal (Norit), 0.2% dextran T-70 (Pharmacia) in assay buffer) which will bind to any free leptin. The assay tubes were mixed, incubated on ice for 10 minutes and centrifuged at 10,000 x g for 12 minutes at 4°C so that any leptin bound to the dextran would be pelleted. The supernatant was removed to a fresh tube and counted on an automatic γ-counter (Wallac).

2.8.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For size analysis, proteins were resolved by molecular weight using one-dimensional SDS-PAGE. This technique uses discontinuous chloride and glycine ion fronts to form moving boundaries that stack and then separate SDS-coated polypeptides by molecular weight (Laemmli, 1970).

2.8.9 Sample preparation for SDS-PAGE

17µl sample was diluted with 33µl sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol) and heated at 95°C for 5 minutes before being applied to a pre-prepared gel.

2.8.10 Gel preparation and running conditions

The Mini-Protean III cell system from Bio-Rad Laboratories was used. Two 4-15% Tris-HCl gradient gels (Biorad) were utilised per cell system, in 400ml of 1x running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Electrophoresis conditions consisted of a constant voltage of 200V for 45 minutes.

2.8.11 Protein Silver staining

This protein staining method is a modification of Blum's rapid silver stain procedure (Blum *et al.*, 1987). The chemical basis for the image development relies on the differential reduction of silver ions bonded to amino acid side chains enabling protein visualisation. The method is reversible and sensitive with reported limits of detection of 1-5ng protein per band.

Each gel was placed in fixative (10% acetic acid v/v, ethanol 30% v/v) for 2 hours and rinsed in 20% v/v ethanol for 20 minutes followed by a 10 minute rinse in distilled water. Subsequently the gel was soaked in sensitiser solution (0.02% w/v sodium thiosulphate) for 1 minute and then rinsed three times in distilled water. Then the gel was soaked in silver nitrate solution (0.2% w/v silver nitrate) for 45 minutes, rinsed in distilled water and soaked in developer solution (0.3% w/v sodium carbonate, 0.025% v/v formaldehyde and 0.004% v/v sodium thiosulphate) until the intensity of the bands was adequate. To stop gel development, the gel was soaked in stop solution (5% w/v Tris Base) for 5 minutes. The gel image was captured using a Sony MVC-FD75 digital camera.

2.9 Statistical Analysis

2.9.1 Hardy-Weinberg equilibrium

The frequency of genotypes observed from a polymorphic locus should remain constant from generation to generation assuming random mating has occurred. If this is so, then the population is in Hardy-Weinberg equilibrium for that locus. Two alleles of a biallelic locus, for example, A_1 and A_2, are observed at a frequency p and q respectively. As such, $p + q = 1$ and the expected genotype frequencies can be calculated as,

Frequency A_1 $A_1 = p^2$ x sample size (n)

Frequency A_1 $A_2 = 2pq$ x n

Frequency A_2 $A_2 = q^2$ x n

And $p^2 + 2pq + q^2 = 1$.

Subsequently, a chi-squared analysis (χ^2) (with 1 degree of freedom) is used to determine if the expected genotype frequencies determined from this equation differ significantly from those values observed experimentally. If there is no statistically significant difference then the observed balance of genotypes does not differ from that expected of a population in Hardy-Weinberg equilibrium. If there is a difference then the cohort studied may have incorrectly assigned genotypes.

2.9.2 Chi- squared (χ^2) analysis of contingency tables

χ^2 analysis enables measurement of the extent to which observed numbers in a contingency table deviate from the expected values. χ^2 distribution tables then show if the observed value deviates by a greater amount than would be expected by chance alone based on a null hypothesis that postulates no difference between observed and expected distributions. 2 (column) x 2 (row) contingency tables were used to compare alleles between different groups (i.e. control versus anorexic individuals).

The χ^2 value is obtained by the equation: $\chi^2 = \Sigma (o-e)^2 / e$

Where o = observed number and e = expected number

The number of degrees of freedom for χ^2 is given by (number of columns -1) x (number of rows-1). The null hypothesis is rejected and the test statistic considered significant if it is assigned a p (probability) value which is less than 0.05. Such a value indicates that the probability of the findings occurring by chance is less than 5%.

An example of a 2x2 contingency table to calculate χ^2 is demonstrated in table 2.13.

		Variable 1		
		0	1	Total
Variable 2	0	a	b	a+b
	1	c	d	c+d
Total		a+c	b+d	a+b+c+d

Table 2.13: An example of a 2x2 contingency table.

Subsequently, χ^2 can be calculated by the following equation:

$\chi^2 = (ad-bc)^2 (a + b + c + d) / (a + b) (c + d) (b + d) (a + c)$

2.9.3 Carriage rates

The frequency of an allele in a population can be increased by the presence of many homozygotes. To assess whether having one copy of a particular allele is sufficient for susceptibility to a disorder, carriage rates are determined. The carriage rate of an allele is the proportion of a population who carry at least one copy of that allele.

2.9.4 Haplotype analysis

The frequency of specific haplotypes in control and patient samples was calculated using the EH program (Xie and Ott 1993), including the assignment of haplotypes with unknown phase by a permutation function in the program. The FASTEH software (Zhao and Sham, 2002) was employed to obtain a p-value via the χ^2 statistic, comparing the distribution of haplotype frequencies between case and control individuals. The null hypothesis that there was no significant difference in haplotype frequency between groups was rejected if $p<0.05$.

2.9.5 Coefficient of linkage disequilibrium (D')

Using a biallelic example at two loci (a_1, a_2 and b_1, b_2), D is measurement of the departure from linkage equilibrium as determined by the following calculation:

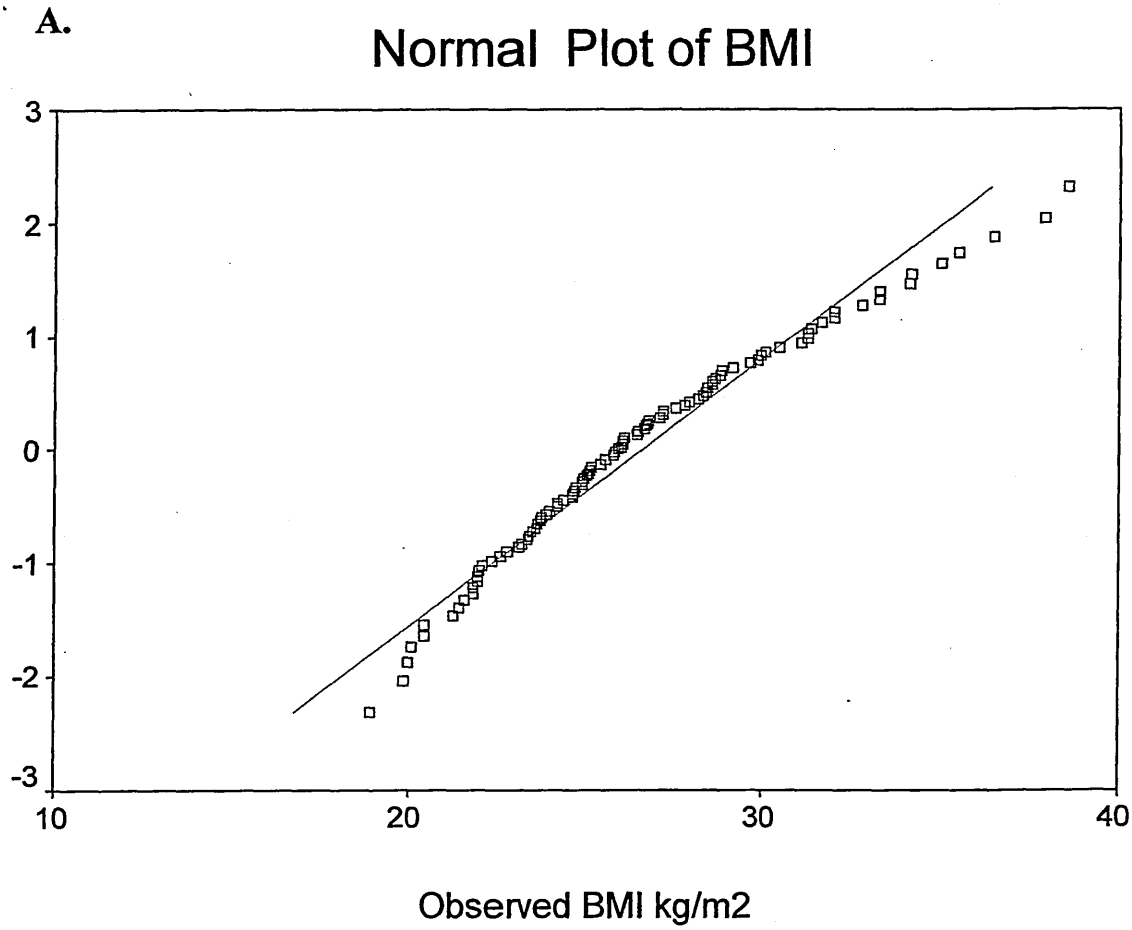
$D = Pa_1b_1P\ a_2b_2 - P\ a_1b_2P\ a_2b_1$ (where P equals frequency)

The 2LD program used the haplotype frequencies obtained from the EH program and calculated D' which is a ratio between the actual value of D, and the maximum value it could possibly be for the given allele frequencies.

2.9.6 Tests for normality

The Normal distribution represents the phenomenon whereby continuous variable data (i.e. data for which there an infinite number of values the variable can take) are distributed via a bell-shaped curve, symmetric about the mean, so that the mean equals the median value. Data that are distributed in this fashion can be subjected to parametric statistical tests. Data which are not distributed in this way, may be analysed by non-parametric tests. Continuous variable data were tested to determine whether the data were normally distributed. The Normal Probability Plot (Normal plot) was constructed by ranking observations into ascending order (e.g. of BMI values) and then plotting this ranked data against the corresponding Normal Score (which is the number of standard deviations above or below the mean that we would expect the observations to lie if the data were Normally distributed). Graphically, the straighter the Normal plot, the more closely it follows a Normal distribution. The Shapiro-Wilk and Kolmogorv-Smirnov tests were used to assess the straightness of the Normal plot. The test calculates the probability that the data were normally distributed. The null hypothesis for the test being that the distribution is normal, and a p-value of less than 0.05 rejects the null hypothesis. Figure 2.1 demonstrates an example of a Normal plot in which the data were found not to be normally distributed.

A.



B.

Tests of Normality

	Kolmogorov-Smirnov[a]		
	Statistic	df	Sig.
VAR00001	.095	97	.030

a. Lilliefors Significance Correction

Figure 2.1: **A.** Normality plot of BMI data constructed by ranking observed BMI values and plotting them against the corresponding Normal score (the number of standard deviations above or below the mean value that each data point would lie if it was normally distributed). **B.** Kolmogorov-Smirnov test result for normality of plotted BMI data detailing the test statistic value of 0.095 and p-value of 0.03. Therefore the variable is not normally distributed.

2.9.7 Mann-Whitney U test

The Mann-Whitney U test was used to compare the mean values between two independent samples of continuous data which were not normally distributed (for example the mean BMI values between two genotype groups for a particular SNP). The null hypothesis for testing such cases stipulates that there is no significant difference in means between the two samples. A p-value of less than 0.05 leads to rejection of the null hypothesis.

2.9.8 Box and whisker plot

Graphical representation and comparison between groups of a particular continuous variable (e.g. BMI between different genotype groups for a particular SNP) was achieved using a box and whisker plot. This consists of a box whose ends correspond to the interquartile range, i.e. the 25^{th} and 75^{th} centiles. A line inside the box marks the median. The length of the two straight lines (whiskers) drawn outwards from the ends of the box represent 1.5 times the interquartile range rolled back to where there is data. The ends of the whiskers are named as the lower and upper fences. Data points outside the fences are plotted individually and may be regarded as outlier (open circle) and extreme (star) values. Box and whisker values more accurately represent the spread and concentration of data points compared to scatter plots. If values of a continuous variable (for example BMI) are identical between different individuals they are simply superimposed on top of each other on a scatter plot. See figure 2.2 for an example of a box and whisker plot.

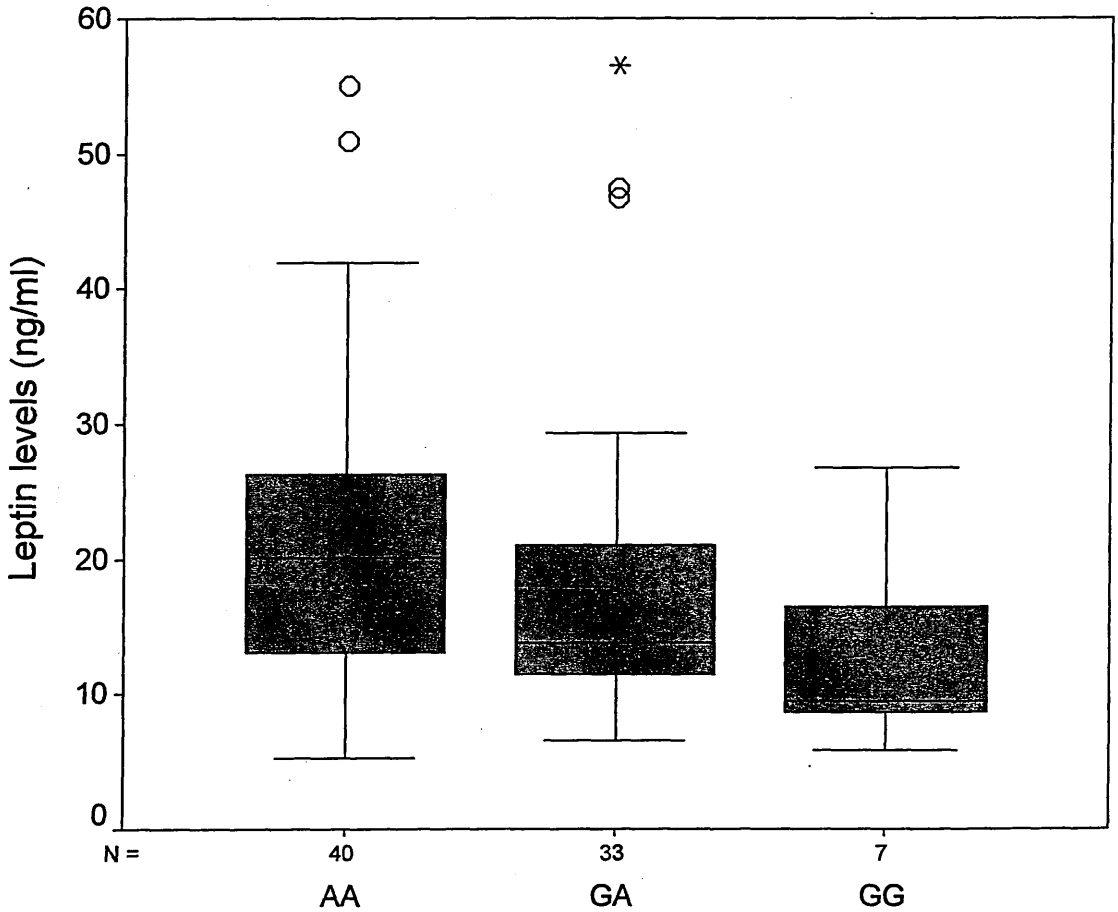


Figure 2.2: Box and whisker plot of leptin levels between genotype groups of the LYS109ARG leptin receptor SNP. The Shaded boxes represent the interquartile range for each data set and the region between the upper and lower fences represent 1.5 x the interquartile range. Open circles represent outlier data points and stars represent extreme value data points.

Chapter 3
Genetic studies of SNPs in the *LEPR* gene in relation to indicators of adiposity

3.1 Introduction

Single nucleotide polymorphisms (SNP) are the most common and evenly distributed genetic variations in the human genome, occurring as frequently as one every 300-1000bp (Schork. *et al.*, 2000).

In fact it has been estimated that 85% of all exons are within 5kb of at least one SNP (The International SNP Map Working Group 2001). Such a level of frequency and the ability to alter protein structure or expression levels as an end result of their sequence variation makes SNPs a potential source of susceptibility factors for disease. Furthermore, via linkage disequilibrium, they may act as 'beacons' for nearby disease-causing genetic targets.

Linkage disequilibrium occurs when a combination of alleles of a group of linked genetic markers (a haplotype), occurs at a frequency that differs significantly from that normally expected in a population. This phenomenon indicates that the alleles have been inherited together, because the physical distance between the loci is small reducing the likelihood for recombination to occur within that region. An allele for a particular SNP may be associated with a particular phenotype. This SNP may be in linkage disequilibrium with alleles of other markers (including SNPs). Indeed, a particular haplotype may be associated with the phenotypic change. One or more of these alleles may have a functional effect on the phenotype or reflect linkage disequilibrium with nearby polymorphisms that are having a functional effect. In the case of SNPs in the *LEPR* gene, it may be that a certain SNP is associated with indicators of adiposity or is in linkage disequilibrium with another SNP in the gene that is exerting an effect or indeed that more than one SNP in a haplotype is involved.

Inter-individual differences in BMI are highly heritable (Austin *et al.*, 1997, Rice *et al.*, 1999), and this could be due, in part, to variation in the *LEP* and *LEPR* receptor genes. Several SNPs have been discovered throughout the *LEPR* gene (Considine *et al.*, 1996, Gotoda *et al.* 1997, Thompson *et al.* 1997). Studies have been undertaken to determine whether these SNPs are implicated as indicators of adiposity. The GLN223ARG SNP has been linked to fat mass in Caucasians (Chagnon *et al.*, 1999). Homozygosity of the G allele for the GLN223ARG SNP has been associated with fat mass and BMI in middle-aged men and post-menopausal women (Chagnon *et al.*, 2000, Yiannakouris *et al.*, 2001 and Quinton *et al.*, 2001). The LYS656ASN SNP has been associated with waist circumference in postmenopausal overweight females (Wauters *et al.*, 2001) and the LYS109ARG SNP has been linked to BMI and fat mass in Caucasians (Chagnon *et al.*, 2000). This study expands upon the initial work by Quinton *et al* (2001) by analysing the LYS109ARG and LYS656ASN SNPs (in addition to the GLN223ARG SNP) for associations with adiposity in a population of postmenopausal women from the Sheffield area (ages ranging from 59.2 to 70.1 years old).

It has been observed that there are differences in the leptin system between males and females. Women have higher leptin levels than men even after adjustment for fat mass (Hickey *et al.*, 1996, Saad *et al.*, 1997). Leptin levels also vary significantly during the menstrual cycle (Hardie *et al.*, 1997). Therefore limiting the study cohort to postmenopausal women only, may limit hormonal effects.

Any associations found with a particular SNP could be explained by a direct effect on the protein function or reflect linkage disequilibrium with other polymorphisms within the same gene or nearby. Serum leptin binding activity (LBA) data are available from this cohort. The technique measures the ability of soluble leptin receptor, present in the individuals serum, to bind radioactively labelled leptin. Any differences in LBA

associated with a certain allele may reflect functional differences in ligand binding or receptor dimerisation due to the fact that the SNPs code for amino acid changes in the extracellular domain of the receptor. As all the SNPs genotyped in this study are expressed in the leptin receptor, they will all be present in the soluble form of the receptor.

3.1.1 Aims of the study

Two *LEPR* SNPs were studied, LYS109ARG and LYS656ASN. The data were combined with information previously amassed for the GLN223ARG SNP. The aims of the study were to:

- genotype postmenopausal subjects for the two SNPs;
- seek associations between these SNPs and BMI, fat mass and leptin levels;
- analyse the potential effects of the amino acid changes on ligand binding via the LBA data;
- determine the extent of linkage disequilibrium between these SNPs and also the GLN223ARG SNP.

3.2 Study Design

Blood for genotyping studies was collected from a community-based cohort of Caucasian postmenopausal women, from the Sheffield area (by Professor R Eastell from the Northern General Hospital, as part of an osteoporosis epidemiological study funded by the Arthritis Research Campaign) (n=200). However, complete clinical data were only available for a sub-set of these women, and only these women were analysed for associations. Blood was spotted onto standard neonatal blood cards and dried

overnight before storage at room temperature. Clinical details were recorded at the time of sample collection. Subjects gave informed consent and the studies had the approval of the North Sheffield Local Research Ethics Committee. The sub-group of women included in the study of leptin levels and LBA were selected as they were not undergoing hormone replacement therapy or using corticosteroids. Serum leptin levels had been measured by Allison Lee (at the University of Sheffield) using the Linco Research Co. RIA kit. Serum LBA had been measured by Naomi Quinton (at Sheffield Hallam University) by incubating serum samples, stripped of endogenous leptin, with iodinated leptin in the presence (to measure non-specific binding) or absence (to measure total binding) of 'cold' leptin. Radioactivity counts were determined using a gamma counter. The specific binding obtained was expressed as a percentage of the total [^{125}I] -leptin counts per minute incubated in 50μl of serum.

Genotyping was carried out as described in chapter 2, sections 2.2.1 and 2.2.2. An A to G base change codes for the LYS to ARG amino acid substitution at codon 109 and a G to C base change codes for the LYS to ASN amino acid change at codon 656. The GLN223ARG SNP was not typed in this present study, instead it was typed at a prior date by by Naomi Quinton. In this SNP, an A to G base change codes for the GLN to ARG amino acid substitution at codon 223. Visualisation of the alleles for LYS109ARG and LYS656ASN SNPs are shown in figure 3.1.

3.2.1 Statistical Analysis

The distribution of genotypes was tested for deviation from Hardy-Weinburg equilibrium by χ^2 analysis (see chapter 2, section 2.9.2).

The continuous variable data (BMI, leptin levels, fat mass, lean mass and LBA) were analysed for normal distribution using SPSS software (see chapter 2, section 2.9.5). The data were not normally distributed due to the small number of study samples with with data values generally deviating from the tails of a normal distribution (see section 2.9.6, figure 2.1). As the data deviated from a normal distribution, the Mann-Whitney U test was used to compare the difference in means between genotype groups for a given variable instead of the 2 sample t-test, which is used when the data is normally distributed. Furthermore, mean values were compared between individuals carrying and not carrying the most common allele. To do this, genotype groups including the most common allele (AA and AG for the LYS109ARG SNP, and GG and GC for the LYS656ASN SNP) were combined into one group and the mean value of a given variable for this combined group was compared to the mean value for the group not carrying the most common allele (GG and CC for the LYS109 ARG and LYS656ASN SNPs respectively). This particular procedure has been repeatedly used in the literature including studies by Quinton and colleagues (2001). Mammes and colleagues (2001) and Hoffstedt and colleagues (2002). Data were displayed graphically in a boxplot format using SPSS software (see chapter 2, section 2.9.7). Haplotype frequencies were determined using the EH program (Xie and Ott, 1993) and D and D' constants were calculated using the 2LD program (Zhao, 2002) to gauge the degree of linkage disequilibrium between alleles for each SNP (see chapter 2, sections 2.9.3 and 2.9.4).

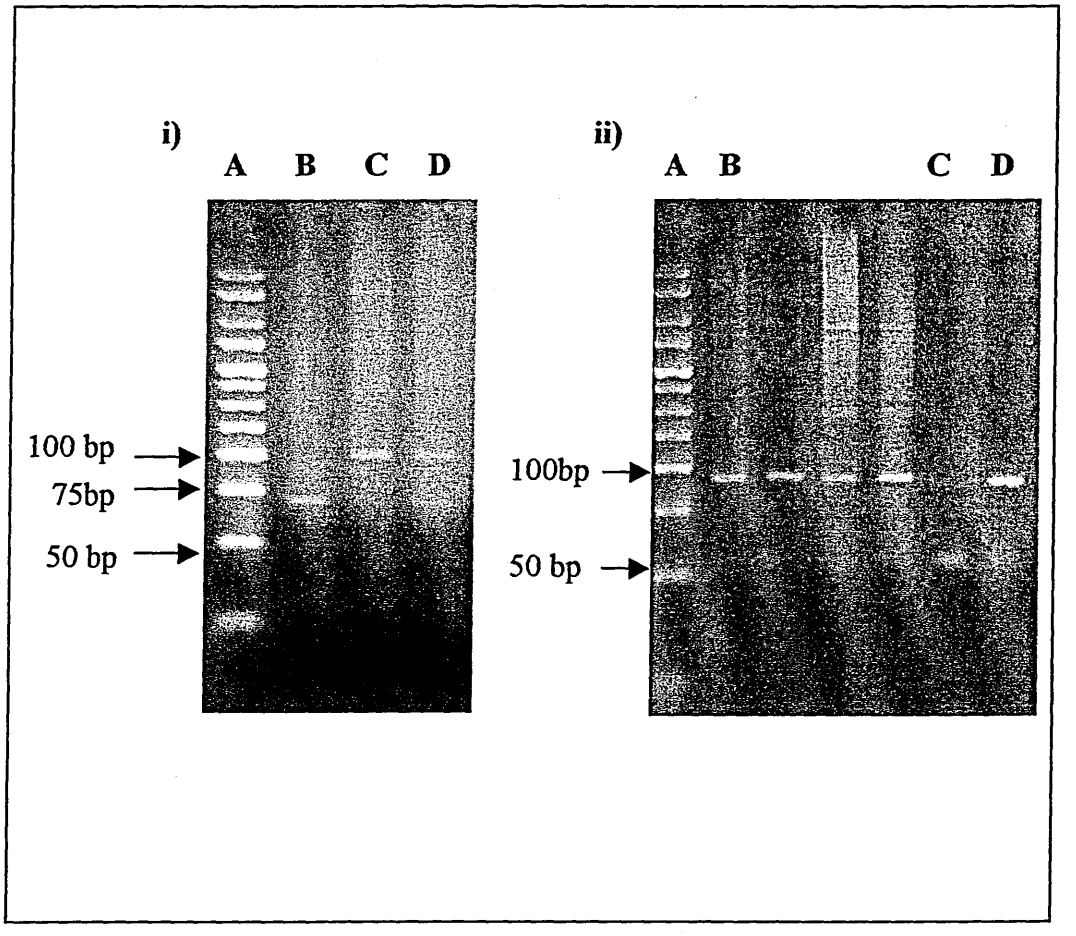


Figure 3.1: **i)** Picture of the LYS109ARG SNP genotypes on a 3% agarose gel displaying, **A.** hyperladder IV DNA marker (Bioline), **B.** homozygous A genotype (70bp), **C.** homozygous G genotype (101bp) and **D.** heterozygous AG genotype,.

ii) Picture of the LYS656ASN genotypes on a 3% agarose gel displaying, **A.** hyperladder IV DNA marker (Bioline), **B.** homozygous G genotype (87 bp), **C.** homozygous C genotype (55bp) and **D.** heterozygous GC genotype (87 + 55bp)

3.3 Results

3.3.1 LYS109ARG polymorphism

To determine allele and genotype frequencies, 184 subjects have been genotyped for the LYS109ARG SNP. The calculated allele frequencies (p = 0.74, q = 0.26) agree well with previous studies (see table 3.1). The distribution of homozygotes and heterozygotes observed, do not differ significantly from that expected of a population in Hardy-Weinberg equilibrium (see table 3.2).

All continuous variable data, which were grouped to a corresponding genotype, was analysed to determine whether it fitted a normal distribution. All such data were not normally distributed and so the non-parametric Mann-Whitney U test was carried out to compare the means for each variable between genotype groups (table 3.3). Boxplots of the different variables are shown in figures 3.2-3.6. For comparison, the data acquired by Quinton and colleagues (2001) for the GLN223ARG SNP in the same cohort is displayed in table 3.4.

Allele	**Post-menopausal women n=184 (This study)**	Lean British males (n=132) *Gotoda et al.*,1997	Obese British males (n=190) *Gotoda et al.*,1997	Caucasian males and females (n=190) *Chagnon et al.*,2000
A	**0.74**	0.74	0.73	0.73
G	**0.26**	0.26	0.27	0.27

Table 3.1: Comparison of allele frequencies for the LYS109ARG SNP with published results

	Observed genotype frequency	**Expected genotype frequency**
	n	n
Heterozygotes	73	71
Homozygous A	100	101
Homozygous G	11	12
Total	184	184

Table 3.2: Predicted genotype values from the Hardy-Weinberg equation for LYS109ARG using the determined allele frequencies. The χ^2 p-value (1 degree of freedom) comparing the expected to observed genotype frequencies was 0.627.

	Genotype			P value
	AA	AG	GG	
BMI (kg/m^2)	27.03 ± 0.52 [1]	26.15 ± 0.73	23.16 ± 0.88	(AA+AG v GG) 0.017
N=	n=54	n=31	n=7	(AA v GG) 0.008
				(AA v AG) ns
Fat mass (g)	28587 ± 1215	24714 ± 1227	18519 ± 3753	0.014
N=	n=46	n=32	n=6	0.009
				0.035
Lean mass (g)	36156 ± 791	36381 ± 908	33894 ± 1442	ns
N=	n=46	n=32	n=6	ns
				ns
Serum leptin concentration	20.76 ± 1.88	18.31 ± 2.10	25.27 ± 2.83	ns
ng/ml N=	n=40	n=33	n=7	ns
Serum LBA (%)	10.41 ± 0.50	10.37 ± 0.54	11.53 ± 2.36	ns
N=	n=35	n=18	n=4	ns

Table 3.3: Mean levels of BMI, fat mass, lean mass, serum leptin levels and serum LBA for each genotype of the LYS109ARG SNP. P values were calculated by comparing non-carriage of the A allele (AA+AG v GG), AA v GG and AA v AG genotypes using a Mann-Whitney U test. [1] ± standard error of mean

	Genotype			P value
	AA	AG	GG	
BMI (kg/m^2)	26.2 ± 0.07 [1]	26.75 ± 0.7	24.3 ± 0.88	(AA + AG versus GG)0.009
N=	n=24	n=37	n=15	
Fat mass (g)	26393 ± 1325	28859 ± 1411	22051 ± 1875	0.006
N=	n=30	n=43	n=15	
Lean mass (g)	35990 ± 1049	36613 ± 1047	36186 ± 1138	ns
N=	n=30	n=43	n=15	
Serum leptin concentration	18.3 ± 2	23.8 ± 2.5	12.6 ± 1.6	0.0001
ng/ml N=	n=26	n=43	n=15	
Serum LBA (%)	10.5 ± 0.50	9.7 ± 0.5	11.6 ± 0.9	0.04
N=	n=21	n=18	n=4	

Table 3.4: Mean levels of BMI, fat mass, lean mass, serum leptin levels and serum LBA for each genotype of the GLN223ARG SNP (Quinton *et al.*, 2001). P values were calculated by comparing non carriage of the A allele (AA + AG versus GG).
[1] ± standard error of mean

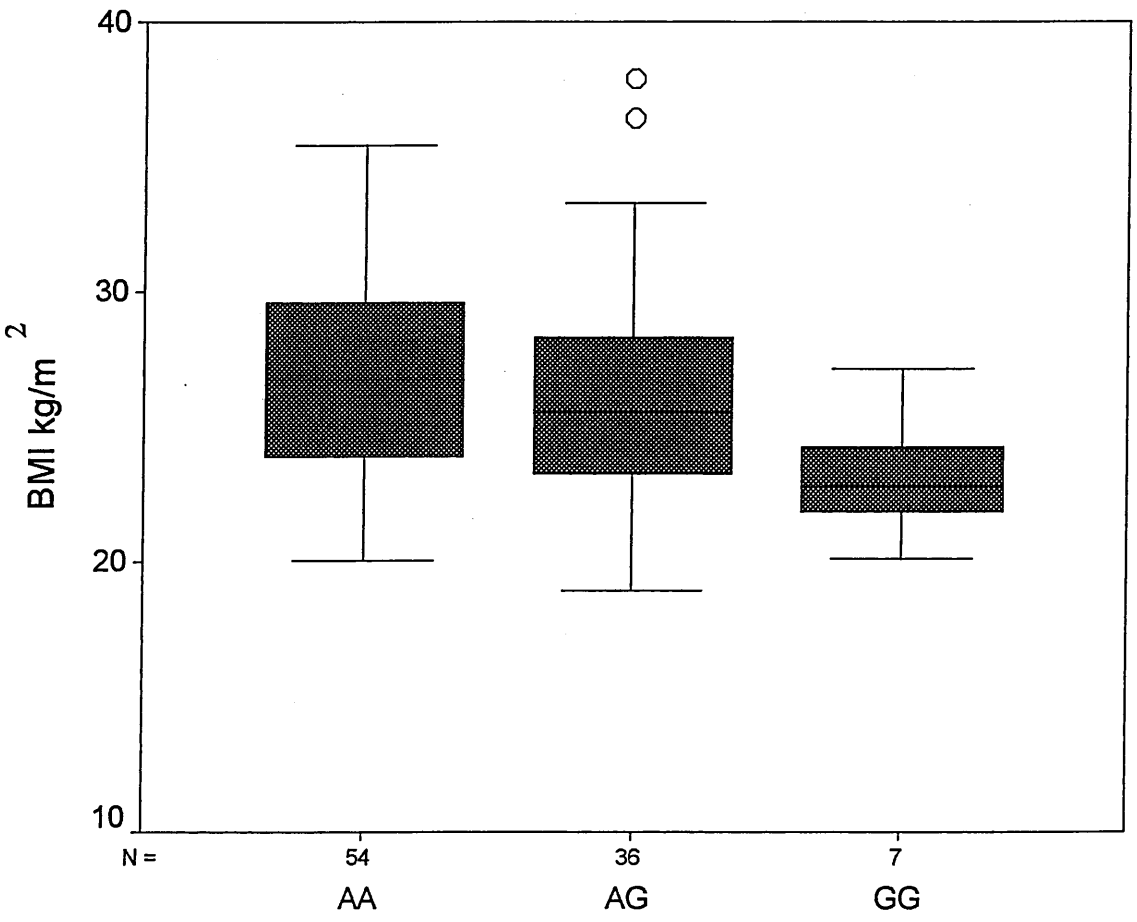


Figure 3.2: Boxplot of BMI data grouped to AA, AG and GG genotypes of the LYS109ARG SNP. The mean of the GG homozygous group (non-carriage of the A allele) is significantly lower than the mean of the combined AA/AG genotype groups ($p = 0.017$). The mean of the GG group is also significantly lower than the AA genotype group ($p=0.008$).

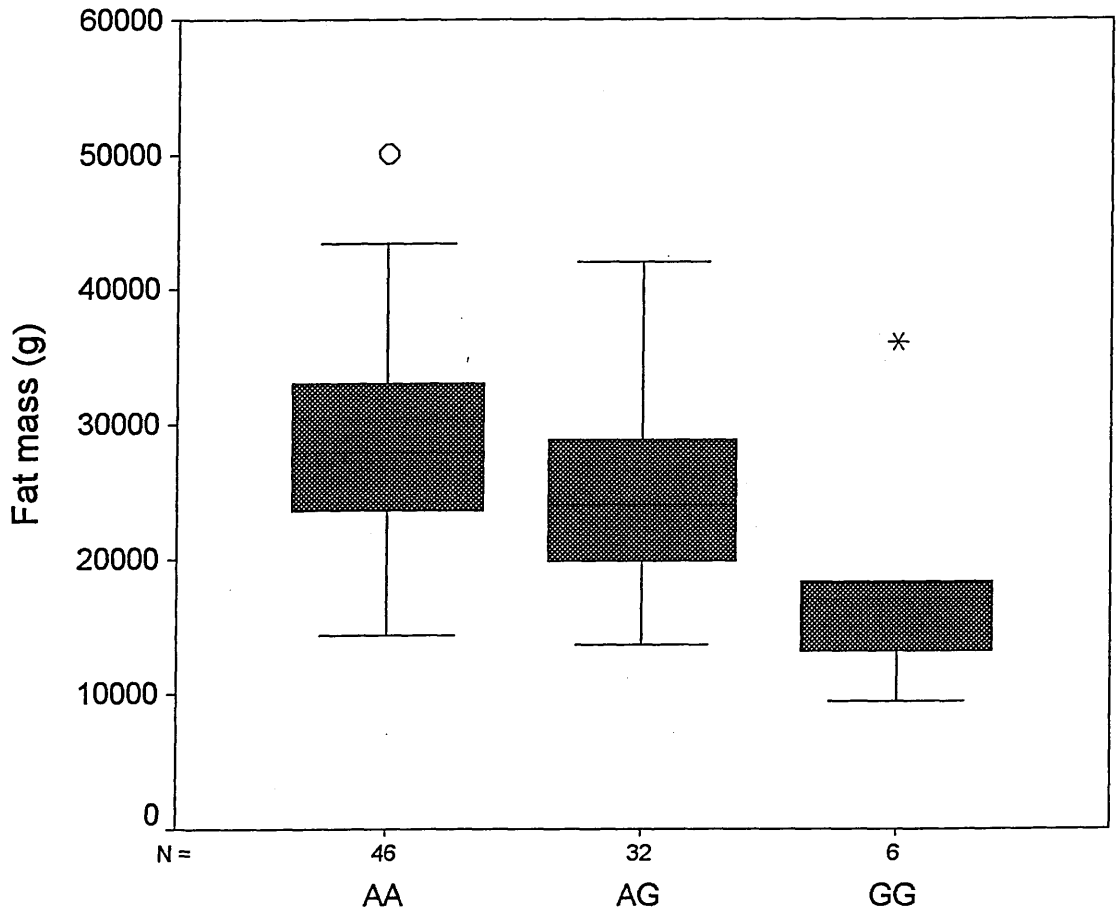


Figure 3.3: Box plot displaying fat mass levels for individuals grouped to the AA, AG and GG genotypes for the LYS109ARG SNP. The mean of the GG homozygous group (non-carriage of the A allele) is significantly lower than the mean of the combined AA/AG genotype groups ($p = 0.014$). The mean of the GG group is significantly lower than the AA genotype group ($p=0.009$). The mean of the GG group is also significantly lower than the AG genotype group (0.035).

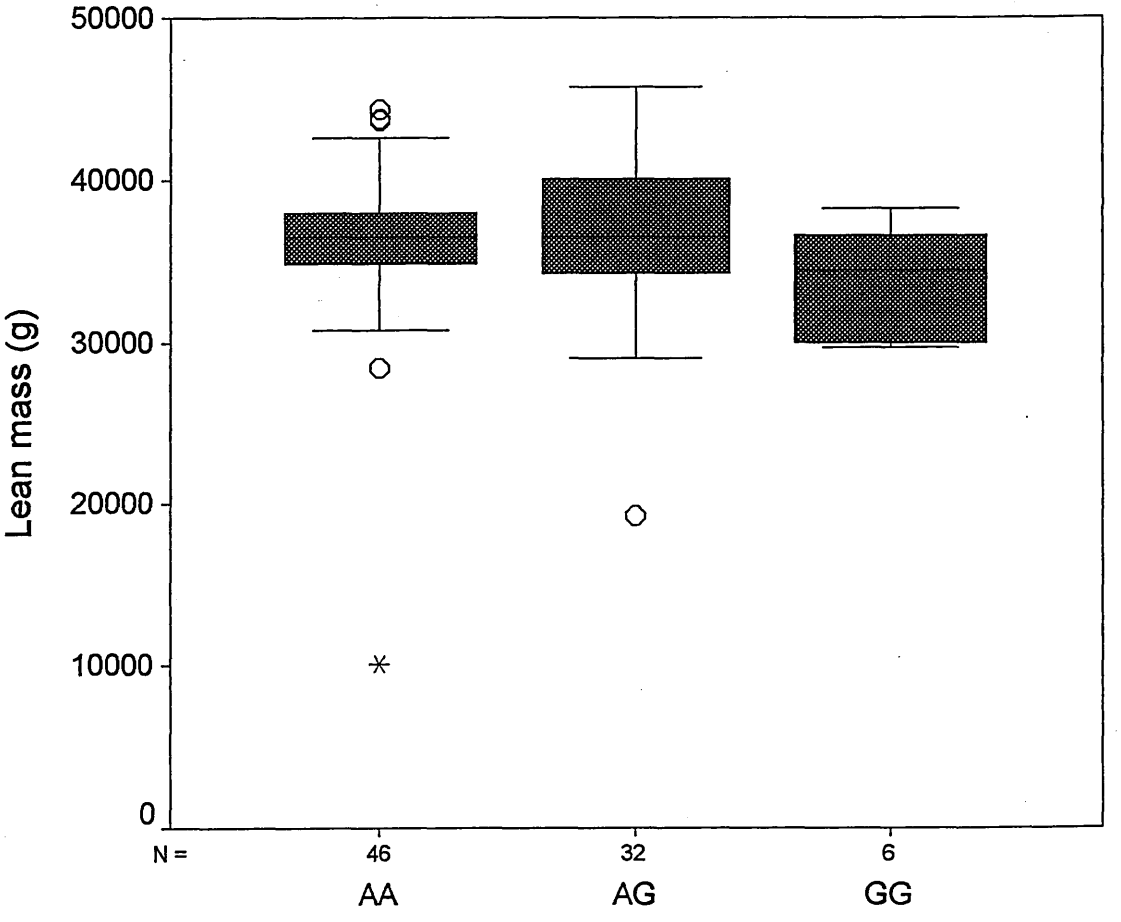


Figure 3.4: Box plot displaying lean mass levels for individuals grouped to AA, AG and GG genotypes for the LYS109ARG SNP. There is no significant difference in mean levels between groups.

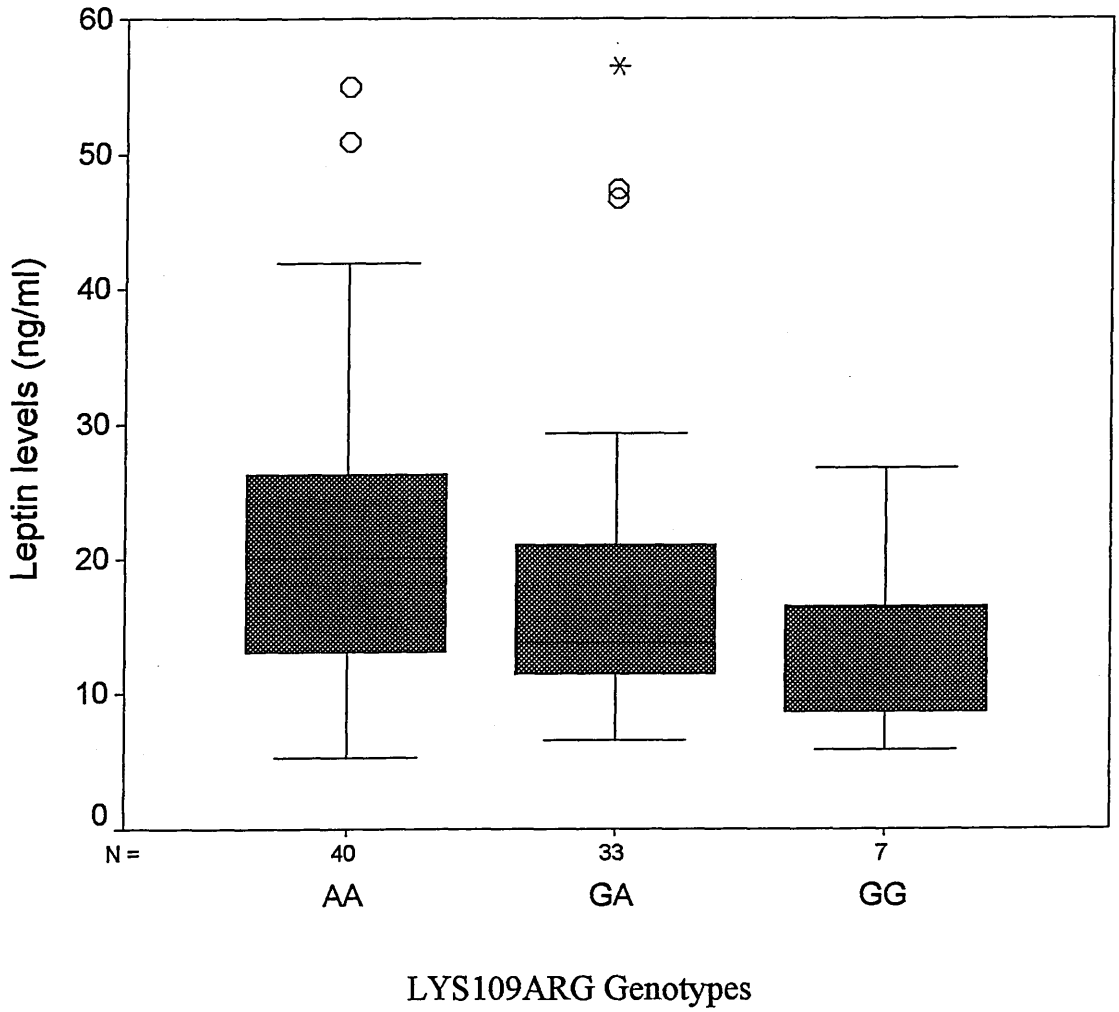


Figure 3.5: Box plot displaying leptin levels for individuals grouped to AA, AG and GG genotypes for the LYS109ARG SNP. There is no significant difference in mean levels between groups.

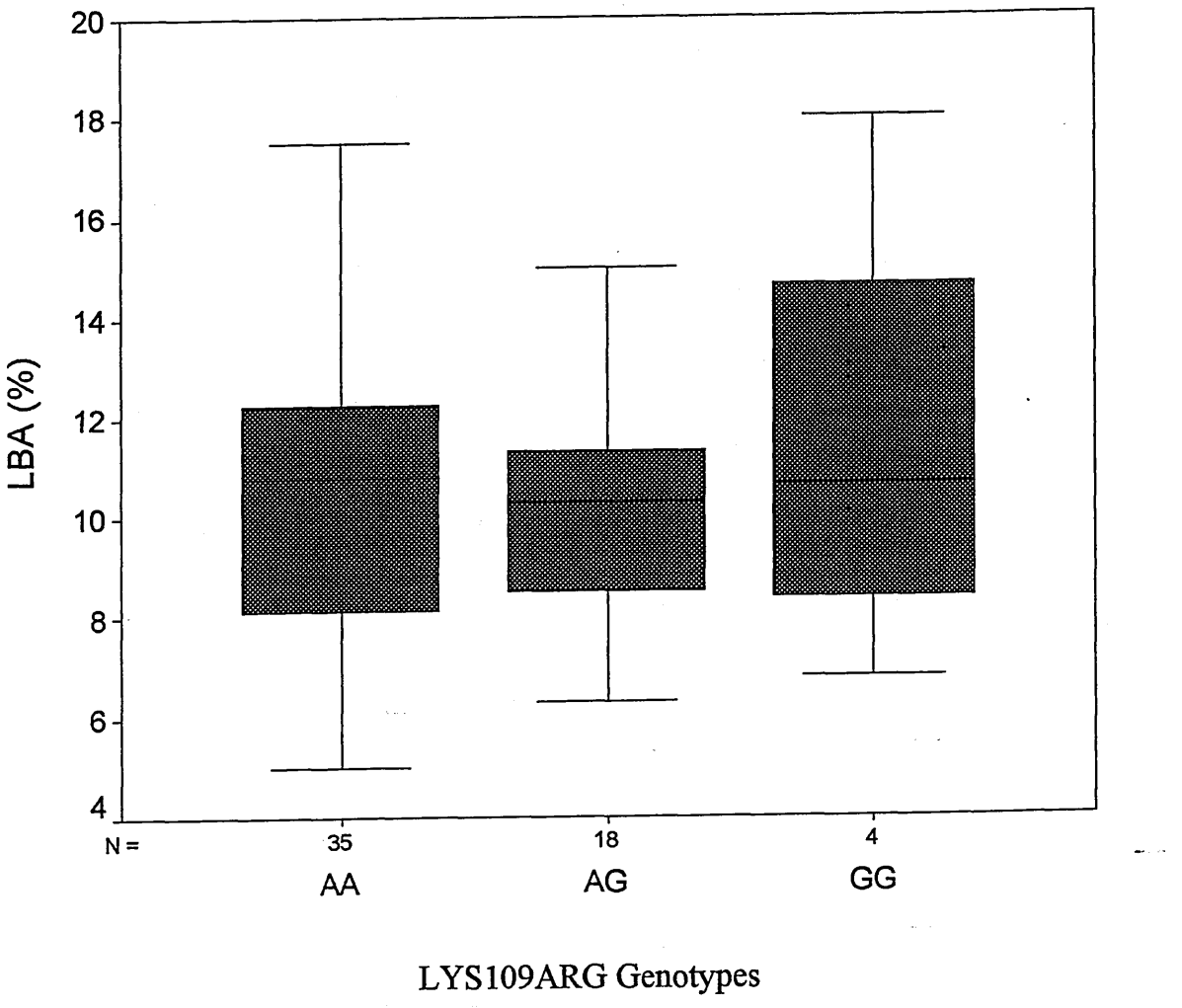


Figure 3.6: Box plot displaying LBA for individuals grouped to AA, AG and GG genotypes for the LYS109ARG SNP. There is no significant difference in mean levels between groups.

3.3.2 LYS656ASN polymorphism

To determine allele and genotype frequencies, 191 subjects have been genotyped for the LYS656ASN SNP. The calculated allele frequencies (p = 0.77, q = 0.23) agree with previous studies (see table 3.5). The observed balance of homozygous and heterozygous genotypes for this SNP did not differ significantly from that expected of a population in Hardy-Weinburg equilibrium (see table 3.6).

Mann-Whitney U tests were again used to compare mean values for each variable between genotype groups (table 3.7). Boxplots for each variable are shown in figures 3.7-3.11.

Allele	**Post-Menopausal women n=191 (This study)**	Lean British Males (n=132) *Gotoda et al.*, 1997	Obese British Males (n=190) *Gotoda et al.*, 1997	Caucasian Males (n=178) *Chagnon et al.*, 2000
G	**0.77**	0.84	0.82	0.82
C	**0.23**	0.16	0.18	0.18

Table 3.5: Comparison of allele frequencies for LYS656ASN with published results

	Observed genotype frequency	**Expected genotype frequency**
	n	n
Heterozygotes	62	68
Homozygous G	116	113
Homozygous C	13	10
Total	191	191

Table 3.6: Predicted genotype results obtained from for Hardy-Weinburg equation for LYS656ASN using the determined allele frequencies. The p-value for the χ^2 analysis (1 degree of freedom) comparing the expected to the observed genotype frequencies was 0.242.

	Genotype			P value
	GG	**GC**	**CC**	
BMI (kg/m^2)	26.04 ± 0.56 [1]	26.74 ± 0.78	26.10 ± 1.44	(GG+GC v CC) ns
N=	n=54	n=31	n=8	GG v CC ns
				(GC v CC) ns
Fat mass (g)	25602 ± 1063	27299 ± 1929	28785 ± 3676	ns
N=	n=50	n=24	n=8	ns
				ns
Lean mass (g)	36290 ± 765	35724± 703	35631 ± 2621	ns
N=	n=50	n=24	n=8	ns
Serum leptin				ns
concentration	17.68 ± 1.50	20.90 ± 2.54	25.27 ± 9.22	ns
ng/ml N=	n=52	n=22	n=5	ns
				ns
Serum LBA (%)	10.08 ± 0.55	10.90 ± 0.66	10.91 ± 1.02	ns
N=	n=24	n=23	n=7	ns

Table 3.7: Mean levels of BMI, fat mass, lean mass, serum leptin levels and serum LBA for each genotype of the LYS656ASN SNP. P values were calculated by comparing non-carriage of the G allele (GG+GC v CC), GG v CC and GC v CC genotypes using a Mann-Whitney U test. [1] ± standard error of mean

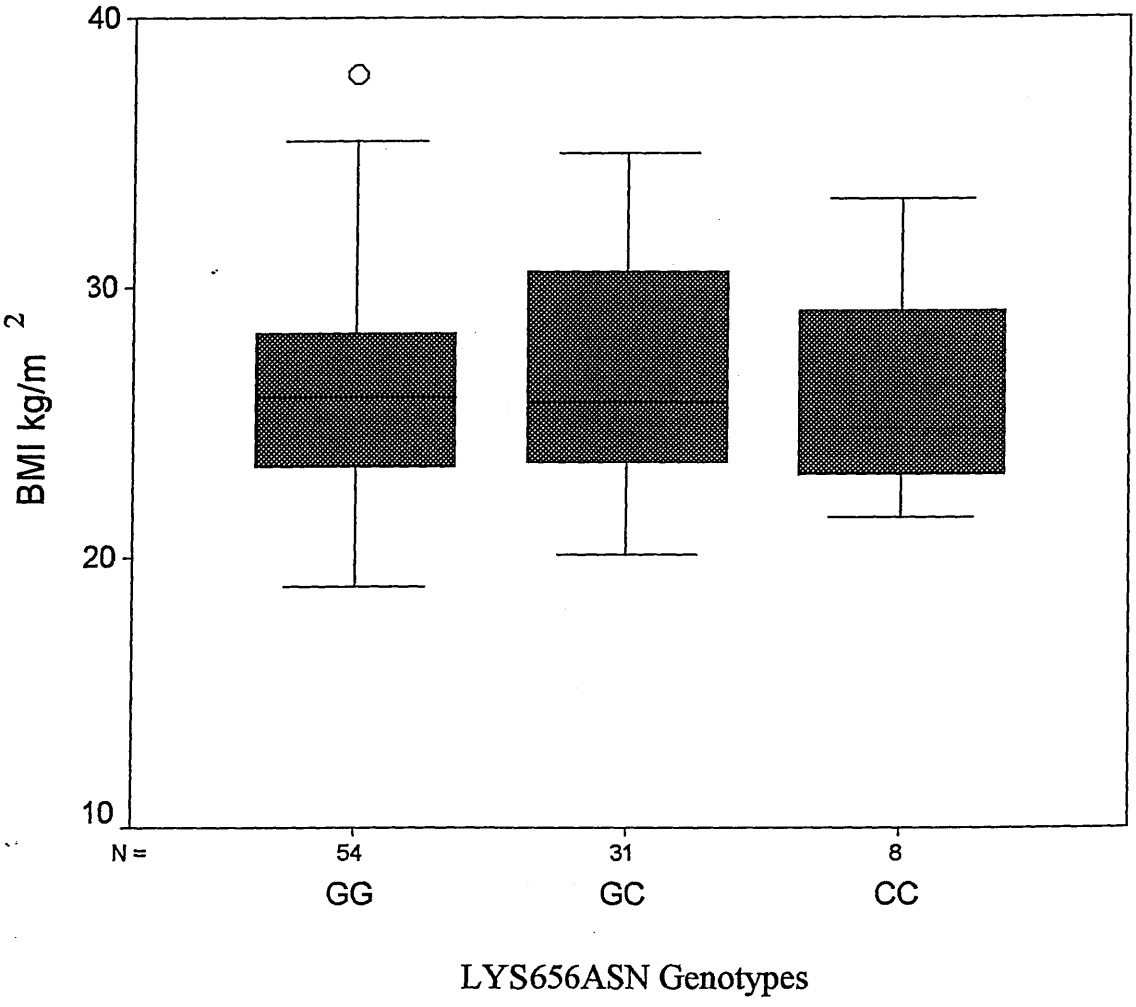


Figure 3.7: Boxplot of BMI data grouped to GG,GC and CC genotypes of the LYS656ASN SNP. There is no significant difference in mean levels between groups.

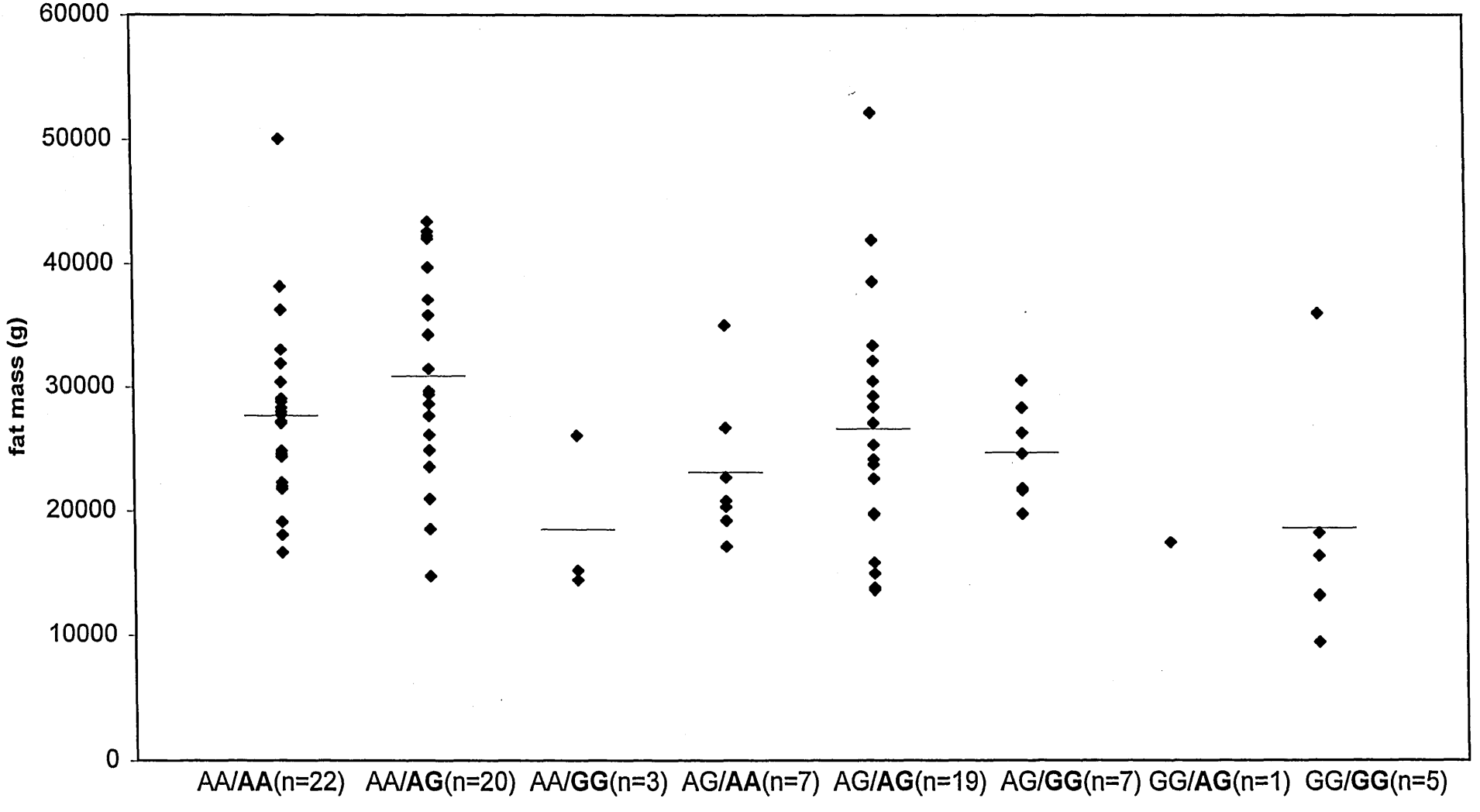


Figure 3.12: Graph displaying the fat mass of individuals with different combinations of the LYS109ARG and GLN223ARG genotypes (GLN223ARG genotype shown in bold and the horizontal bars represent the mean value for each group).

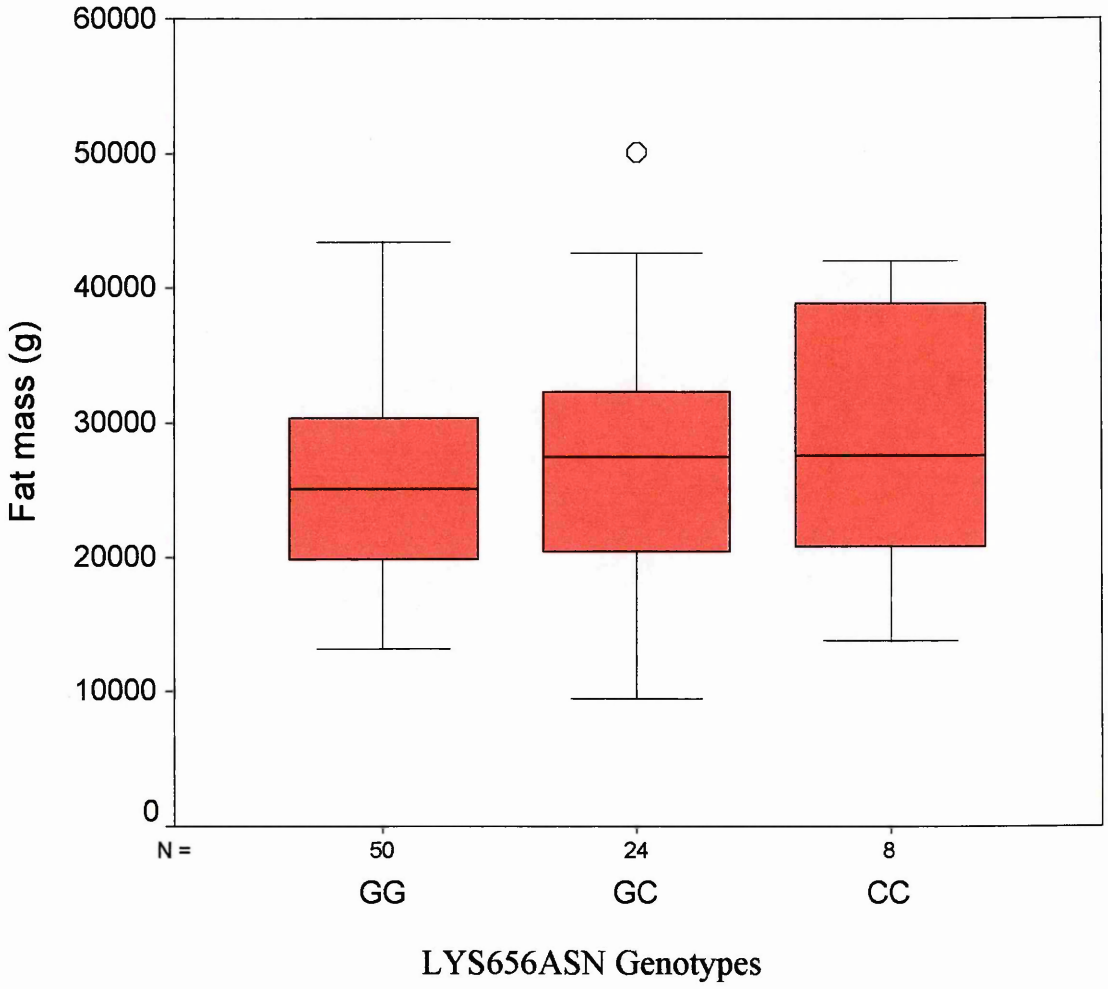


Figure 3.8: Box plot displaying fat mass levels for individuals grouped to GG, GC and CC genotypes for the LYS656ASN SNP. There is no significant difference in mean levels between groups.

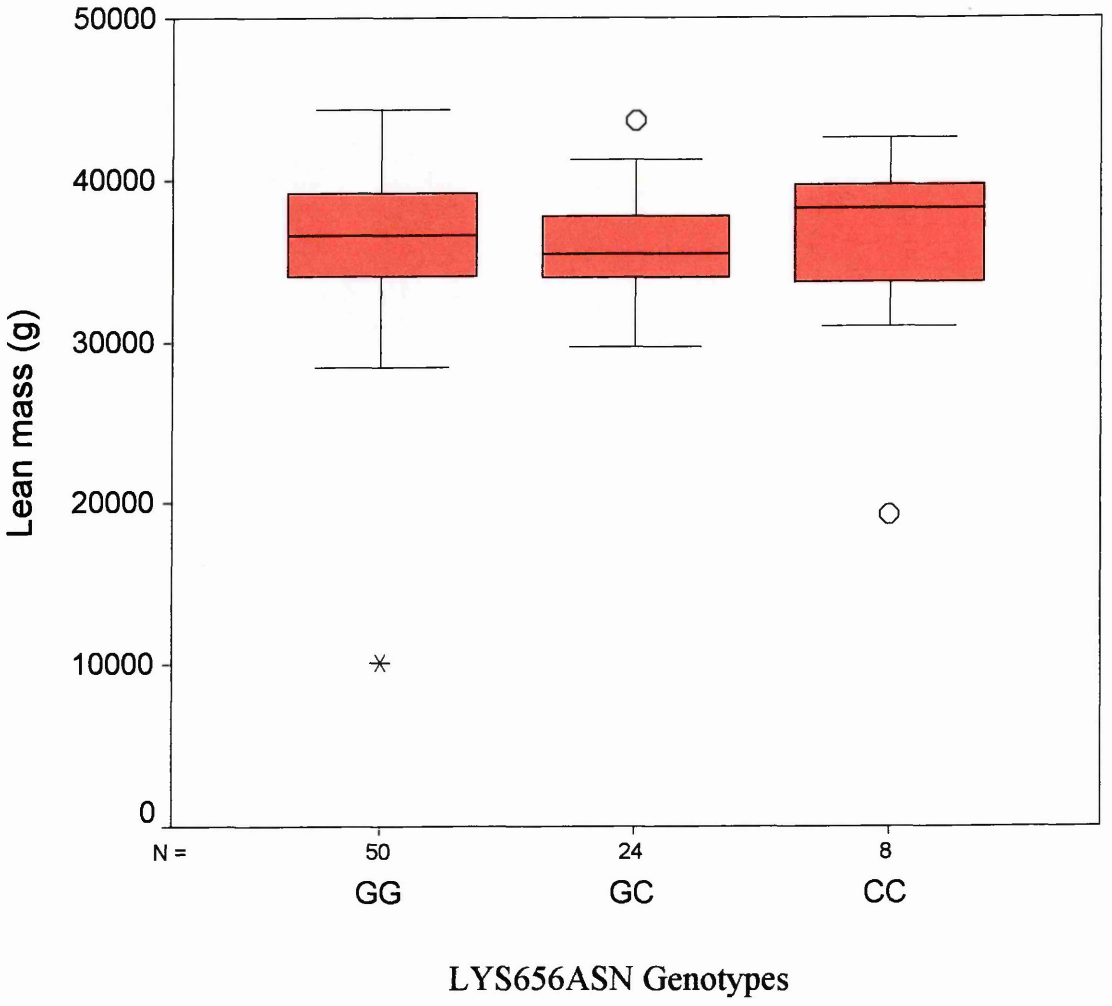


Figure 3.9: Box plot displaying lean mass levels for individuals grouped to GG, GC and CC genotypes for the LYS656ASN SNP. There is no significant difference in mean levels between groups.

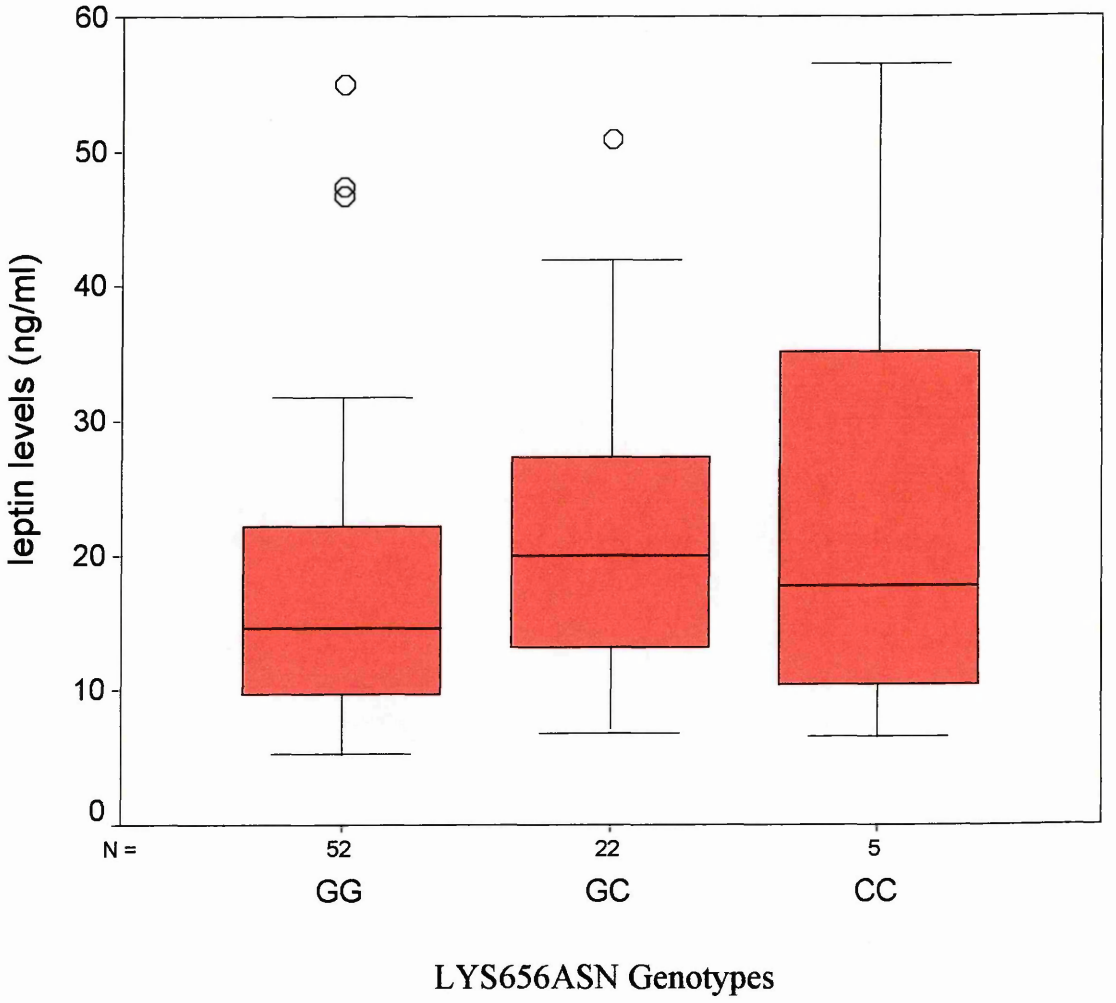


Figure 3.10: Box plot displaying leptin levels for individuals grouped to GG, GC and CC genotypes for the LYS656ASN SNP. There is no significant difference in mean levels between groups.

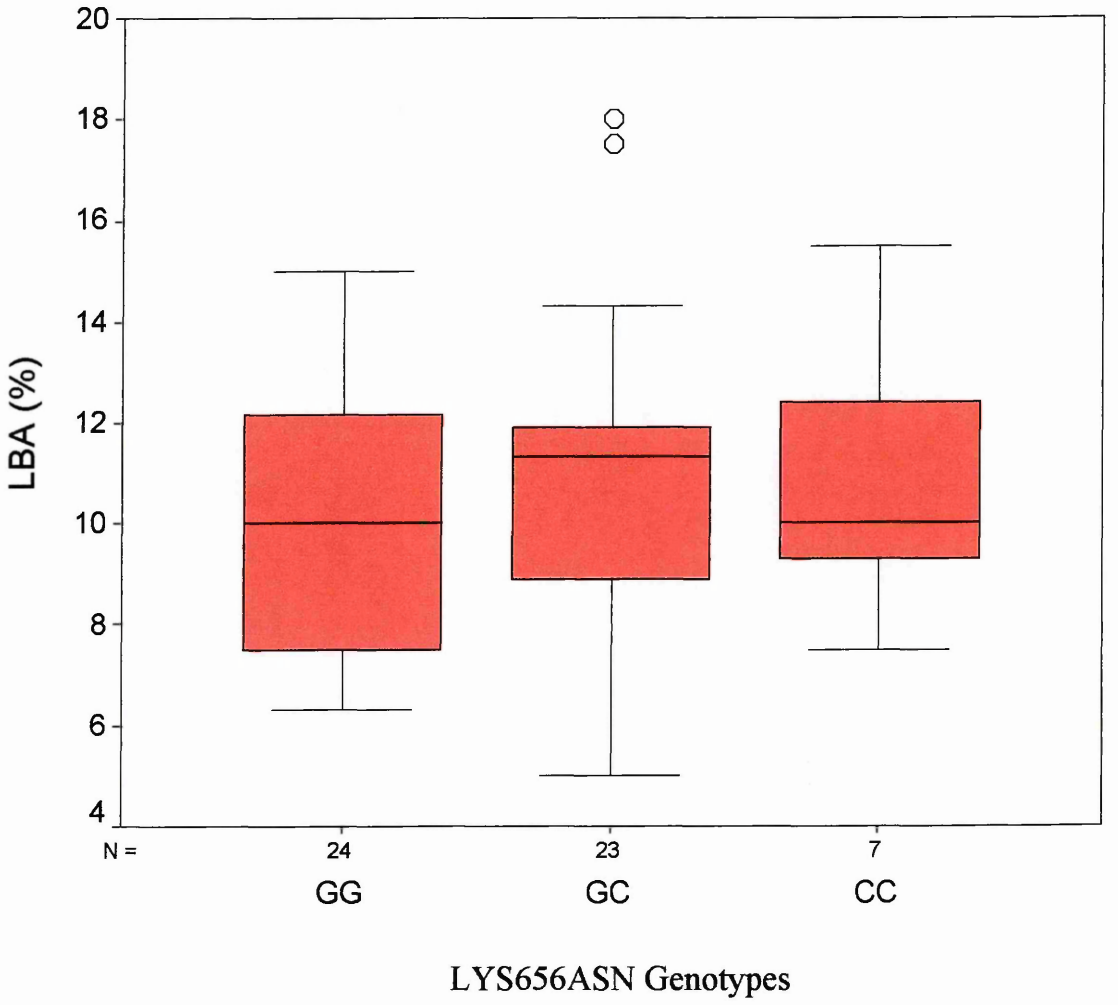


Figure 3.11: Box plot displaying LBA activity for individuals grouped to GG, GC and CC genotypes for the LYS656ASN SNP. There is no significant difference in mean levels between groups.

3.4 Haplotype results

Other genetic studies have detected that *LEPR* SNPs are in linkage disequilibrium (Thompson *et al.*,1997, Gotoda *et al.*, 1997).

Following genotyping for the LYS109ARG and LYS656ASN SNPs (in addition to the previously typed GLN223ARG), it is possible to determine whether alleles of the three SNPs are in linkage disequilibrium in this population. Firstly, the haplotype frequencies were calculated for individuals typed for all three SNPs using the EH software (see table 3.8). Using the 2LD software, D' was subsequently calculated for the different pairwise SNP combinations (table 3.9). The largest value (0.64), indicating the most significant linkage disequilibrium, was obtained from the genotypes of the LYS109ARG and GLN223ARG SNPs. A χ^2 analysis of D between SNPs revealed that linkage disequilibrium between alleles of the LYS109ARG and GLN223ARG SNPs was statistically significant ($p<0.00001$), whilst the other pairwise combinations were not (table 3.10).

As described, the mean fat mass was seen to be statistically different for individuals homozygous for the G allele of the LYS109ARG SNP compared to the AG heterozygotes or the A homozygotes. The data were analysed in conjunction with the genotypes previously obtained for the GLN223ARG SNP for which an association with the G allele and fat mass was observed. The mean fat mass was lowest in individuals who were homozygous for the G allele in both SNPs compared to the other genotype combinations for the LYS109ARG and GLN223ARG SNPs (see figure 3.12).

Haplotype			Cohort
LYS109ARG	GLN223ARG	LYS656ASN	
A	A	G	0.41
A	A	C	0.13
A	G	G	0.14
A	G	C	0.08
G	A	G	0.05
G	A	C	0
G	G	G	0.17
G	G	C	0.03

Table 3.8: Estimated haplotype frequencies determined for study cohort

SNP combination	D'
109 + 223	0.64
109 + 656	0.34
223 + 656	0.03

Table 3.9: D' values calculated by pairwise analysis of SNPs by 2LD software

SNP combination	p value
109 + 223	<0.00001
109 + 656	0.12
223 + 656	0.76

Table 3.10: P values for the D values which were calculated by pairwise analysis

3.5 Discussion

Possible genetic associations with indicators of adiposity in a cohort of postmenopausal European Caucasian women were investigated. The availability of this cohort was advantageous as postmenopausal women may be less susceptible to hormonal influences. Sex hormones could modify leptin levels and adiposity in women who go through the reproductive cycle. Furthermore, a gender specific study may also clarify analysis. For example, Mammes and colleagues (2001) suggested a gender-gene interaction when they reported an association between the 'silent' SER343SER SNP and fat mass in the *LEPR* gene with Caucasian women only, from a mixed gender cohort. As this is a silent change they hypothesised that the SNP may be in association with a functional polymorphism lying in the regulatory region of the *LEPR* gene.

No associations were detected between the LYS656ASN SNP and the various, continuous variable data. However, associations were detected for both fat mass and BMI with the G allele of the LYS109ARG SNP (which codes for the arginine amino acid). That associations were highlighted for both these indicators of adiposity was advantageous, as BMI measurements do not readily distinguish between fat and lean mass. Therefore fat mass is a more accurate measurement of adiposity.

Linkage disequilibrium analysis suggests that the LYS109ARG and GLN223ARG SNPs are in linkage disequilibrium.

Quinton *et al.*, (2001) highlighted positive associations with fat mass, BMI and leptin levels and the GLN223ARG SNP. Because linkage disequilibrium has been established between the two SNPs it is unclear which SNP is having an effect or whether both are having an effect to varying degrees although the multiple associations might suggest that the GLN223ARG is of prime importance. One strategy to help clarify this issue is to carry out functional studies. The LBA assay goes some way towards addressing

this. LBA was significantly associated with GLN223ARG SNP in Quinton's study but not with the LYS109ARG SNP in this study of the same cohort. The LYS to ARG amino acid substitution is a conservative substitution with the same overall charge being maintained which would presumably have a minimal affect on protein function. Nevertheless, the LBA data are not conclusive as the number of individuals analysed for LBA was less than the number of individuals with recorded BMI and fat mass data. For the LYS109ARG SNP, fifty seven individuals were analysed for LBA whilst fat mass and BMI data were available for 84 and 92 individuals respectively. For the GLN223ARG SNP, LBA data were available for 60 individuals whilst fat mass and BMI data were available for 88 and 76 individuals respectively. Furthermore, other proteins in the serum (which could bind to leptin) may contribute to the LBA readings. Also, the amount of soluble leptin receptor was not quantified in the serum samples. It is possible, therefore, that the LBA data correlates with the GLN223ARG SNP because that SNP may be associated with higher levels of soluble receptor protein expression which would increase the LBA count, potentially masking any functional differences at the amino acid level.

Finally, conservative amino acid changes in the leptin receptor have been shown to create an effect. The GLN to PRO substitution at codon 269 (*fa* mutation) in the homologous rat leptin receptor protein maintains a neutral charge (at pH 6-7) but alters the signalling qualities of the receptor (Chua *et al.*, 1996, Phillips *et al.*, 1996). Clearly, association studies can contribute to our understanding of a condition but to augment and further clarify the situation, functional studies should be considered. *In vitro* analysis of ligand binding and / or receptor dimerisation characteristics, of proteins expressing different combinations of the SNPs would be one way of gaining further insights into the issue.

Chapter 4

Functional studies of the leptin receptor

4.1 Introduction

In the mid 1990s, ground-breaking studies involving the identification and characterisation of entities implicated in obesity took place. This was an essential precedent to our current, and evolving, understanding of the way humans regulate food intake and energy expenditure.

Following the discovery of the mouse *ob* gene product, leptin, by Zhang *et al,* (1994) the next step was to identify high affinity leptin-binding sites that would effectively locate the leptin receptor. Tartaglia and colleagues (1995) achieved this by generating leptin-AP (alkaline phosphatase) fusion proteins which located binding sites in the choroid plexus tissue of the mouse brain. This enabled screening of a cDNA expression library of the tissue, again with leptin-AP fusion proteins and identification of a 5.1kb cDNA insert that codes for the leptin receptor in mice. The open reading frame encoded a single membrane-spanning protein with a mature extracellular domain predicted to contain 816 amino acids (minus a 22 amino acid secretion signal sequence). A 23 amino acid transmembrane domain and an intracellular domain of 34 amino acids were also predicted. Afterwards, Chua and colleagues (1996) isolated clones coding for intracellular domains of varying length. Using the cDNA insert as a probe, a human cDNA homologue was identified and the predicted amino acid sequence was deduced to be 78% identical to that of the mouse form.

Subsequent work further characterised the properties of the leptin receptor. Lua and colleagues (1997) expressed and characterised both a full length and putative high affinity human soluble leptin receptor lacking the transmembrane and intracellular domains. The purified soluble protein product was sized at 130-150kDa as determined by Western blot analysis and Coomassie staining. As the native protein was predicted

to be around 94kDa, the protein was assumed to be extensively glycosylated. Expression of the soluble form of the receptor in CHO cells and subsequent de-glycosylation studies highlighted a heavily N-glycosylated protein with minimal O-glycosylation (Haniu *et al.*, 1998).

As any mutations in the receptor were a potential molecular basis for dysregulation of body weight, studies were conducted to detect mutations in the human *LEPR* gene which, in particular, caused obesity. Although few gross mutations were discovered (Clement *et al.*,1998), several SNPs were discovered, some of which code for amino acid substitutions throughout the protein (Gotoda *et al.*, 1997, Thompson *et al.*, 1997). Of the amino acid substitutions that occur in the extracellular domain, one SNP codes for a LYS109ARG amino acid substitution, that is present in a cytokine homology domain of the protein. Another codes for the GLN223ARG substitution found within a loop region that is located between cytokine homology and fibronectin type III domains. Finally, a LYS656ASN amino acid change resides within a further fibronectin type III domain.

In genetic studies, these SNPs have been associated with traits of obesity. Specifically, the GLN223ARG SNP has been highlighted by Chagnon *et al.*,1999, Chagnon *et al.*, 2000, Yiannakouris *et al.*,2001 and Quinton *et al.*, 2001. The LYS109ARG SNP has been highlighted in this present study and the LYS656ASN SNP has been highlighted by Wauters *et al.*, 2001. Due to the phenomenon of linkage disequilibrium, however, it is not clear-cut whether any positive associations found for one specific SNP are functionally significant or are actually due to another nearby SNP or a group of SNPs acting in unison. Quinton and colleagues (2001) measured leptin binding activity in serum from individuals (using a radiolabelled leptin ligand) who had been genotyped for the GLN223ARG SNP in the *LEPR* gene. Greater activity was associated with the

ARG amino acid. However the contribution of other proteins in the serum cannot be discounted from the radioactivity count.

One potential way of tackling these issues is to engineer and then purify variant leptin receptor proteins, expressing different combinations of the SNPs, which would allow them to be analysed individually.

4.1.1 Aims of study

The aims of this study are:

1. to engineer variants of the leptin receptor protein that include different amino acids corresponding to SNPs in the coding region of the *LEPR* gene;
2. to characterise these protein variants;
3. to measure the ability of these variants to bind leptin via a radioactive leptin binding assay.

4.2 Study Design

4.2.1 PCR amplification and cloning of soluble *LEPR*

In the first instance, the coding DNA sequence for the soluble form of the human leptin receptor protein was obtained from HEK293 cells (human embryonic kidney cells) as previous work in the laboratory established that these cells express the protein (unpublished observations). RNA was obtained from these cells and RT-PCR carried out to obtain the cDNA for the soluble form of *LEPR* (See chapter 2, sections 2.4.1 and 2.4.2). The PCR primers were designed to amplify a product of 2457bp. This contains sequence coding for codons 23 to 841 of the extracellular domain of the

LEPR cDNA sequence with the forward primer containing additional sequence to generate a *Bam*HI restriction enzyme site for ease of cloning. Figure 4.1 shows an agarose gel of the PCR reaction highlighting a single band of the predicted size representing the soluble *LEPR* cDNA whilst no DNA is seen in the lane containing a control sample lacking a cDNA template.

This DNA was subsequently cloned into the pGEM -T vector (Promega) (see chapter 2, section 2.5.1). This vector is amenable to the cloning of PCR products because it is provided in a linearised form due to a restriction enzyme digest, after which single thymine base overhangs are added to the 5' positions. During PCR, *Taq* DNA polymerase adds adenine base overhangs when the enzyme reaches the end of a DNA fragment, so that PCR products have an unpaired adenine at the 3' ends. As a result, during a DNA ligation reaction, the adenine and thymine base overhangs of the PCR product (insert) and vector can form complementary base pairings. Once the PCR product has been ligated into the plasmid, subsequent digestions, to enable sub-cloning for example, are greatly facilitated because restriction enzymes work most efficiently when a sufficient length of DNA flanks the target restriction sequences. Therefore, in this scenario, it can be assured that the insert DNA sequence will have undergone complete digestion when compared to enzyme digestion of sites towards the termini of PCR fragments. A plasmid map of the pGEM-T vector and its multiple cloning site region is shown in figure 4.2. Figure 4.3 shows an agarose gel containing restriction digestions of recombinant pGEM-T vector. It displays the successful cloning of the *LEPR* cDNA insert into the pGEM-T vector, with lane B showing 2 bands representing the vector and insert respectively and lane C showing a band representing the linearised form of the vector + insert.

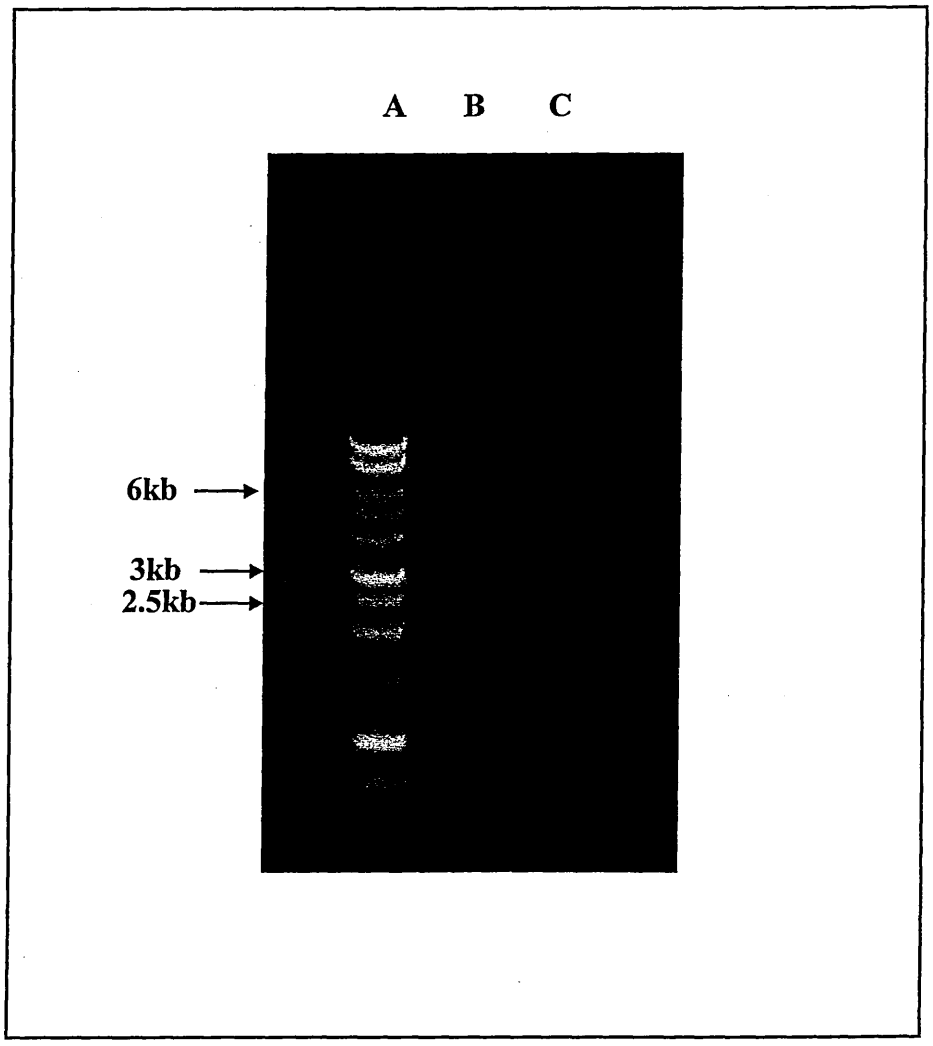
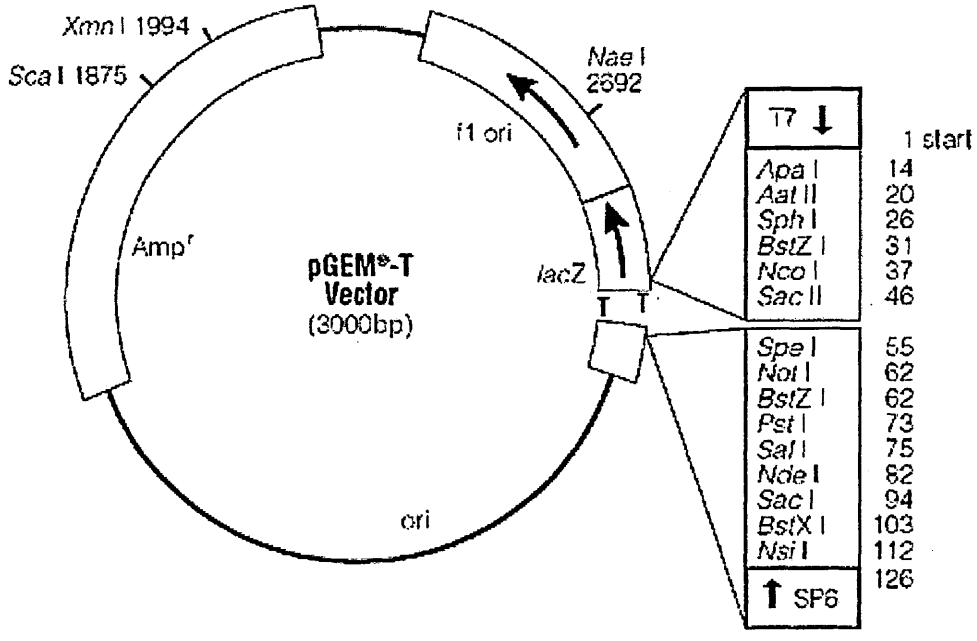


Figure 4.1: Photograph of *LEPR* RT-PCR product on a 1% agarose gel containing, **A**: 1kb DNA marker (Promega), **B**: *LEPR* RT-PCR product (2547bp) and **C**: negative control lacking a DNA template.



pGEM-T Multiple Cloning Site Region

T overhang *Bam* HI *Spe* I

CCATG GCCGC GGGATT (GGATCC CLONED INSERT) ATCAC

Pst I *Sal* I

TAGTG CGGCC GCCTG CAGGT CGAC C

Figure 4.2: Plasmid map of pGEM-T vector and DNA sequence of its multiple cloning site. The red and green highlighted sequences display the restriction enzyme sites used to sub-clone the *LEPR* cDNA sequence into the pCMV Tag-2B vector.

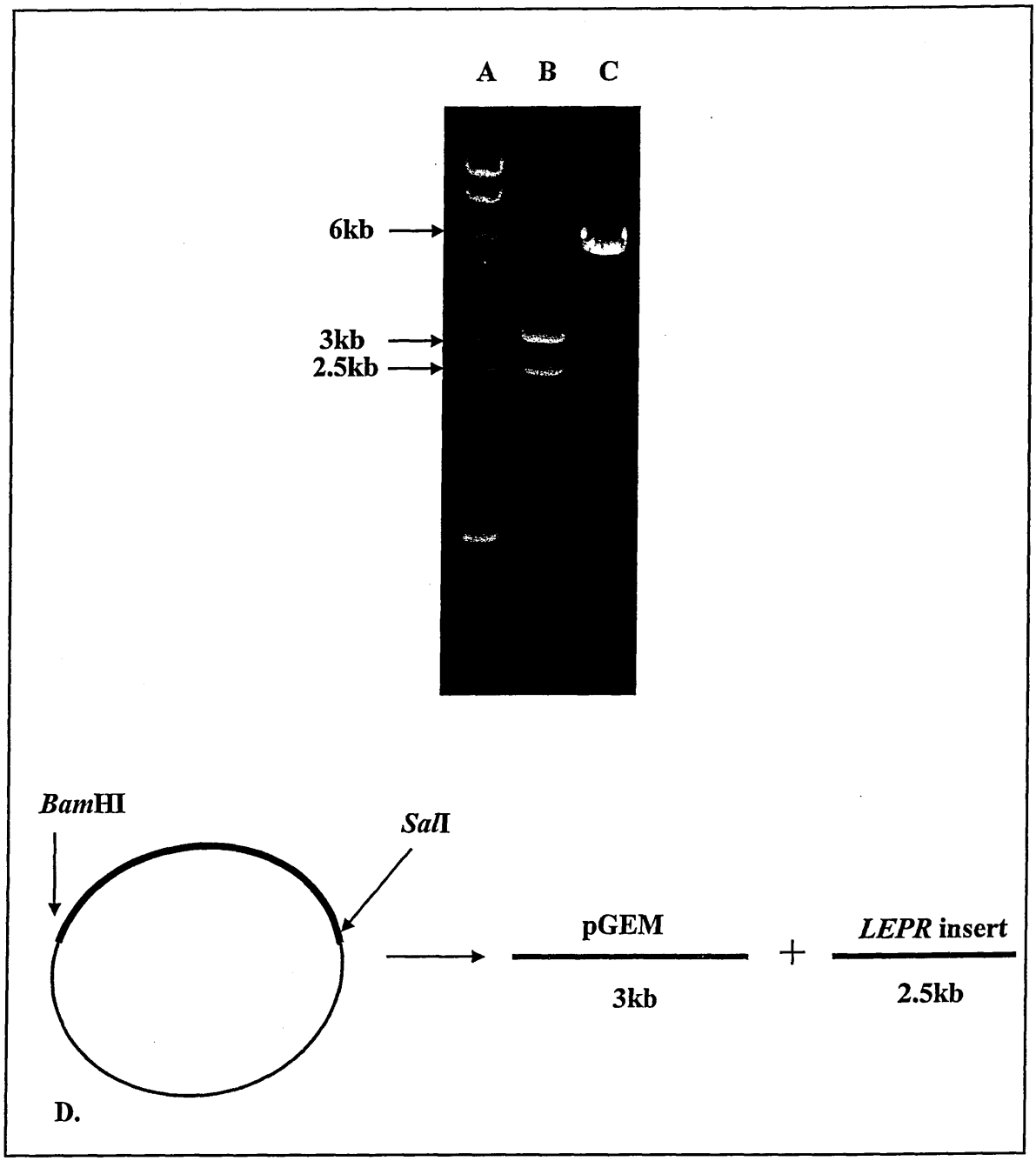


Figure 4.3: Photograph of recombinant pGEM-T vector on a 0.8% agarose gel containing, **A:** 1kb DNA marker (Promega), **B**: *Bam*HI and *Sal*I restriction enzyme digestion of recombinant pGEM-T vector and **C**: *Bam*HI restriction enzyme digestion of recombinant pGEM-T vector (5.5kb). Item **D** is a schematic representation of the *Bam*HI and *Sal*I restriction enzyme digestion of the recombinant pGEM-T vector.

The *Bam* HI restriction enzyme site present at the 5' end of the cloned *LEPR* cDNA insert and the *Sal* I restriction enzyme site present in the pGEM-T multiple cloning site region were used to sub-clone the insert into the pCMV-Tag 2B vector (see chapter 2, section 2.5.2). This is a eukaryotic expression vector able to facilitate the expression of cloned inserts in mammalian cells. Figure 4.4 depicts a map of the plasmid alongside its multiple cloning site region. The plasmid contains an SV40 origin of replication that enables it to replicate in large copy numbers in COS-7 cells because they express the viral SV40 large T antigen. A cytomegalovirus (CMV) promoter sequence to initiate translation is also present. Furthermore, the plasmid contains sequence coding for a FLAG tag (adjacent to the cloned insert) which is a hydrophobic sequence enabling the FLAG/insert fusion protein product to be affinity purified with monoclonal antibodies to the FLAG 'tag'.

Figure 4.5 shows an agarose gel containing restriction digestions of recombinant pCMV-Tag 2B vector DNA. It demonstrates the successful sub-cloning of the *LEPR* cDNA insert into the pCMV-Tag 2B vector with lane B showing a band representing the linearised form of the vector + insert and lane C showing two bands representing the vector and insert respectively.

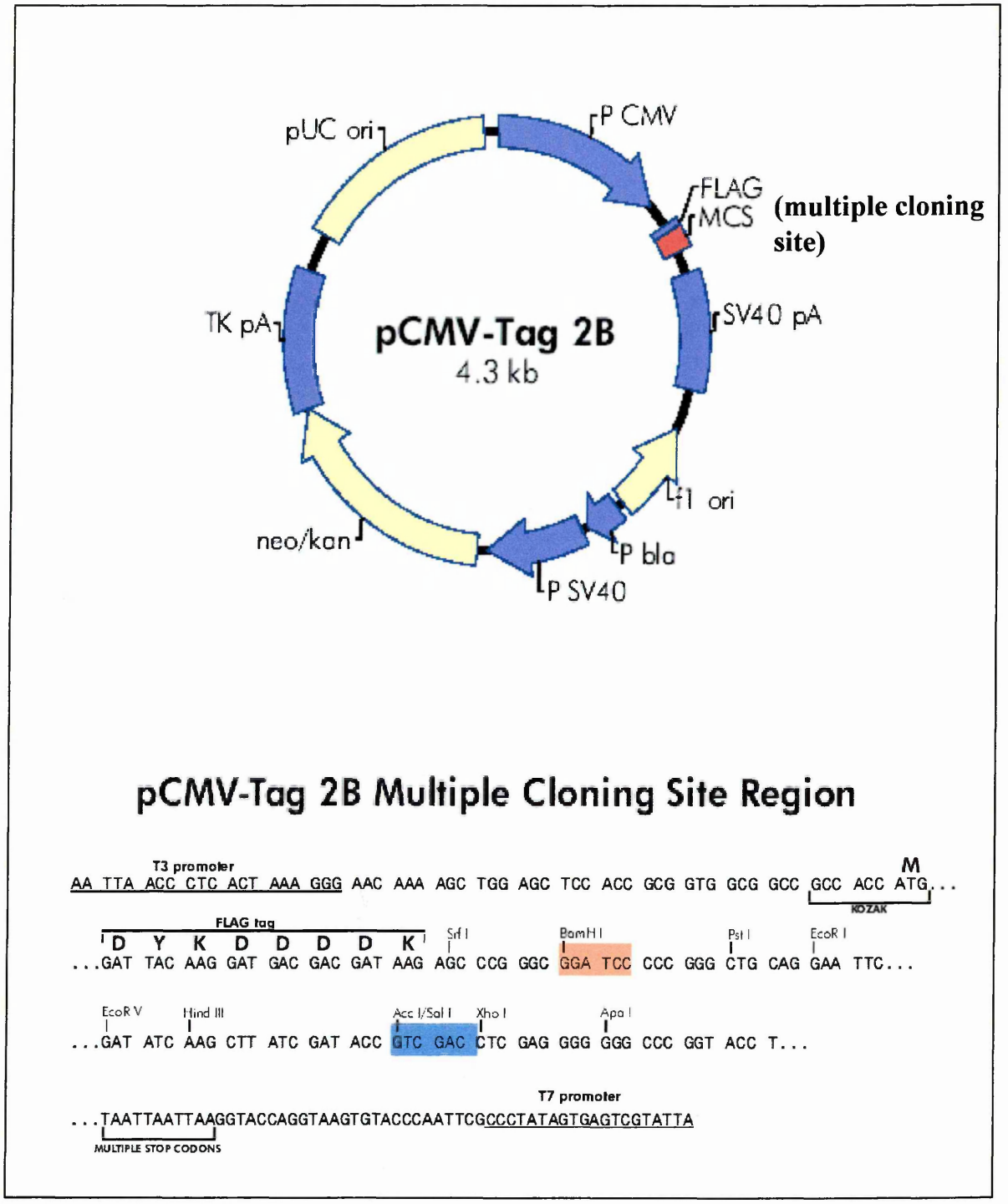


Figure 4.4: Plasmid map of pCMV-Tag 2B expression vector and sequence of multiple cloning site region containing restriction sites, highlighted in red and blue, used to insert *LEPR* cDNA.

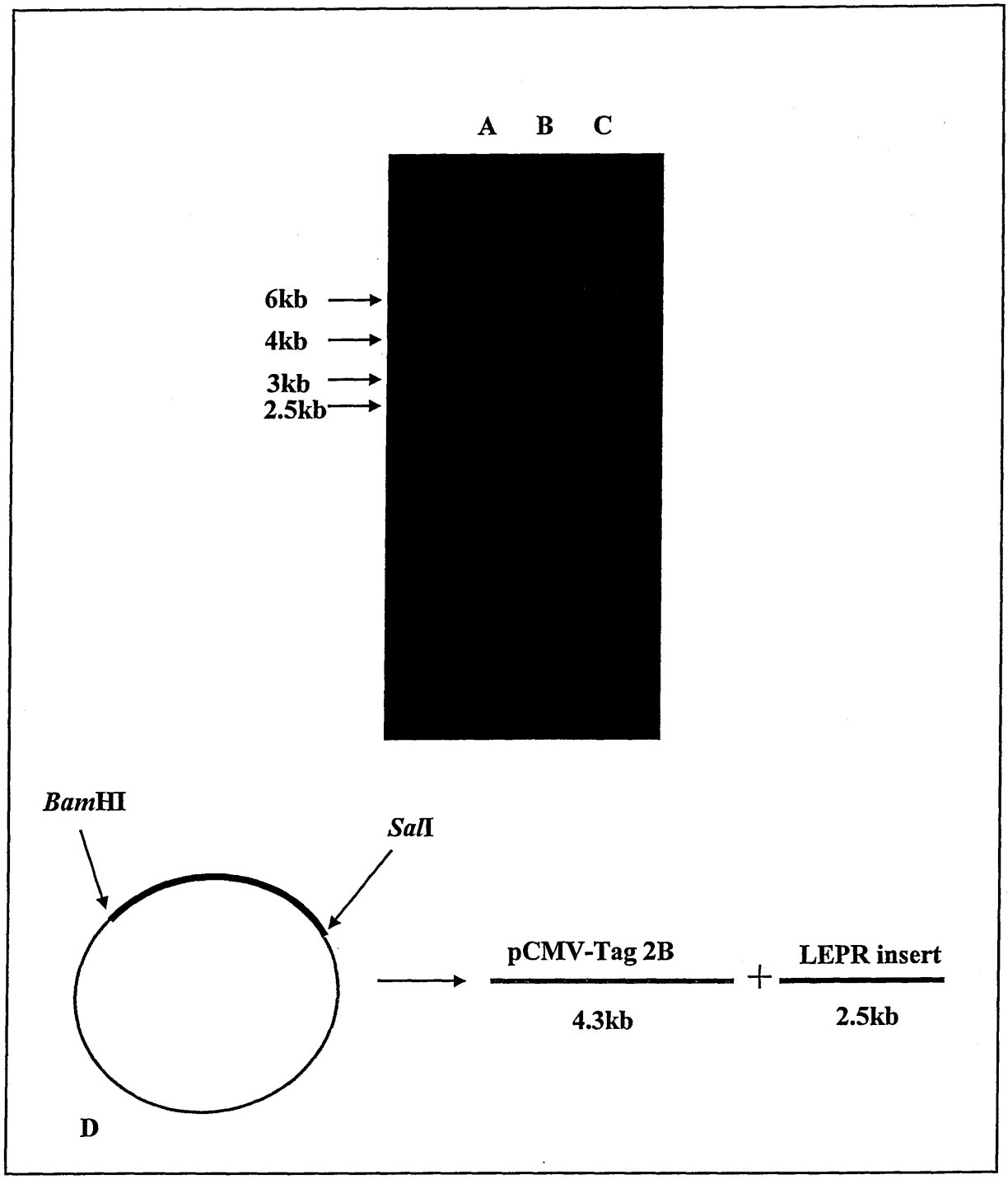


Figure 4.5: Photograph of recombinant pCMV-Tag 2B on a 1.8% agarose gel displaying , **A**:1kb DNA marker (Promega), **B**: *Bam*HI and *Sal*I restriction enzyme digestion of recombinant pCMV-Tag 2B vector and **C**: *Bam*HI restriction enzyme digestion of recombinant pCMV-Tag 2B vector (6.8kb).
Item **D** is a schematic representation of the *Bam*HI and *Sal*I restriction enzyme digestion of recombinant pCMV-Tag 2B.

The recombinant plasmid DNA was sent to the Applied Biotechnology Centre, Imperial College London, for DNA sequencing (see chapter 2, section 2.5.8) to confirm that the DNA sequence was that of the human *LEPR* cDNA sequence and that the insert was cloned in-frame relative to the multiple cloning site of the plasmid. The insert DNA sequence was translated into amino acid sequence using DNASTAR Editseq software (version 4.05). The translated amino acid sequence is shown in figure 4.6 alongside vector-encoded amino acids that would be expressed as part of the fusion protein and also with the different domains of the leptin receptor protein superimposed onto the amino acid sequence. The amino acid sequence is identical to that of codons 23 to 841 of the reference leptin receptor amino acid sequence (NCBI database accession number NP002294) with the exception that the arginine amino acid is present at codon 223 in the clone sequence compared to glutamine in the reference sequence, but this is simply a natural site of variation reflecting a SNP in the relevant position of the DNA sequence. The translated sequence demonstrates that the cloned insert is in fact the extracellular domain of the *LEPR* cDNA and that no mutations have been incorporated into the DNA sequence.

Figure 4.7 shows the DNA sequence across the pCMV-Tag 2B multiple cloning site / insert boundary and confirms that the insert is cloned in-frame in relation to the vector sequence and as such, the sequence will be expressed in the correct reading frame.

Met Asp Tyr Lys Asp Asp Asp Asp Lys Ser Pro Gly Gly Ser Asn Leu Ser Tyr
Pro Ile Thr Pro Trp Arg Phe Lys Leu Ser Cys Met Pro Pro Asn Ser Thr Tyr Asp
Tyr Phe Leu Leu Pro Ala Gly Leu Ser Lys Asn Thr Ser Asn Ser Asn Gly His Tyr
Glu Thr Ala Val Glu Pro Lys Phe Asn Ser Ser Gly Thr His Phe Ser Asn Leu Ser
Lys Thr Thr Phe His Cys Cys Phe Arg Ser Glu Gln Asp Arg Asn Cys Ser Leu Cys
Ala Asp Asn Ile Glu Gly **Lys** Thr Phe Val Ser Thr Val Asn Ser Leu Val Phe Gln
Gln Ile Asp Ala Asn Trp Asn Ile Gln Cys Trp Leu Lys Gly Asp Leu Lys Leu Phe
Ile Cys Tyr Val Glu Ser Leu Phe Lys Asn Leu Phe Arg Asn Tyr Asn Tyr Lys Val
His Leu Leu Tyr Val Leu Pro Glu Val Leu Glu Asp Ser Pro Leu Val Pro Gln Lys
Gly Ser Phe Gln Met Val His Cys Asn Cys Ser Val His Glu Cys Cys Glu Cys Leu
Val Pro Val Pro Thr Ala Lys Leu Asn Asp Thr Leu Leu Met Cys Leu Lys Ile Thr
Ser Gly Gly Val Ile Phe **Arg** Ser Pro Leu Met Ser Val Gln Pro Ile Asn Met Val Lys
Pro Asp Pro Pro Leu Gly Leu His Met Glu Ile Thr Asp Asp Gly Asn Leu Lys Ile
Ser Trp Ser Ser Pro Pro Leu Val Pro Phe Pro Leu Gln Tyr Gln Val Lys Tyr Ser Glu
Asn Ser Thr Thr Val Ile Arg Glu Ala Asp Lys Ile Val Ser Ala Thr Ser Leu Leu Val
Asp Ser Ile Leu Pro Gly Ser Ser Tyr Glu Val Gln Val Arg Gly Lys Arg Leu Asp
Gly Pro Gly Ile Trp Ser Asp Trp Ser Thr Pro Arg Val Phe Thr Thr Gln Asp Val Ile
Tyr Phe Pro Pro Lys Ile Leu Thr Ser Val Gly Ser Asn Val Ser Phe His Cys Ile Tyr
Lys Lys Glu Asn Lys Ile Val Pro Ser Lys Glu Ile Val Trp Trp Met Asn Leu Ala
Glu Lys Ile Pro Gln Ser Gln Tyr Asp Val Val Ser Asp His Val Ser Lys Val Thr Phe
Phe Asn Leu Asn Glu Thr Lys Pro Arg Gly Lys Phe Thr Tyr Asp Ala Val Tyr Cys
Cys Asn Glu His Glu Cys His His Arg Tyr Ala Glu Leu Tyr Val Ile Asp Val Asn
Ile Asn Ile Ser Cys Glu Thr Asp Gly Tyr Leu Thr Lys Met Thr Cys Arg Trp Ser
Thr Ser Thr Ile Gln Ser Leu Ala Glu Ser Thr Leu Gln Leu Arg Tyr His Arg Ser Ser
Leu Tyr Cys Ser Asp Ile Pro Ser Ile His Pro Ile Ser Glu Pro Lys Asp Cys Tyr Leu
Gln Ser Asp Gly Phe Tyr Glu Cys Ile Phe Gln Pro Ile Phe Leu Leu Ser Gly Tyr Thr
Met Trp Ile Arg Ile Asn His Ser Leu Gly Ser Leu Asp Ser Pro Pro Thr Cys Val Leu
Pro Asp Ser Val Val Lys Pro Leu Pro Pro Ser Ser Val Lys Ala Glu Ile Thr Ile Asn
Ile Gly Leu Leu Lys Ile Ser Trp Glu Lys Pro Val Phe Pro Glu Asn Asn Leu Gln
Phe Gln Ile Arg Tyr Gly Leu Ser Gly Lys Glu Val Gln Trp Lys Met Tyr Glu Val
Tyr Asp Ala Lys Ser Lys Ser Val Ser Leu Pro Val Pro Asp Leu Cys Ala Val Tyr
Ala Val Gln Val Arg Cys Lys Arg Leu Asp Gly Leu Gly Tyr Trp Ser Asn Trp Ser
Asn Pro Ala Tyr Thr Val Val Met Asp Ile Lys Val Pro Met Arg Gly Pro Glu Phe
Trp Arg Ile Ile Asn Gly Asp Thr Met Lys **Lys** Glu Lys Asn Val Thr Leu Leu Trp
Lys Pro Leu Met Lys Asn Asp Ser Leu Cys Ser Val Gln Arg Tyr Val Ile Asn His
His Thr Ser Cys Asn Gly Thr Trp Ser Glu Asp Val Gly Asn His Thr Lys Phe Thr
Phe Leu Trp Thr Glu Gln Ala His Thr Val Thr Val Leu Ala Ile Asn Ser Ile Gly Ala
Ser Val Ala Asn Phe Asn Leu Thr Phe Ser Trp Pro Met Ser Lys Val Asn Ile Val
Gln Ser Leu Ser Ala Tyr Pro Leu Asn Ser Ser Cys Val Ile Val Ser Trp Ile Leu Ser
Pro Ser Asp Tyr Lys Leu Met Tyr Phe Ile Ile Glu Trp Lys Asn Leu Asn Glu Asp
Gly Glu Ile Lys Trp Leu Arg Ile Ser Ser Ser Val Lys Lys Tyr Tyr Ile His Asp His
Phe Ile Pro Ile Glu Lys Tyr Gln Phe Ser Leu Tyr Pro Ile Phe Met Glu Gly Val Gly
Lys Pro Lys Ile Ile Asn Ser Phe Thr Gln Asp Asp Ile Glu Lys His Gln Ser Asp Ala
Gly **Ile Thr Ser Ala Ala Ala Cys Arg Ser Thr Ser Arg Gly Gly Pro Val Val Asp**
Leu Glu Gly Gly Pro Gly Thr

Figure 4.6: Protein sequence of translated *LEPR* cDNA clone presenting the domain regions as follows; CK domains in green, loop region in blue , F3 domains in turquoise and the C2 domain in pink. pCMV-Tag 2B vector encoded amino acids are highlighted in black, underlined font and the LYS109ARG, GLN223ARG and LYS656ASN variant amino acids are highlighted in red font.

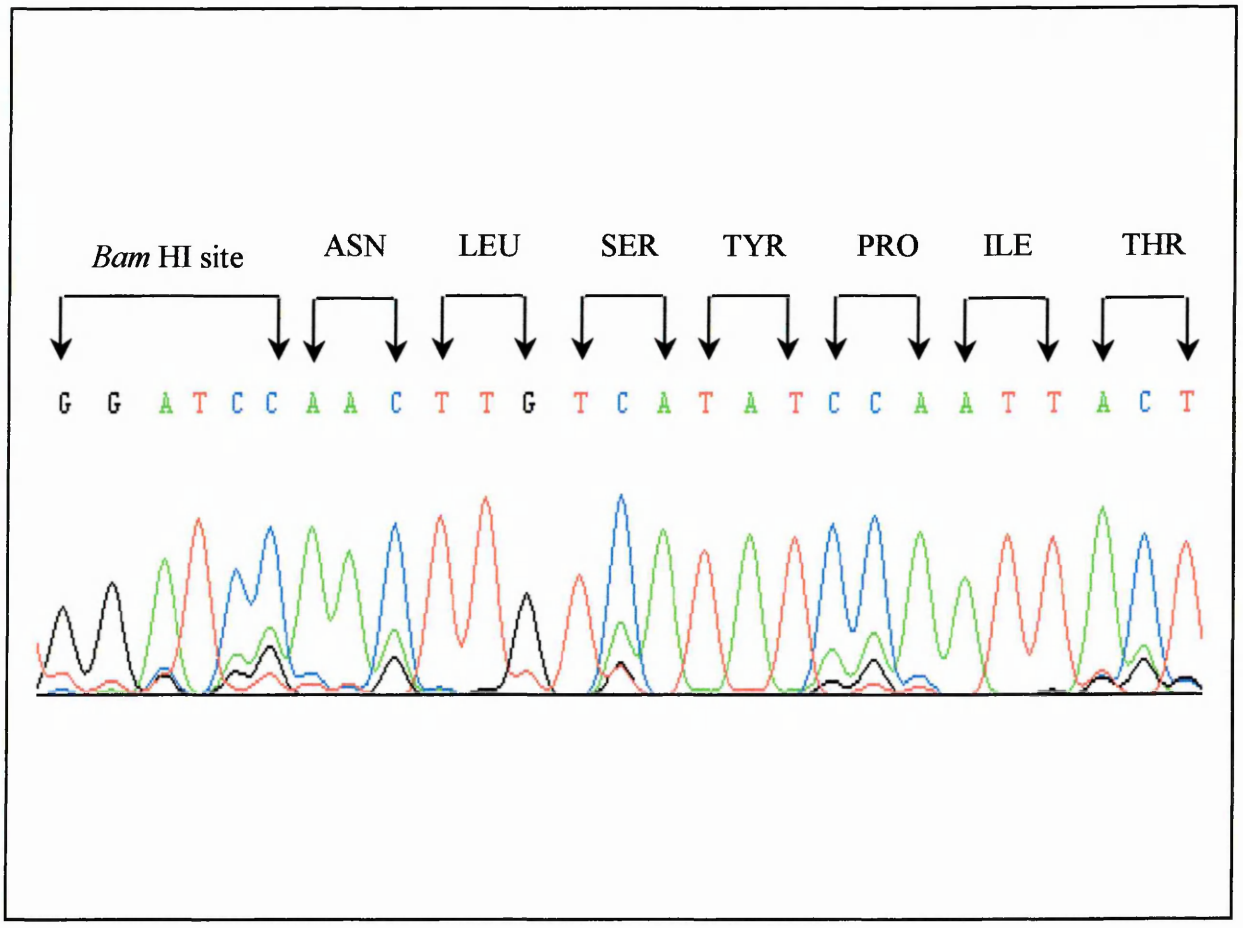


Figure 4.7: DNA sequence chromatogram of recombinant pCMV-Tag 2B vector showing the *BamHI* restriction site present within the multiple cloning site of the vector immediately followed by an ASN codon . This is codon no.23 of the *LEPR* cDNA sequence and demonstrates that the cDNA sequence was cloned in-frame relative to the vector sequence.

4.2.2 Site-directed mutagenesis

To enable expression of the extracellular protein variants coded for by the different combinations of the LYS109ARG and GLN223ARG SNPs in the DNA sequence, site-directed mutagenesis was utilised. Variants of the LYS656ASN SNP were not constructed as results in this thesis indicate that this SNP has not been associated with indicators of adiposity and was not determined to be in significant linkage disequilibrium with the other SNPs (see chapter 3). The method is described in chapter 2, section 2.5.3. Essentially, it relies upon the annealing of two primers (in separate reactions for each SNP) to exactly the same sequence on opposite DNA strands across the region of the recombinant pCMV-Tag 2B plasmid which contains the desired site to be mutated. In the middle of the primer sequences, a single mismatched base is present at the desired site of mutation which, after DNA amplification, will result in the incorporation of this alternative base in the recombinant 'daughter' plasmids. After amplification, the non amplified plasmid DNA (which is methylated) was digested by the enzyme *Dpn* I which preferentially targets methylated DNA leaving the amplified daughter plasmids (which are not methylated) available to be transformed into bacterial cells and spread onto antibiotic agar plates (to select for transformed bacteria). Plasmid DNA was purified from colonies which subsequently grew, and sent to the Applied Biotechnology Centre, Imperial College London for DNA sequencing to confirm that the site-directed mutagenesis reactions were successful.

This methodology allowed the generation of different combinations of the GLN223ARG and LYS109ARG SNPs. Figure 4.8 shows the A to G base change that was required to generate the LYS to ARG amino acid change in the protein sequence at codon 109. Figure 4.9 shows the G to A base change, which was required to

generate the ARG to GLN amino acid substitution at codon 223. Following site-directed mutagenesis, the following combinations were generated;

1. *LEPR* insert in original parental recombinant plasmid containing sequence coding for the LYS amino acid at codon 109 and the ARG amino acid at codon 223.
2. *LEPR* insert in daughter plasmid which contains the sequence coding for the ARG amino acid at codon 109, after changing the DNA sequence for that codon from AAG to AGG, whilst maintaining the ARG amino acid at codon 223.
3. *LEPR* insert in daughter plasmid which contains the sequence coding for the GLN amino acid at codon 223, after changing the DNA sequence for that codon from CGG to CAG, whilst maintaining the LYS amino acid at codon 109.

Note that the combination of ARG at codon 109 and GLN at codon 223 remains to be created.

4.2.3 Recombinant *LEPR* production and protein analysis

Once the mutagenesis reactions were finished and confirmed by sequence analysis, each plasmid was separately transfected into COS-7 mammalian cells to enable expression of the protein variants (see chapter 2, section 2.7.5). Furthermore, as control experiments, the cells were transfected with the native pCMV-Tag 2B vector and flasks were also incubated in the absence of vector.

Following 72 hours incubation, proteins were affinity purified from the lysates using a FLAG tag immunoprecipitation kit (Sigma) (chapter 2, section 2.8.3 and 2.8.4).

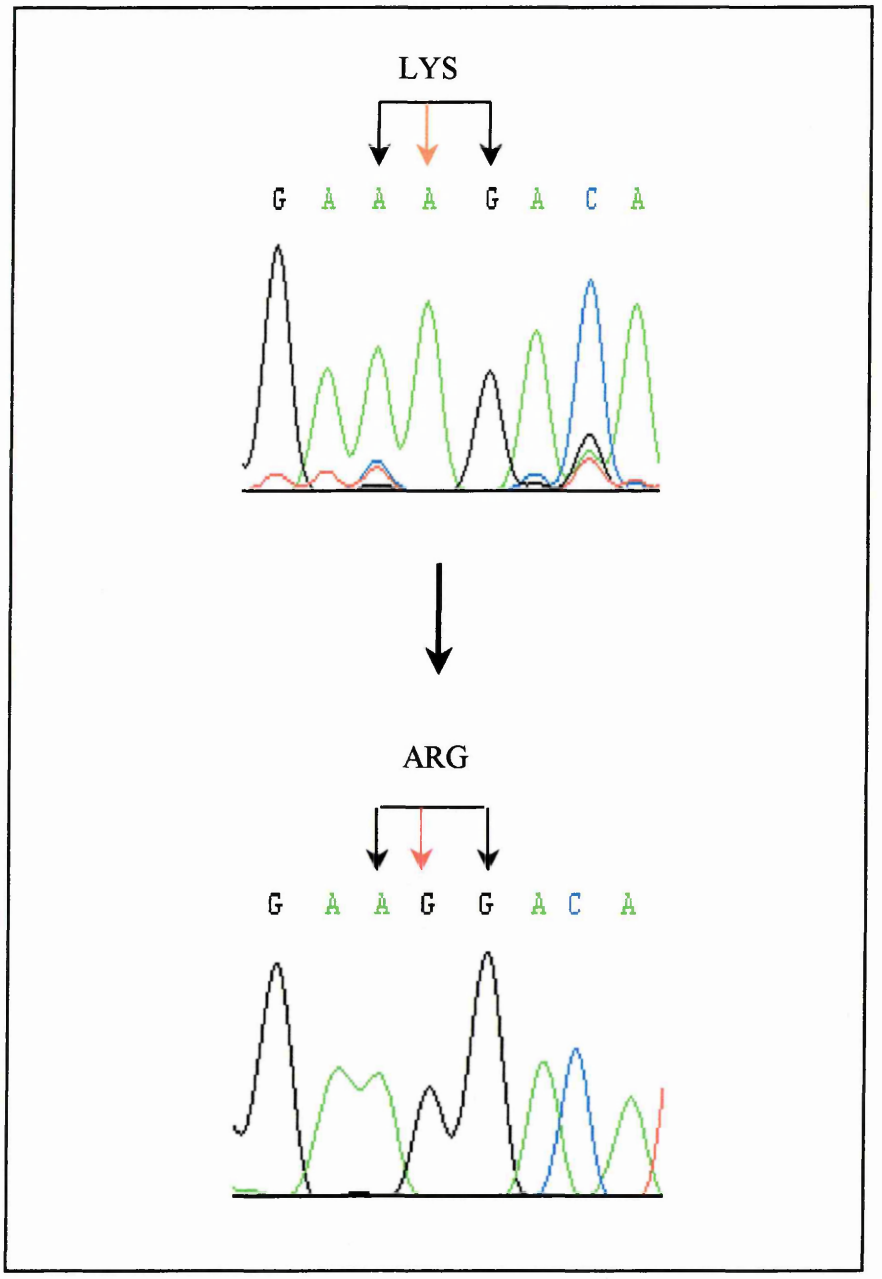


Figure 4.8: DNA sequence chromatogram confirming the A to G base change that codes for a LYS to ARG amino acid substitution at codon 109 of the *LEPR* cDNA sequence.

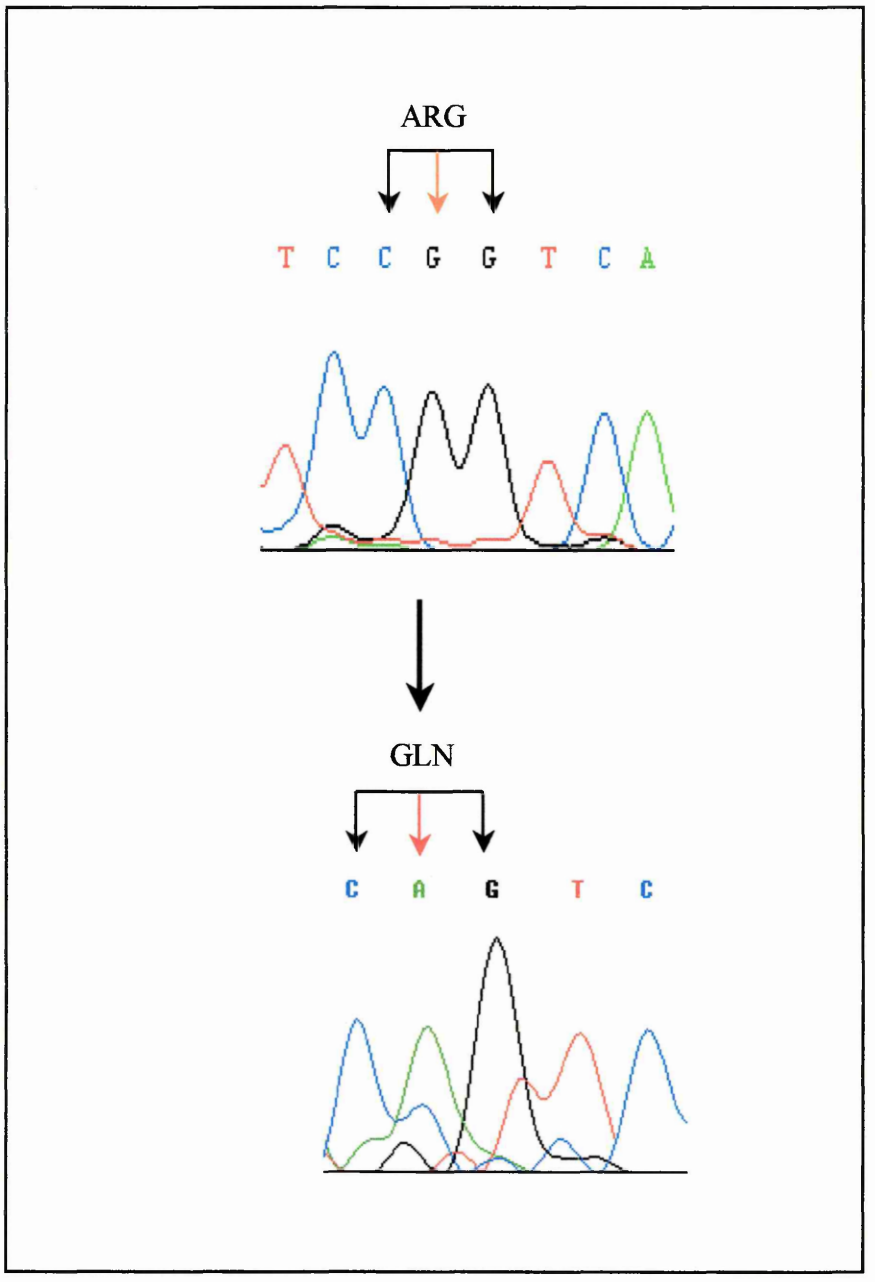


Figure 4.9: DNA sequence chromatogram confirming the G to A base change that codes for an ARG to GLN amino acid substitution at codon 223 of the *LEPR* cDNA sequence.

The protein products were visualised on a 4-15% SDS-PAGE gel (Figure 4.10), and the molecular weight of the purified protein was determined by constructing a standard log graph of the distance migrated in the gel by the SDS-PAGE protein standards (Bio-Rad) (figure 4.11). As such, the relative molecular mass of the protein was estimated to be 125-130kDa. No protein product was observed in cells transfected with the native vector only. Nor was any protein product observed in the cell media.

To confirm that the purified protein was in fact the human leptin receptor, it was analysed via a specific double monoclonal human leptin receptor ELISA (Abcam) (see chapter 2, section 2.8.5). Using the statistical software package, FigP, a standard curve was generated from the absorbances obtained for a series of soluble *LEPR* standards of known concentrations (see figure 4.12). From this graph, the software derived the concentration from the absorbances of the various purified *LEPR* protein variants. The samples were diluted 1 in 3 upon addition to the ELISA plate and each sample and standard was measured in duplicate (Table 4.1).

LEPR variant	Average absorbance for diluted samples (450nm)	Neat sample concentration (U/ml)*
Blank sample	0.017	-
LYS109:ARG223	0.09	8.2
ARG109:ARG223	0.103	9
LYS109:GLN223	0.093	8.3

Table 4.1: ELISA results for *LEPR* protein variants. * 1 unit equals 2ng recombinant leptin receptor standard.

Leptin receptor was not detected in purified samples from cells transfected with the native pCMV-Tag 2B vector or in the media of cells transfected with the recombinant plasmid.

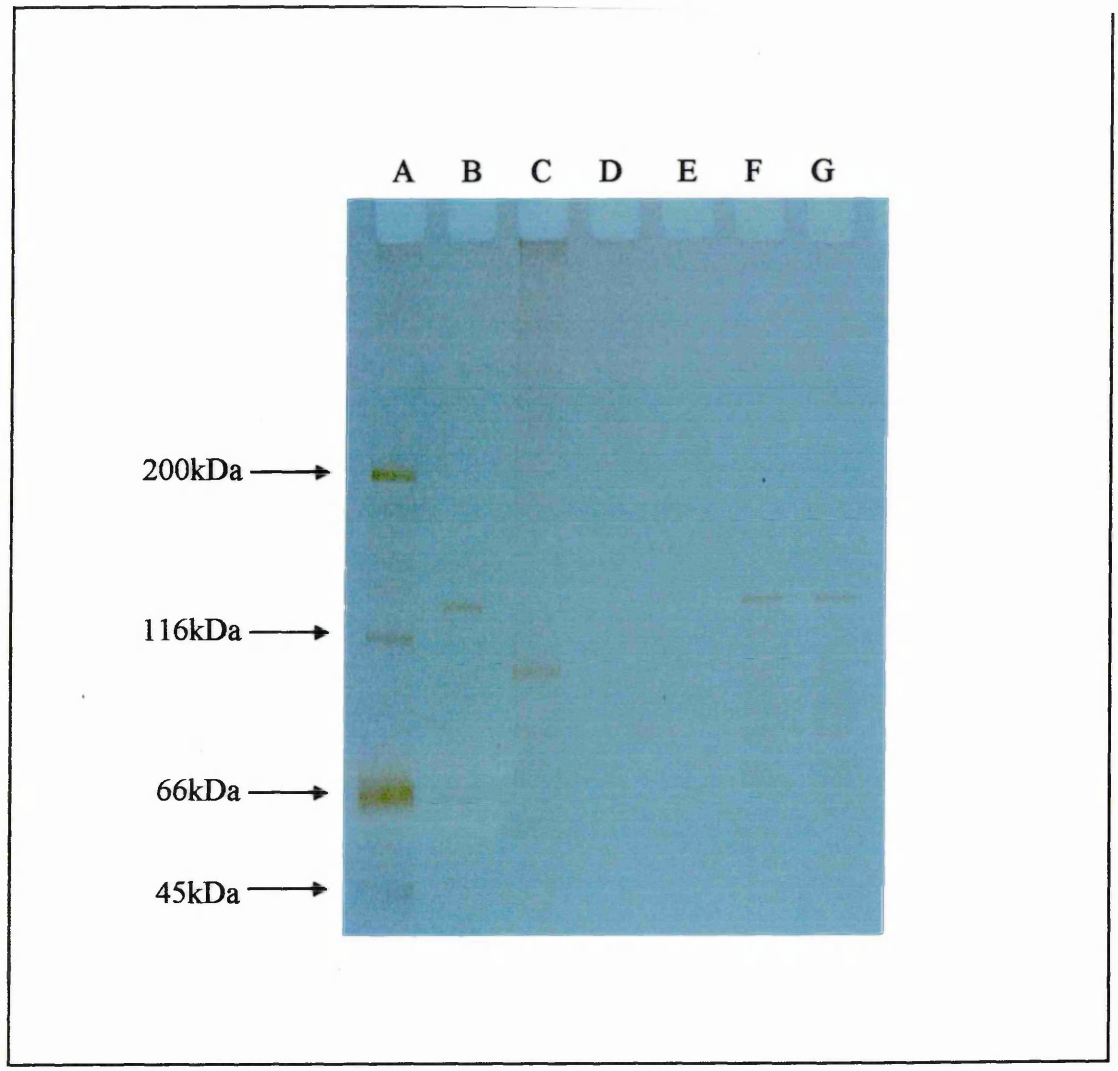


Figure 4.10: Photograph of silver-stained 4-15% SDS-PAGE gel displaying, **A**: High molecular weight protein standard (BioRad), **B**: affinity purified LYS109:ARG223 recombinant leptin receptor protein variant from lysed COS-7 cells, **C**: PnGaseF treated LYS109:ARG223 leptin receptor protein, **D**: affinity purified control sample obtained from COS-7 cells transformed with native pCMV-Tag 2B plasmid only, **E**: affinity purified LYS109:ARG223 leptin receptor variant protein from COS-7 cell media, **F**: affinity purified ARG109:ARG223 leptin receptor protein variant from lysed COS-7 cells and **G**: affinity purified LYS109:GLN223 leptin receptor protein variant from lysed COS-7 cells.

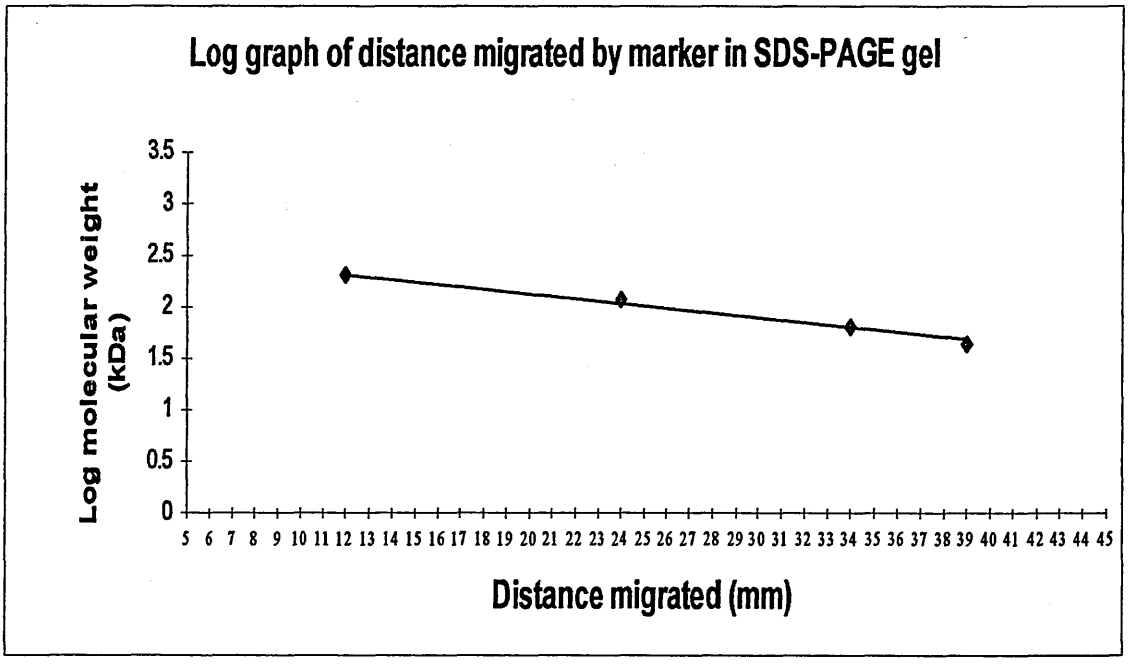


Figure 4.11: Log graph of distance migrated by marker protein bands in 4-15% TRIS-HCL gel.

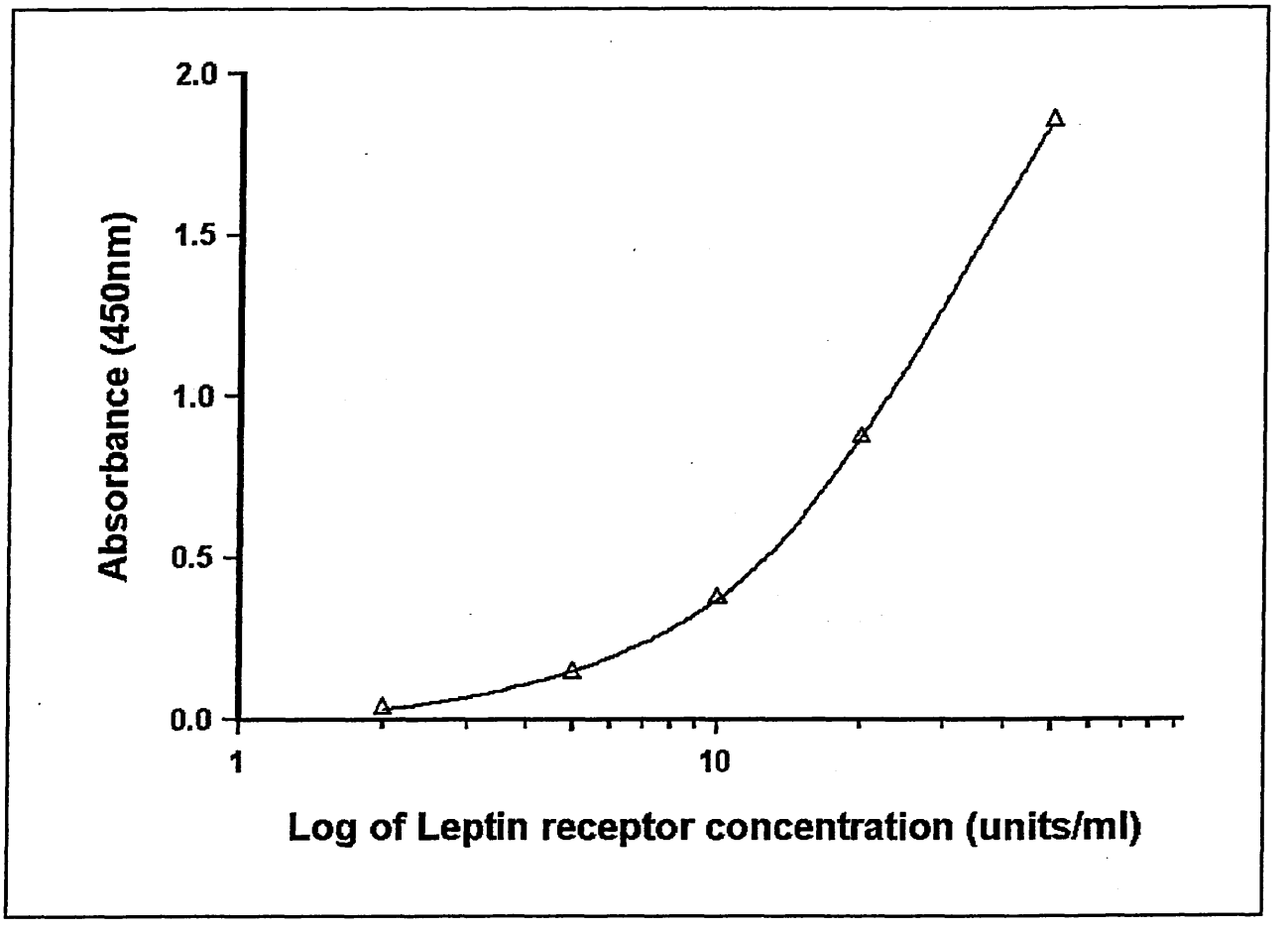


Figure 4.12: Standard curve of absorbances of leptin receptor ELISA protein standards

Recombinant expression of proteins in mammalian cells should contain similar post-translational modifications to the original protein of interest. Such modifications are important, in particular, protein glycosylation which is common on cell-surface receptors is known to facilitate ligand binding (Rudd *et al.*, 1999). To analyse the extent of glycosylation of the recombinant protein, a sample was digested with the enzyme PNGaseF that removes saccharides linked to asparagine amino acids in the protein sequence (see chapter 2, section 2.8.6). Figure 4.10 displays the deglycosylated protein in lane C. As determined from the log graph of the distance migrated by the protein standards in the SDS-PAGE gel, the deglycosylated protein was estimated to be 95-100 kDa.

The expressed protein contains codons 23 to 841 and was engineered to lack the first 22 amino acids of the primary sequence, which is assumed to be the signal sequence. This strategy was followed because, originally, an (unsuccessful) attempt was made to express the cDNA in bacterial cells which lack the cell machinery to cleave signal sequences. The signal sequence directs the protein through the endoplasmic reticulum and towards the cell membrane. Because of this, the remaining amino acid sequence was submitted to software which uses neural network algorithms to analyse the biochemical properties of amino acid strings embedded within the protein that may direct the protein to specific subcellular locations in the absence of the putative signal sequence. The software, TargetP version 1.01 (Emanuelsson *et al.*, 2000), and SignalP (Nielsen *et al.*, 1997) was used to search for such sequences. No specific sequences were detected however.

A method of measuring and comparing the leptin binding of the variant proteins is to subject them to the leptin-binding assay. This procedure is explained in chapter 2, section

2.8.7, but essentially involves incubating the purified leptin receptor sample with I^{125} leptin in the presence or absence of non-radioactive leptin. By subsequently measuring the radioactivity counts on a γ-counter, the total binding of the ligand (sample containing radioactive leptin) minus the non-specific binding (sample containing radioactive and non-radioactive leptin) equals the specific binding of radioactive ligand to the receptor. Specific binding was measured for the LYS109/ARG223 and LYS109/GLN223 variants with 0.5 units (determined from ELISA results where 1 unit is equivalent to 2 ng of leptin receptor) of each variant present in each tube. The total and non-specific measurements were carried out in duplicate. Figure 4.13 shows the specific binding for both variants, alongside a positive (serum) control (to determine that the assay is working) and a blank control (lacking purified leptin receptor protein). Clearly, the samples are specifically binding the radioactive leptin (compared to the blank). These preliminary results suggest that there may be differences in ligand binding associated with the SNPs tested but the experiment would need to be repeated in multiples to statistically validate any results, as at the time there was insufficient sample to repeat the experiments.

Furthermore, due to time constraints towards the end of this body of work, and because more of this particular purified protein was used up during the SDS-PAGE analysis, the ARG109ARG223 protein was not available to be tested via the LBA assay and would therefore need to be tested for future experiments. Also, the ARG109GLN223 was not generated which would have enabled analysis of the LYS109ARG mutation on leptin binding to the receptor.

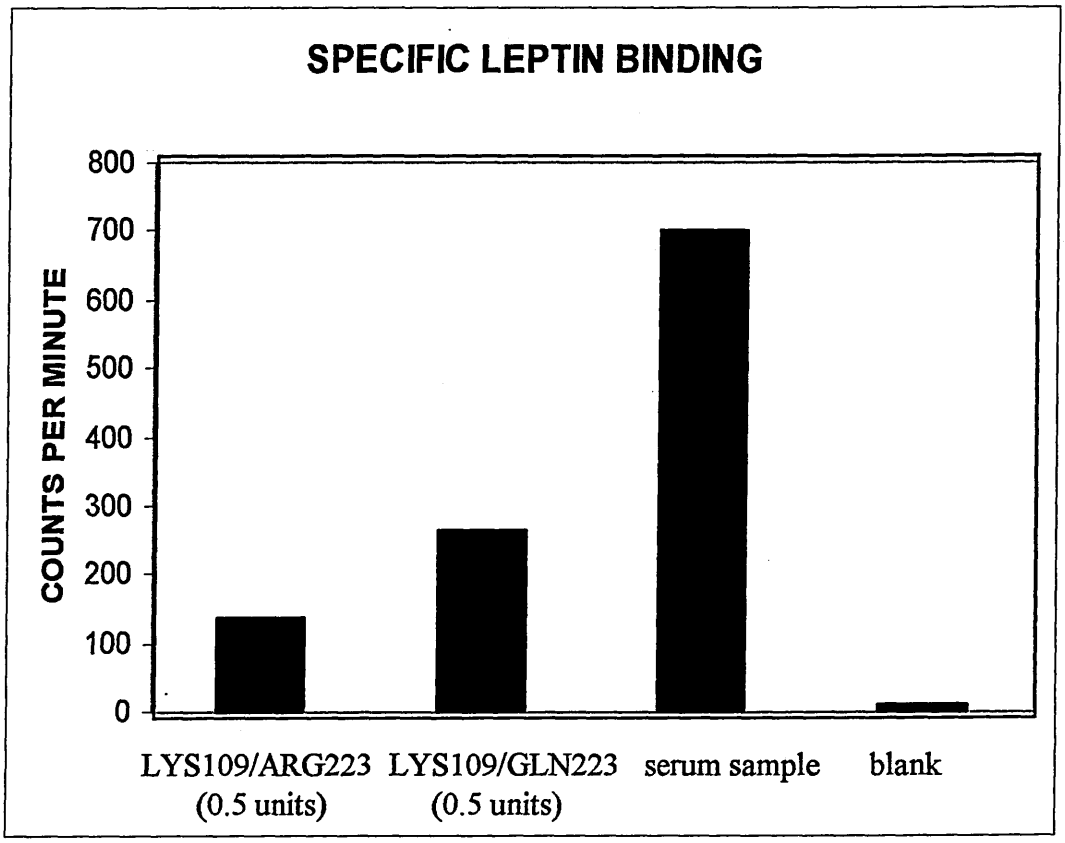


Figure 4.13: Graph displaying radioactive counts per minute emitted from I^{125} leptin specifically bound to leptin receptor protein variants, serum sample and water sample (measurements were carried out in duplicate and the mean counts per minute plotted).

4.3 Discussion

As a result of this study, a series of soluble human leptin receptor protein variants have been created. They are available as a set of tools to compare and contrast their properties. This would be of substantial scientific interest because several genetic studies have sought associations between SNPs in the leptin receptor cDNA sequence and indicators of adiposity. Some of these studies have highlighted an association or linkage with a particular SNP (Chagnon *et al.,* 1999, Chagnon *et al.,* 2000, Yiannakouris *et al.,* 2001, Quinton *et al.,* 2001) whilst others have not (Echwald *et al.,* 1997, Matsuoka *et al.,* 1997, Silver *et al.*, 1997). A limitation of these studies is that it is difficult to distinguish which SNP is having an effect if linkage disequilibrium occurs. Also different population groups make up the sample cohort between studies. As a result, different environmental factors may contribute to varying degrees between studies depending upon population susceptibility. Furthermore, population admixture and stratification may confound results. One way of dissecting the issue would be to compare functional properties of purified leptin receptor variants such as the ability to dimerise or bind leptin using *in-vitro* methodologies. Importantly, it would enable the comparison of specified combinations of SNPs expressed in each variant to potentially assign functionally significant effects to certain SNPs and not others. Using purified protein would also remove the affect of any systemic accessory proteins, which participate in the leptin:leptin receptor interface *in vivo.*

Characterisation of the leptin receptor has been attempted by deletion studies of its extracellular domain (Fong *et al.,* 1998, Sandowski *et al.,* 2002). The extracellular domain contains two repeating CK-F3 domains separated by an amino acid loop sequence. Combined CK-F3 domains form ligand-binding sites in other cytokine

receptors (Livnah *et al.,* 1996, Wells and de Vos, 1996). By comparing binding of radiolabelled leptin to different deletion mutants of the extracellular domain, Fong suggested that the second CK-F3 is required for leptin binding and receptor activation. This region does not contain the LYS109ARG or GLN223ARG variants. However as more than one association study has identified lowered BMI in association with the ARG amino acid of the GLN223ARG variant (Chagnon *et al.,* 2000, Yiannakouris *et al.,* 2001, Quinton *et al.,* 2001), it is possible that this form has enhanced activity. One possible explanation may be that this variant enhances leptin binding or receptor activation and as such would not be detected by Fong's methodology, which only generated mutants which resulted in reduced ligand binding compared to the full-length wild type receptor. Fong comments that the first CK:F3 domain may not affect leptin binding in his experiments because the 'unusually long connecting loop may confer a very high degree of flexibility, preventing the formation of a stable leptin-binding site'. As the GLN223ARG variant is present in this loop and the ARG variant is associated with lowered indicators of adiposity, then this variant may stabilise the loop region, reducing the degree of flexibility and improving leptin binding and / or receptor activation properties. However, it must be noted that the preliminary results from the work presented here do not indicate enhanced leptin binding and if this is held true after more detailed analysis then an alternative explanation would be required to explain the association between this variant and lowered BMI.

Whilst generating these protein variants, incidental observations were made of the molecular properties of the protein. The expressed protein lacked the first 22 amino acids that are a putative signal sequence (Tartaglia *et al.,* 1995). This was so constructed because the protein was initially intended to be expressed in bacterial

cells which lack the protein machinery to cleave signal sequences from pro-peptides. Unfortunately the protein failed to express in a bacterial system and so was tested in a eukaryotic system. No protein was detected in the COS-7 cell media by SDS-PAGE or ELISA analysis but it was detected in the cell lysates. This suggests that the putative signal sequence is real. Of interest is the deglycosylation experiment which suggests that the protein is heavily N-glycosylated as a reduction in size from approximately 130kDa to 95kDa was observed after treatment with the enzyme PNGaseF. The protein size agrees well with expression of the leptin receptor from COS-7 cells by Liu *et al* (1997). They characterised a size of approximately 130kDa by Western blot analysis and Coomassie staining of the purified soluble receptor, and suggested that this was probably the glycosylated form of the receptor. Haniu *et al.*, (1998) expressed the soluble leptin receptor in CHO cells and detected a purified protein of approximately 145kDa which was extensively N-glycosylated at about 36 % of the total protein weight. In this study, the predicted molecular weight from amino acid sequence alone is 97kDa (93.5kDa leptin receptor + 4kDa vector-encoded amino acid sequence). The reduction from about 130kDa to this size following deglycosylation agrees with data produced by Haniu that the protein is extensively glycosylated, even in a different eukaryotic cell system. Signal sequences are generally assumed to direct nascent proteins to the endoplasmic reticulum, where N-glycosylation occurs, and subsequently to the Golgi apparatus where the attached oligosaccharides are modified, before location to the cell surface and secretion out of the cell. Although the signal sequence is missing, it does not appear to have deleteriously affected protein glycosylation levels. It is possible that sorting signals embedded within the receptor amino acid sequence may contribute to locating it to the ER. Software analysis of the receptor protein sequence did not detect any obvious

sorting sequences. It may be that novel sorting sequences are present which carry out the procedure. It should be noted from the ELISA results that the overall level of recombinant protein expression is substantially lower than that commonly observed for transient expression in COS-7 cells (Warren and Shields, 1984; and Seed and Aruffo, 1987). It could be hypothesised, therefore that loss of the signal sequence reduces the amount of recombinant protein sorted to the ER but that the protein which does reach the ER is adequately glycosylated.

Certainly the observations in this study warrant further examination to characterise the phenomenon.

Chapter 5

Investigation of polymorphisms in other relevant cytokine genes in relation to adiposity

5.1 Introduction

As research into eating disorders and obesity has progressed, the interaction of leptin with other members of the cytokine network has become apparent and specific relationships uncovered.

Although BMI correlates with leptin (Considine et al., 1996) and inversely correlates with leptin receptor levels (Ogier et al., 2002), individuals with an equivalent BMI have variable levels of these proteins. It is therefore important to find additional factors that might have an influence on this. The pleiotropic and overlapping nature of cytokine activity means that other cytokines deserve investigation regarding obesity in association with the leptin system as well as on their own merits. In this chapter, polymorphisms in other relevant cytokine genes have been investigated in addition to leptin receptor SNPs previously studied in this thesis.

As discussed in the introduction to this thesis, leptin is a satiety factor that is released into the blood stream from adipocytes and is able to cross the blood-brain barrier and bind specifically to receptors in the hypothalamus where it elicits signals to regulate food intake and energy expenditure.

Screening of the coding region of the *LEP* gene has yielded very few mutations that might contribute to obesity. As a result, studies of the 5' flanking region ensued.

In one study, several sequence variants were found, three of which were found to differ in allele frequencies between obese and non-obese women (W-D, Li *et al.*, 1999). The most significant difference was observed for the G to A -2548 polymorphism with the A allele reported to be associated with higher leptin levels in a study of men and women (Mammes *et al.*, 1998). Mammes and colleagues (2000) went on to show that the G allele was associated with lowered leptin levels and

overweight in men only. However, Le Stunff *et al,* (2000) published results which showed that in young, obese Caucasian girls, the A allele is associated with lower leptin levels between girls of comparable adiposity. More recently, Hoffstedt and colleagues (2002) observed that adipose tissue leptin mRNA levels were higher in AA homozygotes compared to GA/GG subjects in non-obese females.

Another relevant factor is tumour necrosis factor alpha (TNF-α). It was originally called cachexin because it induces cachexia (marked loss of weight and wasting) (for review Beutler and Cerami, 1989). Grunfeld *et al.,* (1996) and Sarraf *et al.,* (1997) have reported that TNF-α administration increases leptin levels in rodents. In humans, Zumbach *et al.,* (1997) demonstrated that TNF-α infusion increases serum leptin levels in patients with solid tumours, whilst Mantzoros *et al.,* (1997) have shown a positive association between TNF- α and leptin levels with BMI in healthy controls and people with non-insulin dependent diabetes. Tumour necrosis factor alpha (TNF-α) belongs to the TNF family of cytokines. It acts predominately through two transmembrane receptors, TNF receptors 1 and 2 (TNFR1 and 2) which are found on a variety of cell types including fibroblasts, endothelial cells and adipocytes and are also present in soluble form. TNF-α is often referred to as the master pro-inflammatory cytokine with cell signalling leading to increased expression of other pro-inflammatory cytokines, such as IL-6 and IL-1.

Within the 5'-flanking region of the TNF-alpha gene, 308 nucleotides upstream from the transcription initiation start site, resides the TNF-alpha G-308 A SNP. The A allele appears to increase promoter activity and has been linked to several diseases, many of which are immune related (Abraham and Kroeger, 1999). With regard to obesity, the A allele is reported to be more frequent in individuals with a higher BMI (Brand *et al.,*

2001) suggesting a susceptibility locus for obesity. Also, Hoffstedt *et al,* (2000) reported an allele specific difference confined to women only, whereby the A allele was again associated with higher BMI and also fat mass.

A further cytokine that may be involved in weight regulation is the interleukin receptor antagonist (IL-1ra). Leptin has been shown to induce expression and secretion of IL-1ra from monocytes (Gabay *et al.,* 2001). The molecular basis for this effect was subsequently investigated (Dreyer et al., 2002). It was discovered that the induction of IL-1ra by leptin required the long form of the leptin receptor and activation of p42/44 MAPKs which binds to a nuclear factor kappaB site of the ILRN promoter.

IL-1ra levels are elevated in obese individuals, with a positive linear correlation with leptin levels, and are reduced after loss of body weight and lowered leptin levels resulting from surgery (Meier *et al.,* 2002). IL-1ra is secreted from many cell-types, but particularly macrophages in unison with IL-1 alpha (α) and IL-1 beta (β). These are released in response to injury or an immunological insult and promote inflammation. IL-1ra binds to the IL-1 type 1 and type 2 receptors, as do IL-1α and IL-1β, but unlike these two molecules, it does not elicit intracellular signalling events. As such, it is a competitive inhibitor of IL-1 activity, acting as an endogenous anti-inflammatory agent.

The IL1RN gene contains a variable number tandem repeat (VNTR), with a core repeat sequence, 86bp in length. Five alleles have been reported (Lennard *et al.,* 1992, Tarlow *et al.,* 1993), with the 2 x 86bp repeat (known as IL1RN*2) implicated in severity of clinical course of various disorders, including systemic lupus erythematosus and ulcerative colitis (Blakemore *et al.,* 1994 and Mansfield *et al.,* 1994). It has been postulated that IL1RN*2 may correlate with a lowered level of production of IL-1ra (Tarlow *et al.,* 1993, Blakemore *et al.,* 1994).

Leptin regulates IL-1ra and also IL-1ra protein levels correlate with leptin levels. It is possible that a variation, in response to leptin, may be partly influenced by the VNTR alleles. As IL-1ra inhibits IL-1 activity, which has cachexic properties, any potential variation in IL-1ra response to leptin may consequently modulate the cachexic effects of IL-1. Alternatively, altered levels of IL-1ra could adjust leptin levels via cytokine cross-talk involving positive and/or negative feedback pathways although no studies have reported that IL-1ra can influence leptin levels.

The apparent clinical, molecular and cell biological observations that implicate the cytokines leptin, TNF-α and IL-1ra with regulation of weight highlight the validity of analysing polymorphisms within their gene sequences to determine any associations with markers of adiposity. This was carried out in a cohort of Caucasian postmenopausal women from the Sheffield area who had previously been analysed for SNPs in the *LEPR* gene in relation to indicators of adiposity.

5.1.1 Study aims

Three polymorphisms were investigated in this study, the -2548 *LEP* promoter polymorphism, the G-308A TNF-alpha polymorphism and the IL1RN VNTR polymorphism.

The aims of these studies were to:

- determine whether there is evidence for associations between these polymorphisms and markers of adiposity
- determine whether the leptin promoter polymorphism effects serum leptin levels (as previously reported in males) in a postmenopausal female study group;
- investigate whether the TNF-α promoter SNP and IL1RN VNTR influence serum leptin levels.

5.2 Study Design

The study design is the same as presented in chapter 3, section 3.2.

Genotyping was carried out as described in chapter 2, sections 2.2.3 and 2.2.4. The genotypes for the *LEP* G -2548 A SNP are shown in figure 5.1, in figure 5.2 for the TNF-alpha G-308A SNP and in figure 5.3 for the IL1RN VNTR.

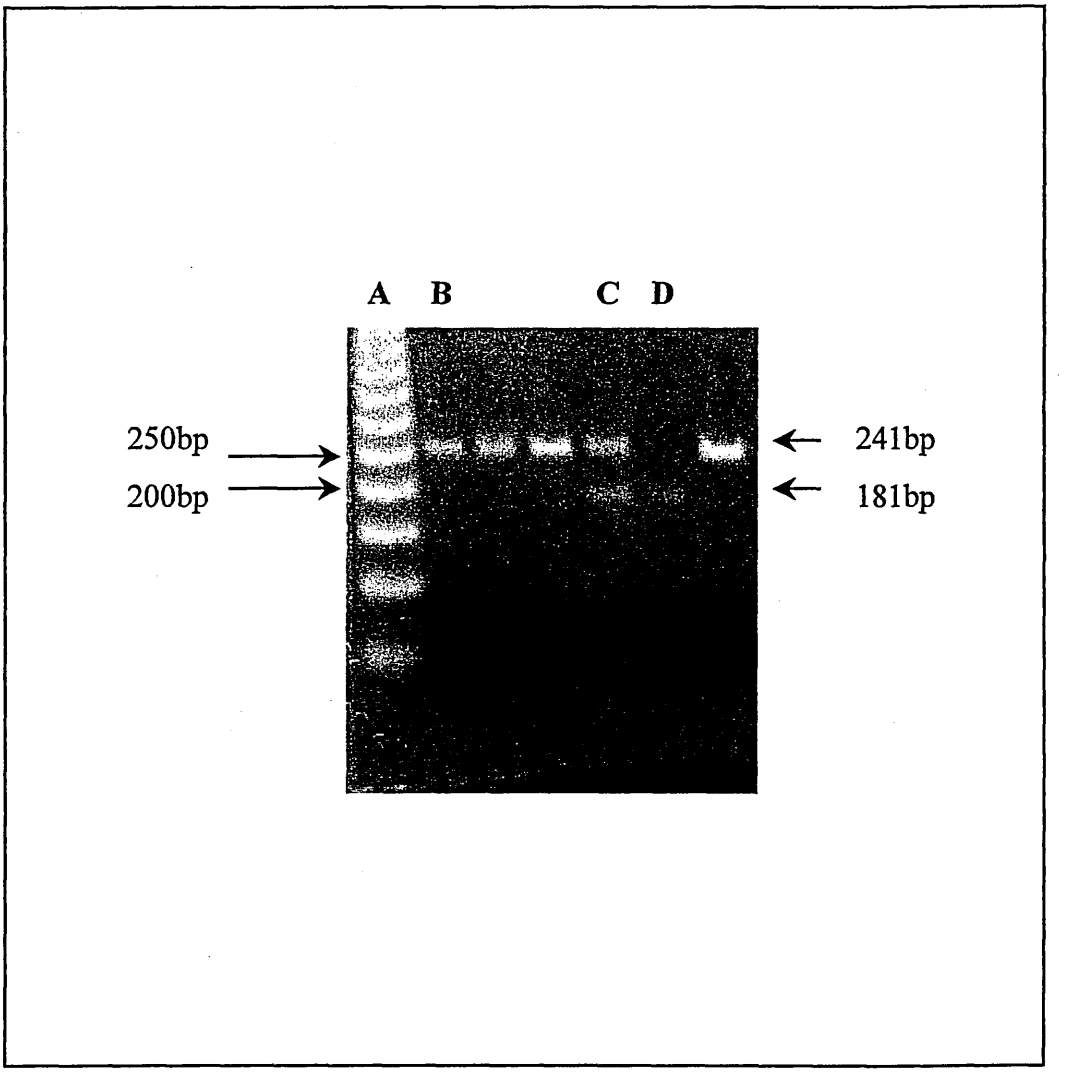


Figure 5.1: Photograph of *LEP* -2548 SNP genotypes on a 3% nusieve agarose gel displaying **A:** 50bp DNA marker (Promega), **B:** AA genotype (241bp), **C:** GA genotype and **D:** GG genotype (181bp).

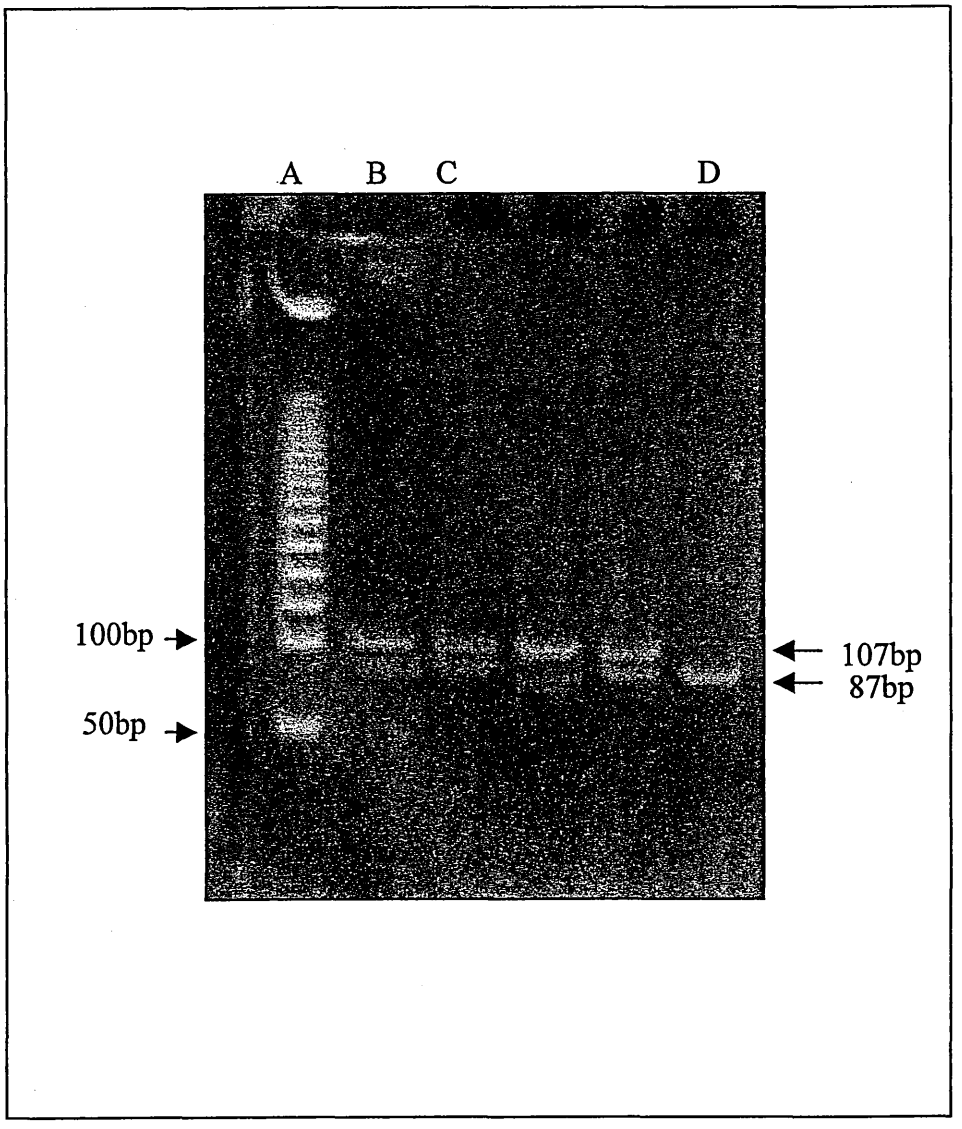


Figure 5.2: Photograph of TNF alpha G-308A SNPgenotypes on a 9% polyacrylamide gel displaying, **A:** 50bp DNA marker (Promega), **B:** the GG genotype (107bp), **C:** the AG genotype and **D:** the AA genotype (87bp).

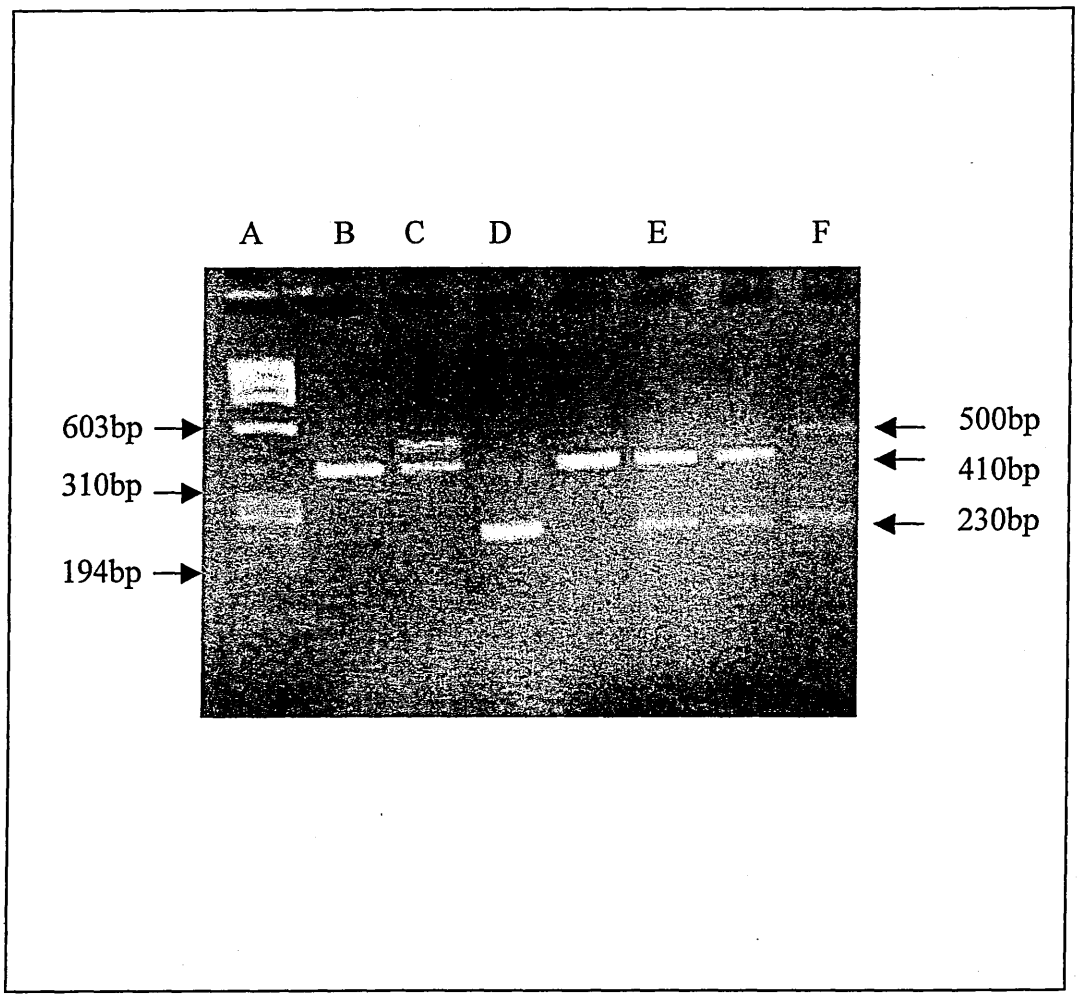


Figure 5.3: Photograph of the IL1RN VNTR genotypes on a 2% agarose gel displaying **A:** ϕX174 *Hae*III DNA marker (Promega), **B:** 4,4 repeat genotype (410bp), **C:** 4,5 repeat genotype, **D:** 2,2 repeat genotype (230bp), **E:** 2,4 repeat genotype and **F:** 2,5 repeat genotype. Note that the 5 repeat is approximately 500bp.

5.2.1 Statistical Analysis

Genotype results were analysed by the Hardy-Weinberg equation to determine if the distribution of heterozygotes and homozygotes fitted that expected of a population in Hardy-Weinberg equilibrium (see chapter 2, section 2.8.1). Continuous variable data were analysed using SPSS software to determine whether it was normally distributed (see chapter 2, section 2.8.6). Statistical comparison of the means of data grouped to each genotype for a particular SNP were analysed by a Mann-Whitney U test (see chapter 2, section 2.8.7). Data were displayed graphically using boxplots, plotted using SPSS software (see chapter 2, section 2.8.8).

5.3 Results

5.3.1 *LEP* G -2548 A SNP

98 individuals were typed for this SNP. The observed genotypes were in Hardy-Weinberg equilibrium and allele frequencies of 0.51 and 0.49 were calculated for p and q (see table 5.1). Whilst this data indicates that the A allele is the most frequent allele in this cohort, due to the small sample size and the closeness in frequencies of the G and A alleles in other groups studied (table 5.1), the G allele is treated as the most common allele for statistical analysis. The continuous variable data were not normally distributed and the Mann-Whitney U test was used to compare the mean value for each variable between genotype groups (see table 5.2 and figures 5.4-5.8).

Allele	**Post-menopausal women (n=98)**	Le Stunff *et al* (2000) Obese Caucasian girls (n=93)	Mammes *et al* (1998) Overweight Caucasian men and women (n=117)	Hoffstedt et al (2002) non-obese Caucasian women
G	**0.49**	0.5	0.56	0.49
A	**0.51**	0.5	0.44	0.51

Table 5.1: Comparison of allele frequencies for the *LEP* G -2548 A SNP with published results

	Observed genotype frequency	Expected genotype frequency
	n	n
Heterozygotes	53	49
Homozygous A	23	25
Homozygous G	22	24
Total	98	98

Table 5.2: Predicted genotype distribution obtained from the Hardy-Weinberg equation for the *LEP*-2548 SNP using the determined allele frequencies. The p-value for the χ^2 analysis of comparing the expected to the observed genotype frequencies was 0.42 (1 degree of freedom).

	Genotype			P value
	GG	**AG**	**AA**	
BMI (kg/m^2)	27.08 ± 0.82	26.75 ± 0.56	25.28 ± 0.89 *	(AA v GG) 0.012 (AA v AG/ GG) 0.045
N=	n=22	n=53	n=23	(AA v AG) NS
Fat mass (g)	28368 ± 2083	26261 ± 1102	25095 ± 2052	NS NS
N=	n=19	n=47	n=19	NS
Lean mass (g)	35285 ± 1671	36545 ± 622	35937 ± 1082	NS NS
N=	n=19	n=47	n=19	NS
Serum leptin concentration ng/ml	20.90 ±10.61	20.05 ± 10.61	14.95 ± 11.29	0.035 0.017
N=	n=18	n=43	n=20	0.015
Normalised serum leptin (ng/ml per kg fat mass)	0.79 ±0.081	0.74 ±0.045	0.57 ±0.059	0.07 0.014 0.015
N=	n=17	n=42	n=18	

Table 5.3: Mean levels of BMI, fat mass, lean mass and serum leptin levels for each genotype of the *LEP* -2548 SNP. P values were calculated by comparing non-carriage of the G allele (GG/AG v AA genotype groups), AA v GG and AA v AG genotype groups using a Mann-Whitney U test * ± standard error of mean

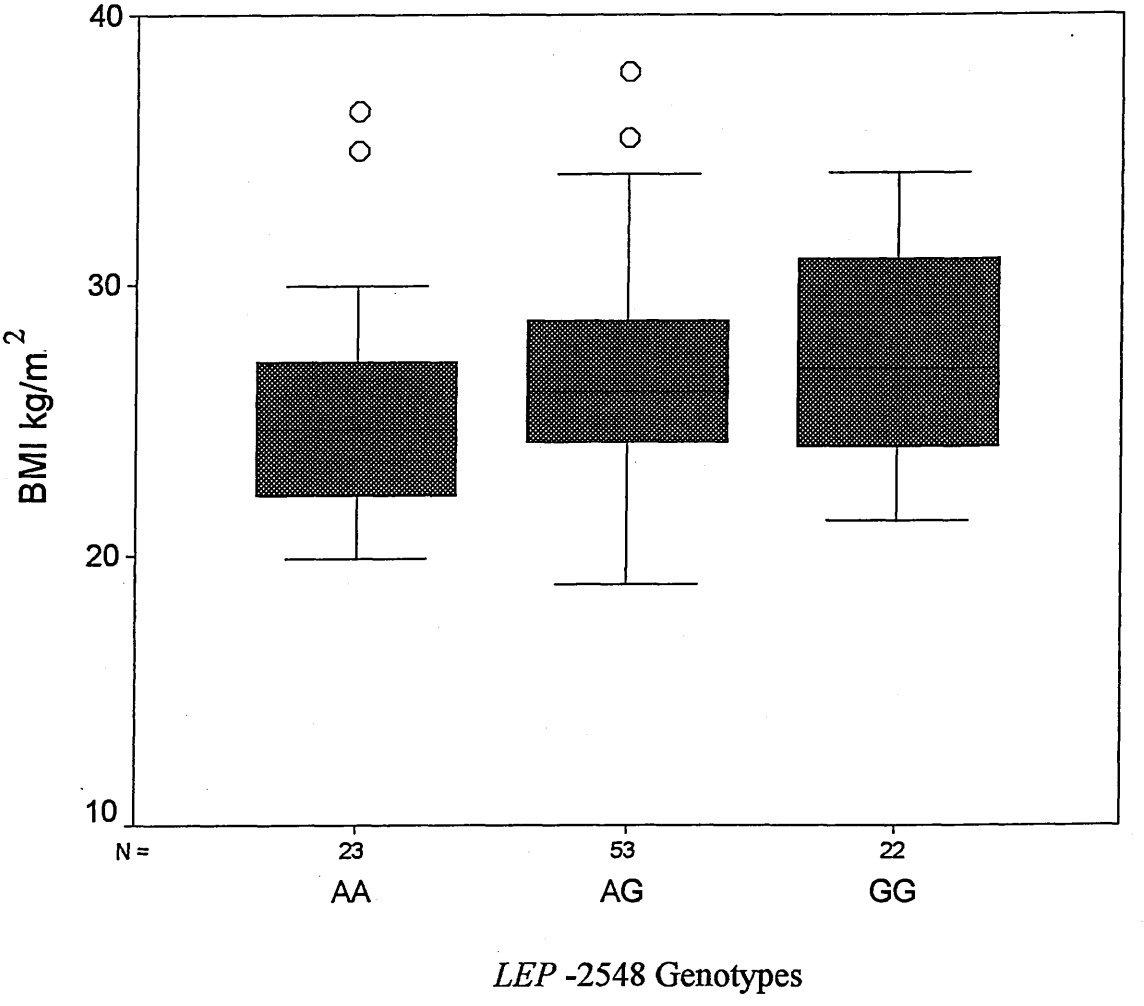


Figure 5.4: Boxplot of BMI levels grouped to the AA, AG and GG genotypes of the *LEP* -2548 SNP. Comparison of the mean value for the AA group with the AA/AG groups showed that it was significantly lower (p=0.045). This group was also significantly lower than the GG genotype group (p=0.012)

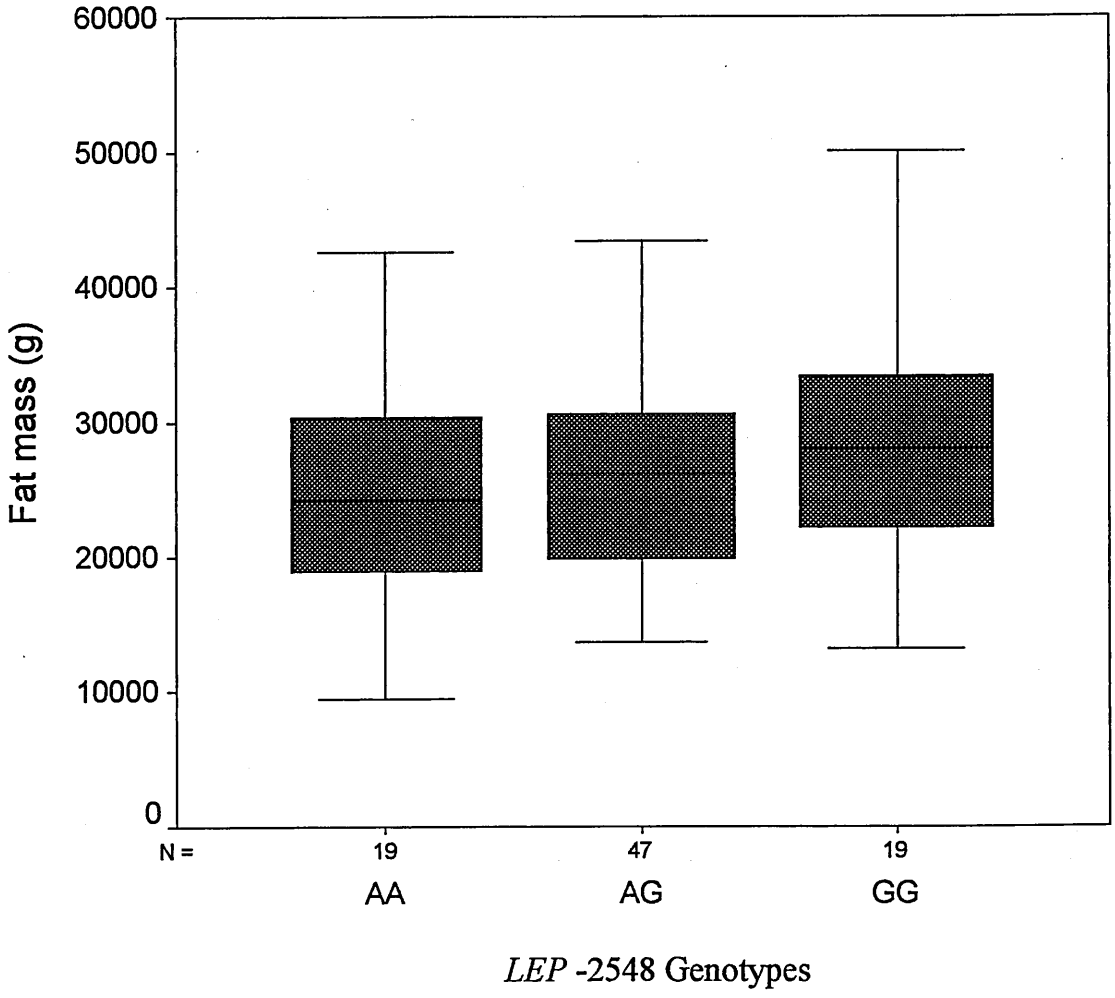


Figure 5.5: Boxplot of fat mass levels grouped to the AA, AG and GG genotypes of the *LEP* -2548 SNP. There is no significant difference between the mean of each groups.

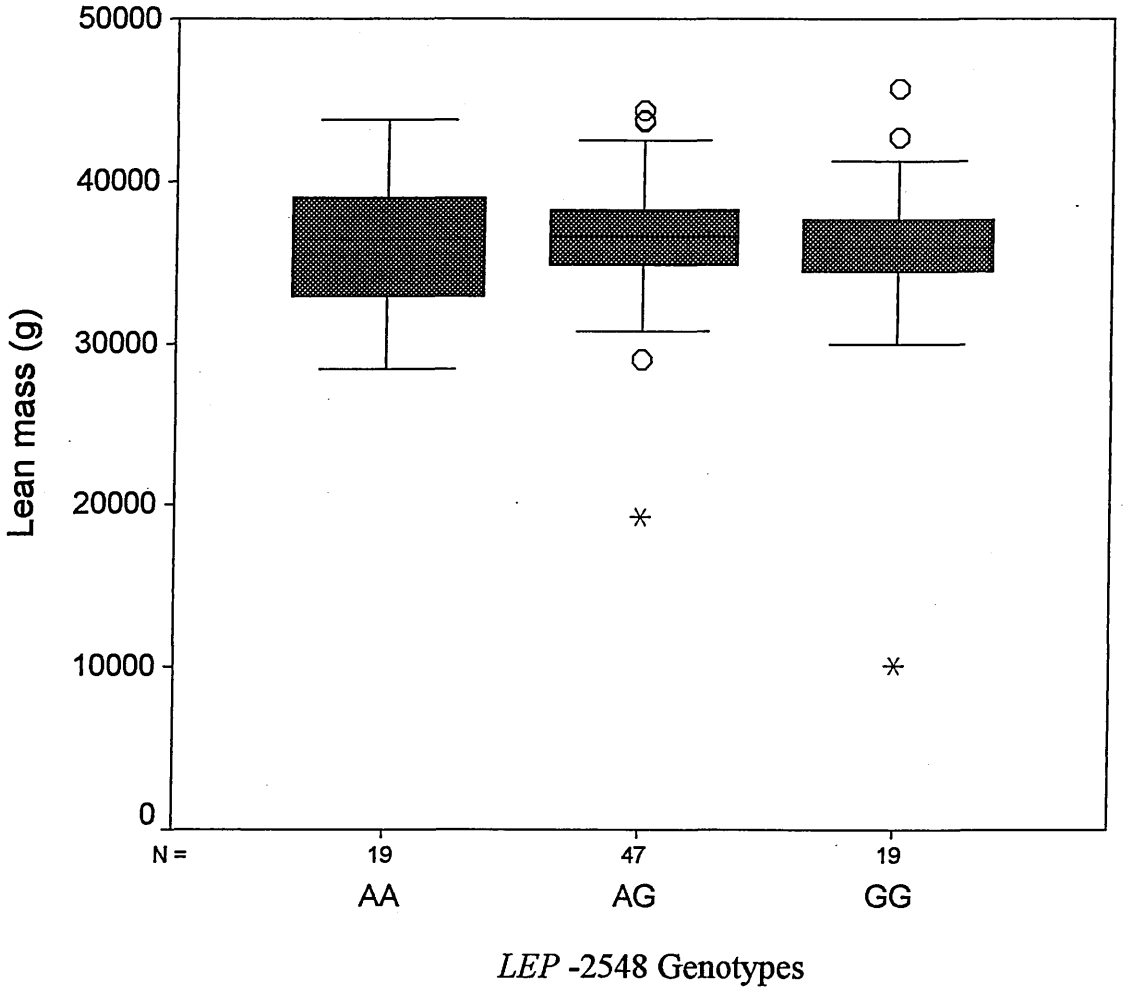


Figure 5.6: Boxplot of lean mass levels grouped to the AA, AG and GG genotypes of the *LEP* -2548 SNP. There is no significant difference between the mean of each groups.

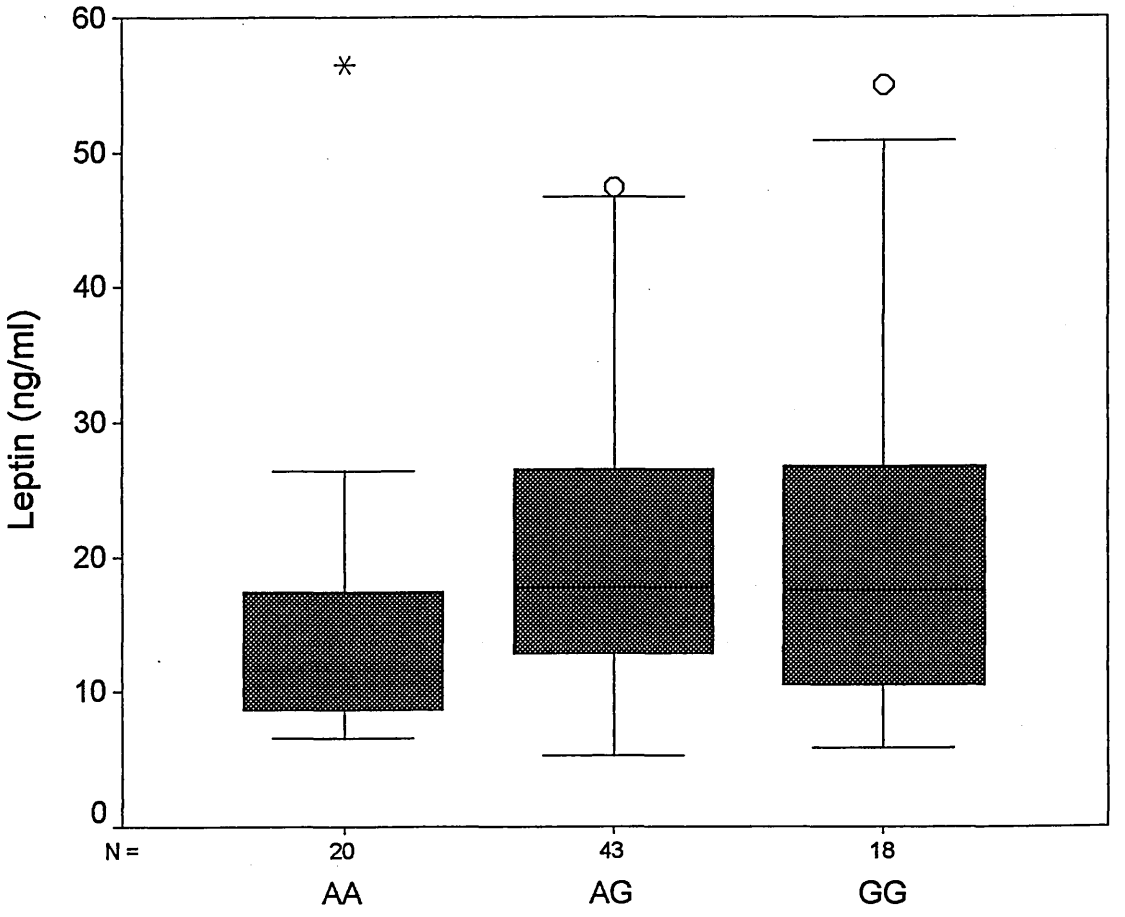


Figure 5.7: Boxplot of serum leptin levels grouped to the AA, AG and GG genotypes of the *LEP* -2548 SNP. Comparison of the mean value of the AA group with the combined other two groups showed that it was significantly lower (p=0.017). This group was also lower than the GG genotype group (p=0.035) and the AG group (p=0.015)

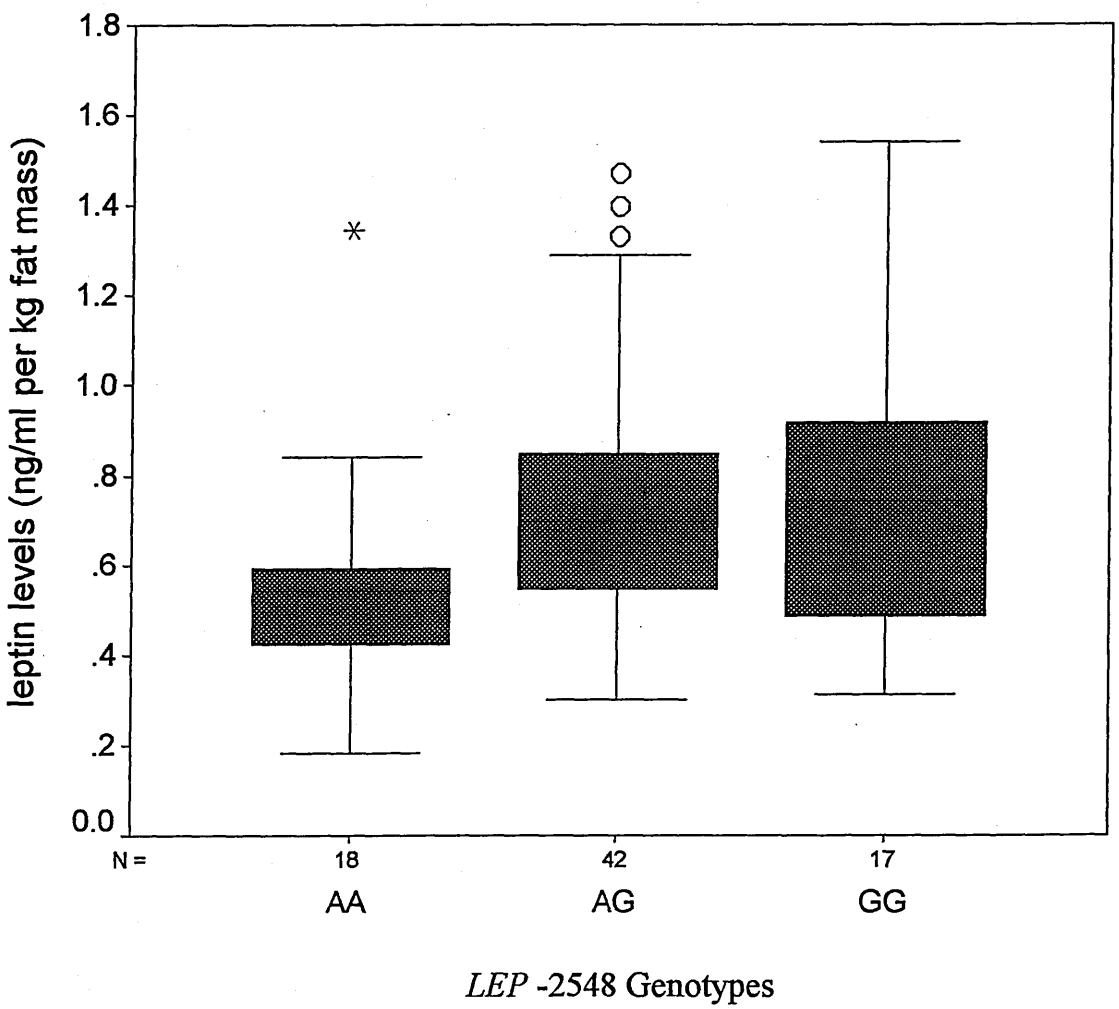


Figure 5.8: Boxplots of normalised serum leptin levels grouped to the AA, AG and GG genotypes of the *LEP* -2548 SNP. Comparison of the mean value of the AA group with the combined other two groups showed that it was significantly lower (p=0.014). This group was also significantly lower than the GG group (p=0.07) and the AG genotype group (0.015).

5.3.2 TNF-alpha G-308A SNP

97 individuals were typed for the -308 polymorphism. The balance of observed genotypes was as predicted by the Hardy-Weinberg equilibrium from the allele frequencies of 0.8 (p) and 0.2 (q) respectively (table 5.3). Mann-Whitney U analysis was used to compare the mean variable value between genotype groups (table 5.4 and figures 5.9-5.13).

Allele	**Post-menopausal Caucasian women n=97 (This study)**	Rasmussen *et al* (2000) Danish Caucasian men and women n=380	Fernandez-Real *et al* (1997) Spannish Caucasian men and women n=38	Brand *et al* (2001) German Caucasian men and women *n =176*
G	**0.80**	0.81	0.76	0.84
A	**0.20**	0.19	0.24	0.16

Table 5.4: Comparison of allele frequencies for the TNF-alpha G-308A SNP with published results

	Observed genotype frequency	**Expected genotype frequency**
	n	n
Heterozygotes	31	31
Homozygous A	4	4
Homozygous G	62	62
Total	97	97

Table 5.5: Predicted genotype distribution obtained from the Hardy-Weinberg equation for the TNF-alpha G –308A SNP using the determined allele frequencies. The p-value for the χ^2 analysis of comparing the expected to the observed genotype frequencies was 0.96 (1 degree freedom).

	Genotype			P value
	GG	AG	AA	
BMI (kg/m^2)	26.75 ± 0.57[1]	26.05 ± 0.65	28.4 ± 2.29	(AA v GG) NS (AA v GG/GA) NS
N=	n=62	n=31	n=4	(AA v AG) NS
Fat mass (g)	27216 ± 1254	24219 ± 1274	28349± 3370	NS NS
N=	n=51	n=29	n=4	NS
Lean mass (g)	36742 ± 514	34558 ± 1314	39047 ± 1138	NS NS
N=	n=51	n=29	n=4	NS
Serum leptin concentration	20.61 ± 1.85	15.57 ± 1.77	20.15 ± 3.56	NS NS
ng/ml N=	n=49	n=27	n=4	NS
Normalised Serum leptin concentration	0.74 ± 0.047	0.63 ± 0.049	0.69 ± 0.057	NS NS
(ng/ml per kg fat mass) N=	n=45	n=27	n=4	NS

Table 5.6: Mean levels of BMI, fat mass, lean mass and serum leptin levels for each genotype of the TNF alpha G –308A SNP. P values were calculated by comparing non-carriage of the G allele (AA v GG/AG genotype groups), AA v GG and AA v AG genotype groups using a Mann-Whitney U test. [1] ± standard error of mean.

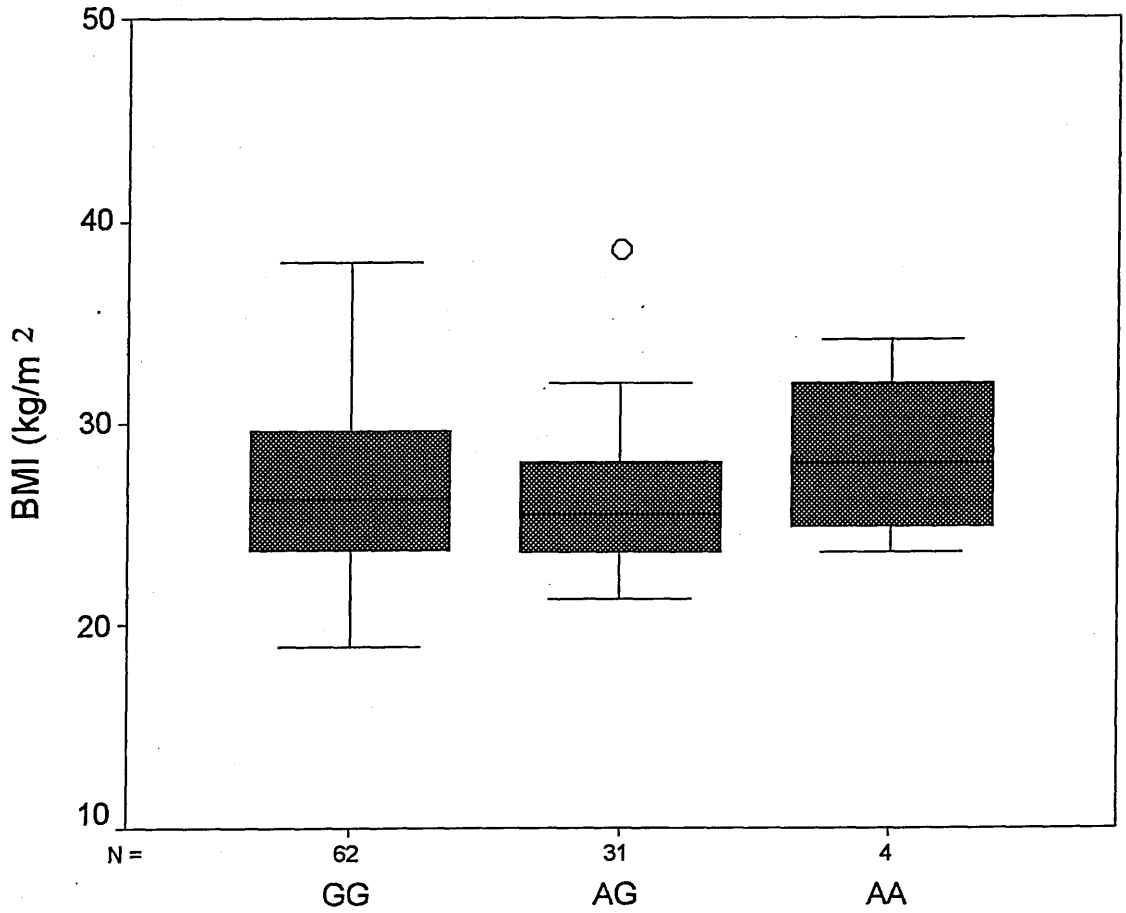


Figure 5.9: Boxplots of BMI mass levels grouped to GG, AG and AA genotypes of the TNF-α -308 SNP. There is no significant difference between the mean of each groups.

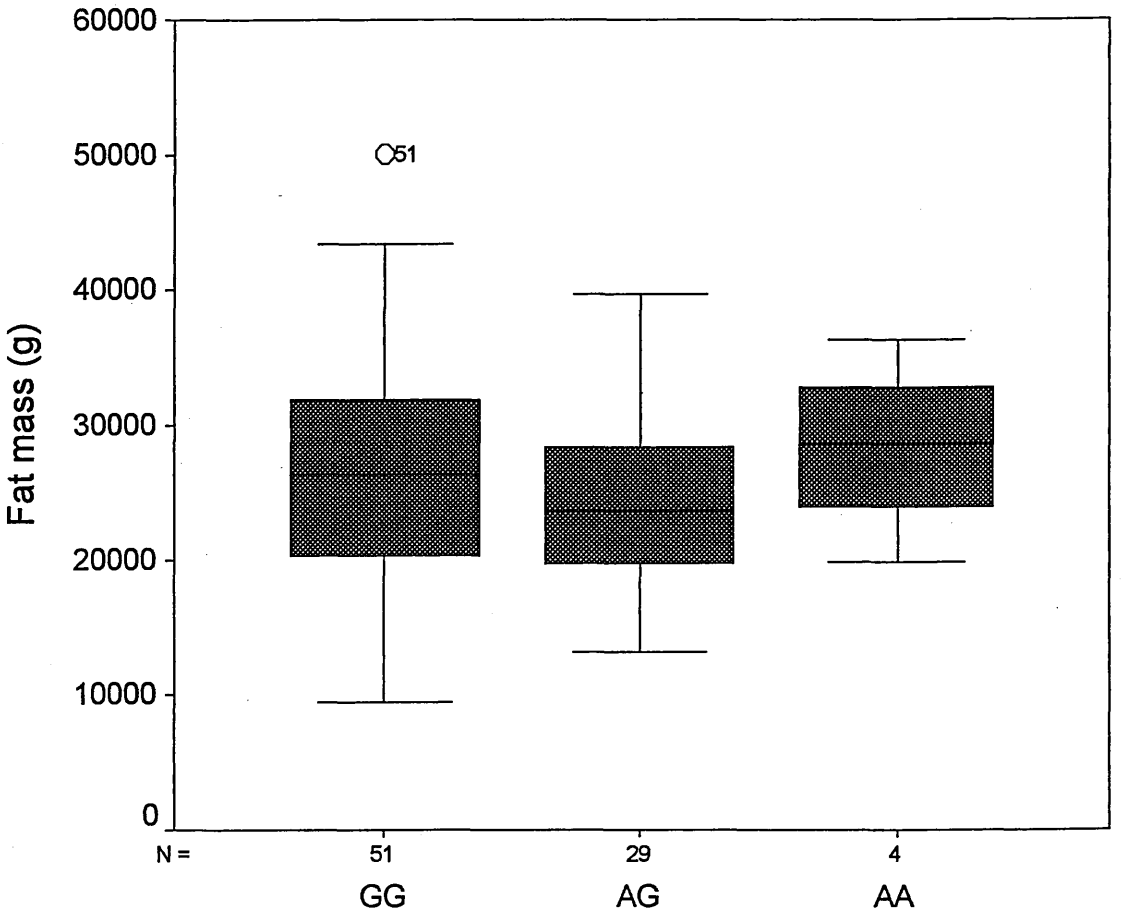


Figure 5.10: Boxplots of fat mass levels grouped to GG, AG and AA genotypes of the TNF-α -308 SNP. There is no significant difference between the mean of each groups.

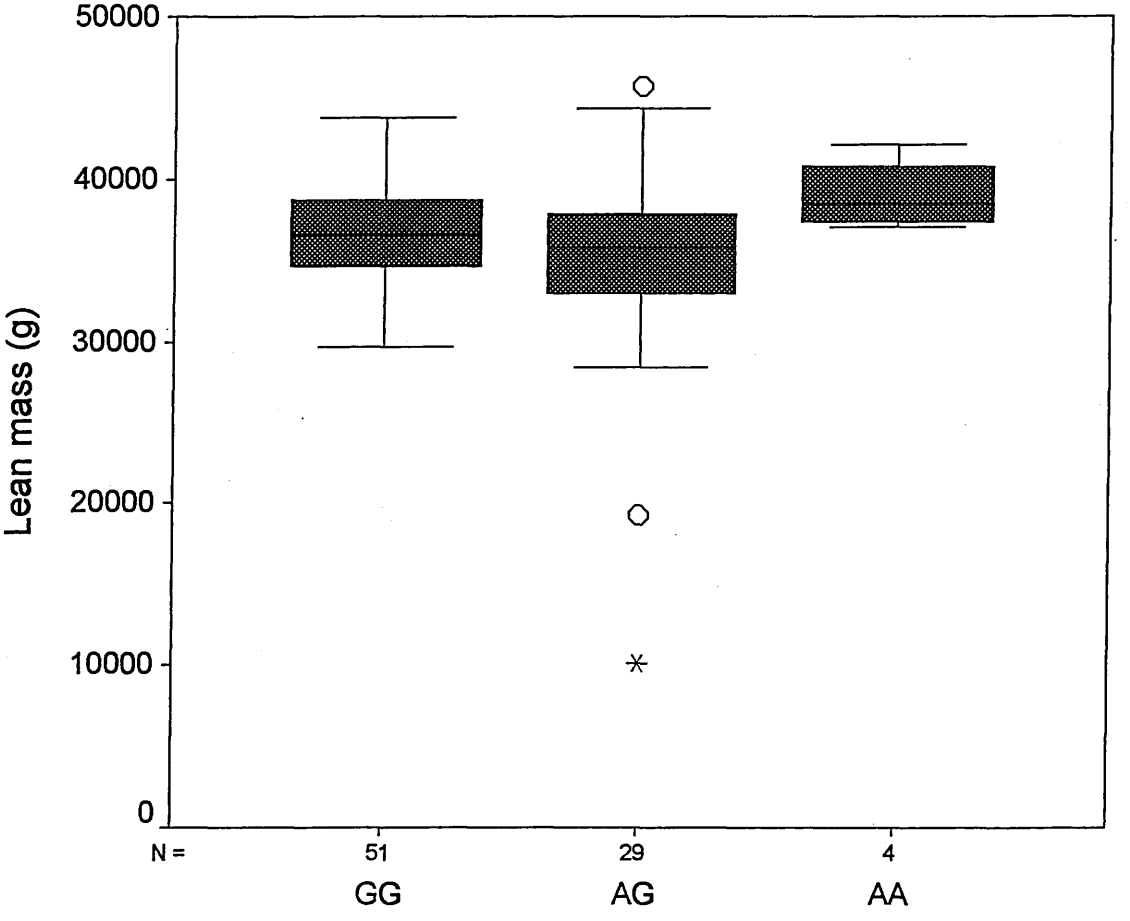


Figure 5.11: Boxplots of lean mass levels grouped to GG, AG and AA genotypes of the TNF-α -308 SNP. There is no significant difference between the mean of each groups.

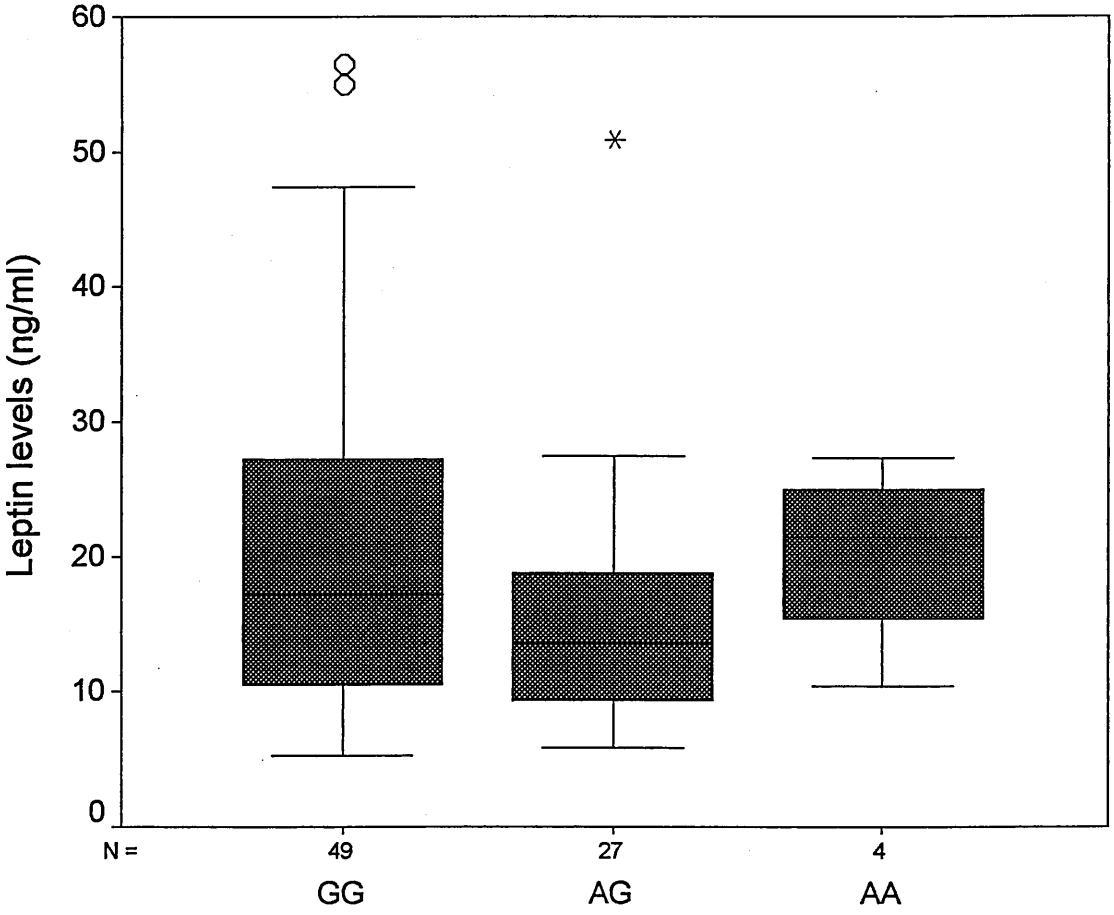


Figure 5.12: Boxplots of serum leptin levels grouped to GG, AG and AA genotypes of the TNF-α -308 SNP. There is no significant difference between the mean of each groups.

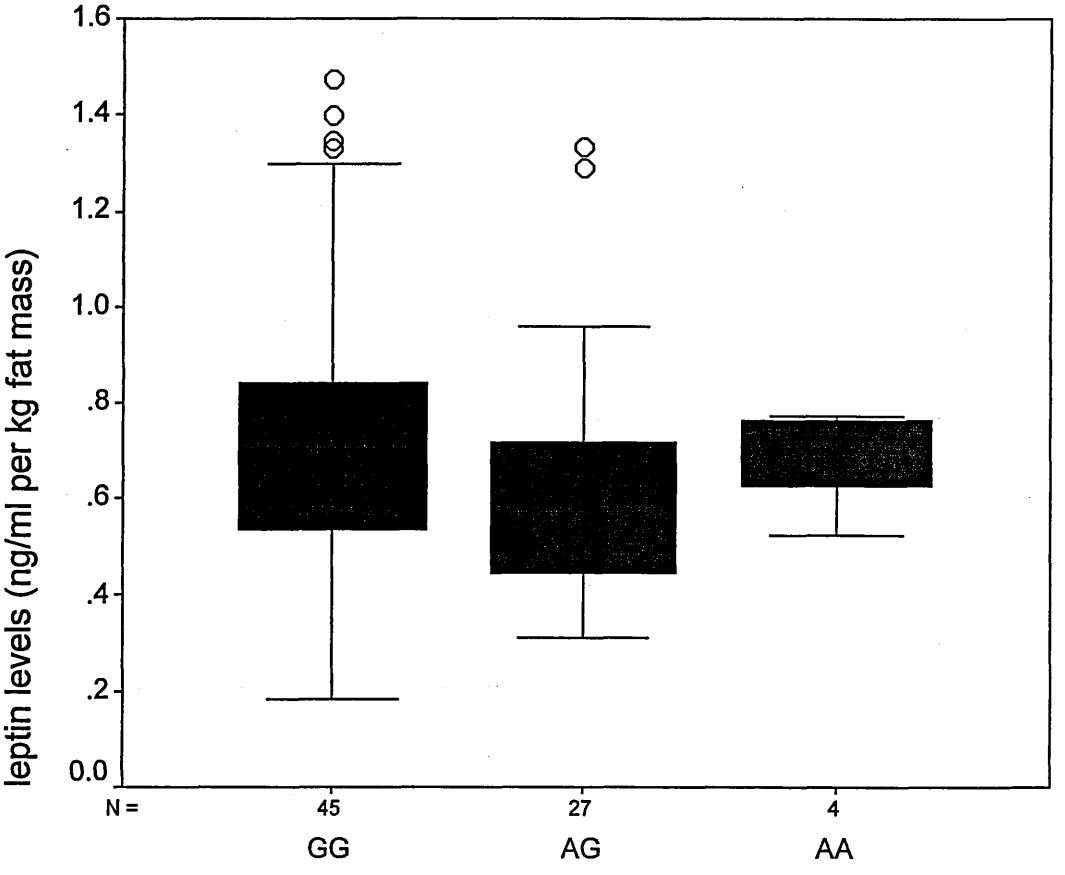


Figure 5.13: Boxplots of normalised serum leptin levels grouped to GG, AG and AA genotypes of the TNF-α -308 SNP. There is no significant difference between the mean of each groups.

5.3.3 IL1RN VNTR

To determine allele and genotype frequencies, 100 subjects have been genotyped for the IL1RN VNTR polymorphism. There have been 5 alleles reported for this polymorphism, each comprising a different number of the 86-bp core repeat sequence. In this study only the 2, 4 and 5 repeat alleles were observed. The frequencies for the 3 alleles are p =0.3, q = 0.67 and r = 0.03 respectively for the 2, 4 and 5 repeats. Due to the relative rarity of the 5 repeat allele, the 4 and 5 repeats are considered as one allele in order to simplify statistical analysis of the continuous variable data via a biallelic format. Furthermore, as this particular strategy has been repeatedly reported in the literature , table 5.7 presents comparisons of carriage of the IL1RN 2 repeat versus non-carriage between this study and published results. The distribution of genotypes observed does not differ from those predicted from a population in Hardy-Weinberg equilibrium (see table 5.5). The Mann-Whitney U test was used to compare the mean value for each variable between genotype groups (see table 5.6 and figures 5.14-.5.18)

Allele	**Postmenopausal Caucasian women This study** **(n=100)**	Healthy Japanese men and women Watanabee *et al* (2002) n= 74	American male and female Caucasians with Type 2 diabetes Marculescu *et al* (2002) n=250	Healthy Czech male and female Caucasians Vencovsky *et al* (2001) n=168
carriage of 2 repeat	**0.45**	0.068	0.27	*0.16*
non carriage of 2 repeat	**0.55**	0.932	0.73	0.84

Table 5.7: Comparison of carriage versus non-carriage of the IL1RN 2 repeat allele between this study and published results.

	Observed genotype frequency	Expected genotype frequency
	n	n
Heterozygotes 2,*	33	41
Homozygous 2,2	12	8
Homozygous *,*	55	51
Total	100	100

Table 5.8: Predicted genotype distribution obtained from the Hardy-Weinberg equation for the IL1RN VNTR in the study group using the determined allele frequencies. The p-value for the χ^2 analysis of comparison between the expected and observed genotype frequencies was 0.06 (degrees of freedom =1). Note that * represents the 4 or 5 repeats placed into a single group.

	Genotype			P value
	,	2,*	2,2	
BMI (kg/m^2)	25.92 ± 0.61[1]	26.9 ± 0.58	26.36 ± 0.87	(2,2 v *,*) NS (2,2 v 2,*/ *,*) NS
N=	n=54	n=31	n=12	(2,2 v 2,*) NS
Fat mass (g)	25166 ± 1334	27183 ± 1395	28910 ± 1878	NS NS
N=	n=46	n=28	n=11	NS
Lean mass (g)	35253 ± 884	37425 ± 770	36416 ± 787	NS NS
N=	n=46	n=28	n=11	NS
Serum leptin concentration (ng/ml) N=	15.54 ± 1.2 n=43	21.67 ± 2.73 n=27	25.83 ± 4.2 n=11	0.011 0.02 NS
Normalised serum leptin (ng/ml per kg fat mass) N=	0.63 ±0 .037 n=40	0.75 ± 0.066 n=27	0.86 ± 0.091 n=10	0.006 0.02 NS

Table 5.9: Mean levels of BMI, fat mass, lean mass and serum leptin levels for each genotype of the IL1RN VNTR. P values were calculated by comparing non-carriage of the * allele (2,2 v 2,* / *,* genotype groups), 2,2 v *,* and 2,2 v 2,* genotype groups.
* = 4 or 5 repeat. [1] ± standard error of mean

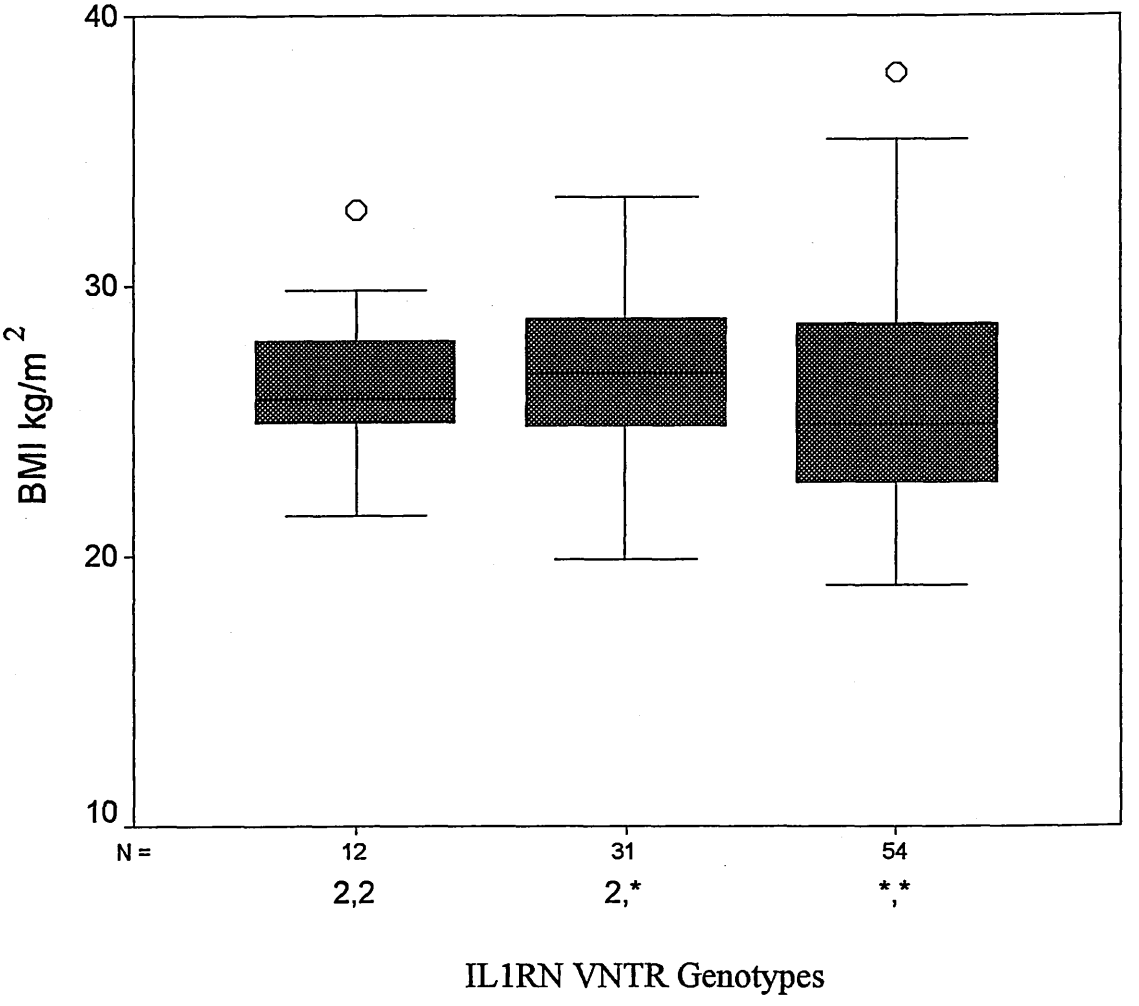


Figure 5.14: Boxplots of BMI data grouped to ILRN VNTR genotypes.

* represents the 4 or 5 repeat allele. There is no significant difference between the mean of each groups.

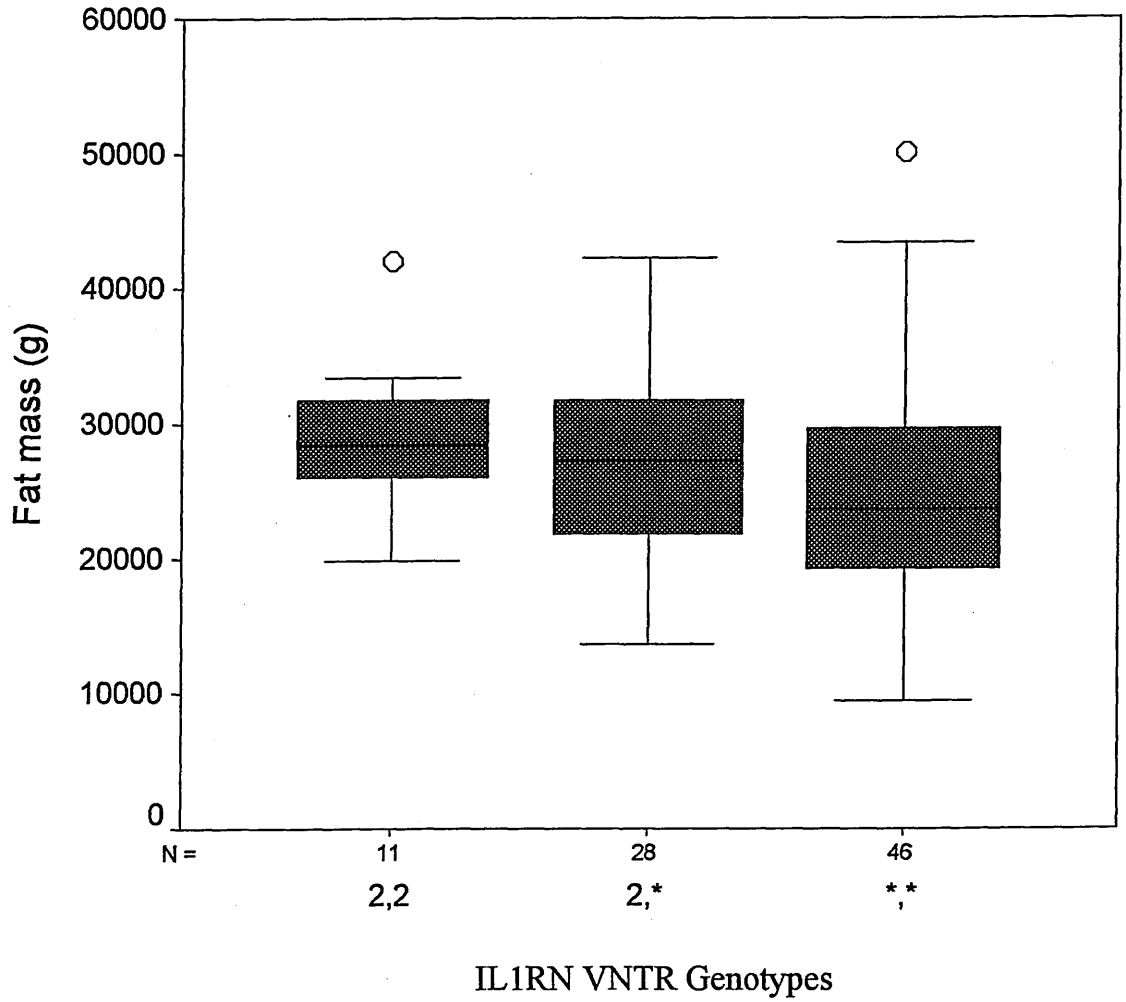


Figure 5.15: Boxplots of fat mass levels grouped to ILRN VNTR genotypes. * represents the 4 or 5 repeat allele. There is no significant difference between the mean of each groups.

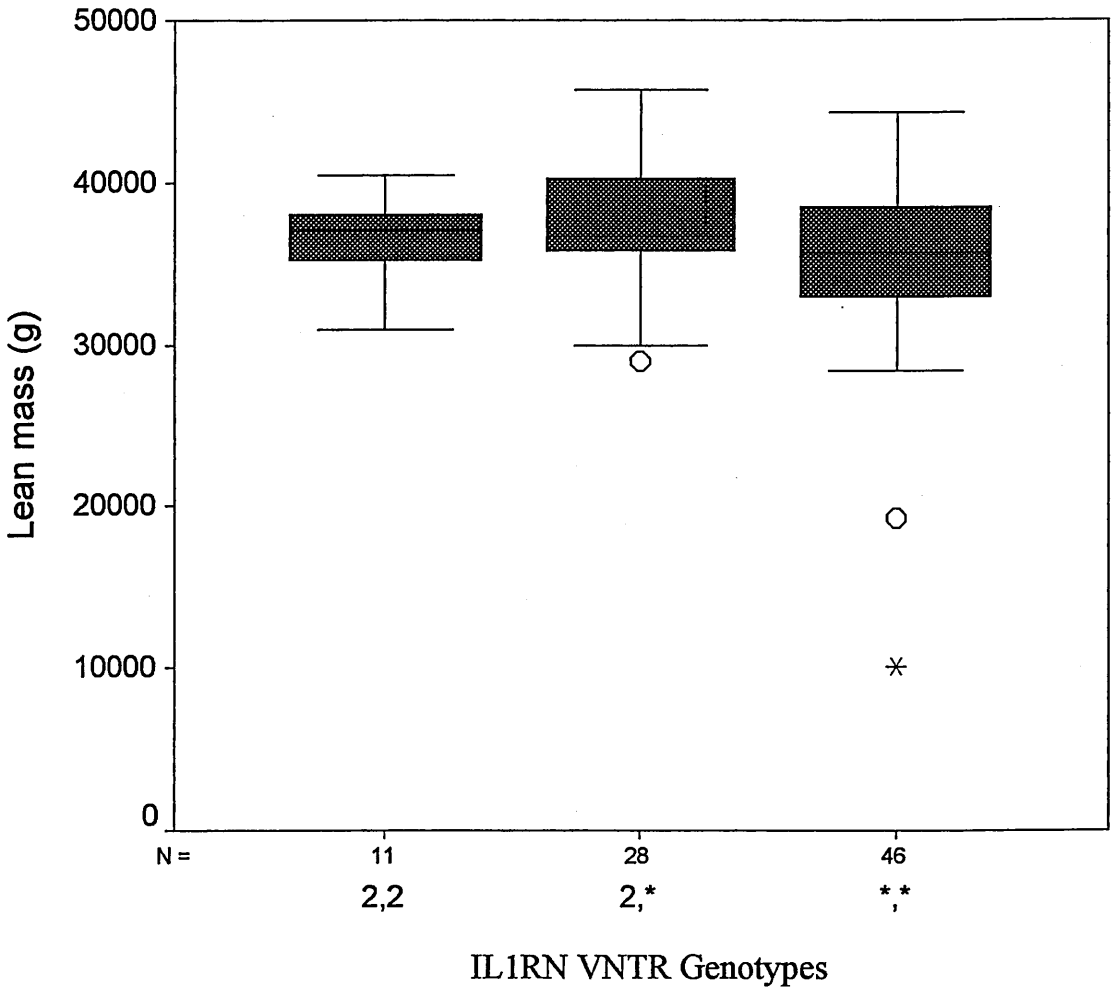


Figure 5.16: Boxplots of lean mass levels grouped to ILRN VNTR genotypes. * represents the 4 or 5 repeat allele. There is no significant difference between the mean of each groups.

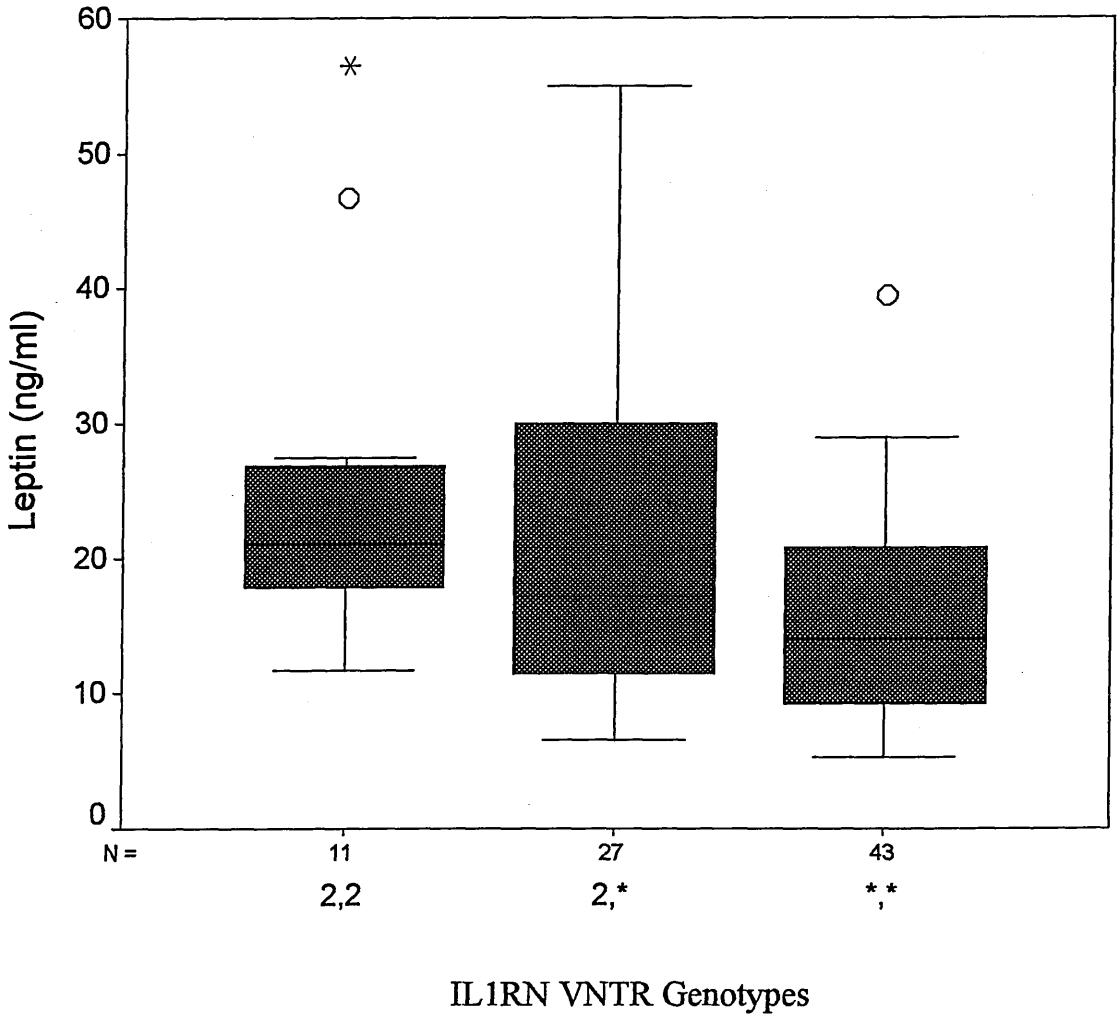


Figure 5.17: Boxplots of serum leptin levels grouped to the ILRN VNTR genotypes. * represents the 4 or 5 repeat allele. Comparison of the mean levels between the *,* and the combined other 2 groups genotype reveals that the mean levels are significantly lower in the *,* group (p=0.02). Also, the mean level is lower in the *,* group compared to the 2,2 genotype group (p=0.011).

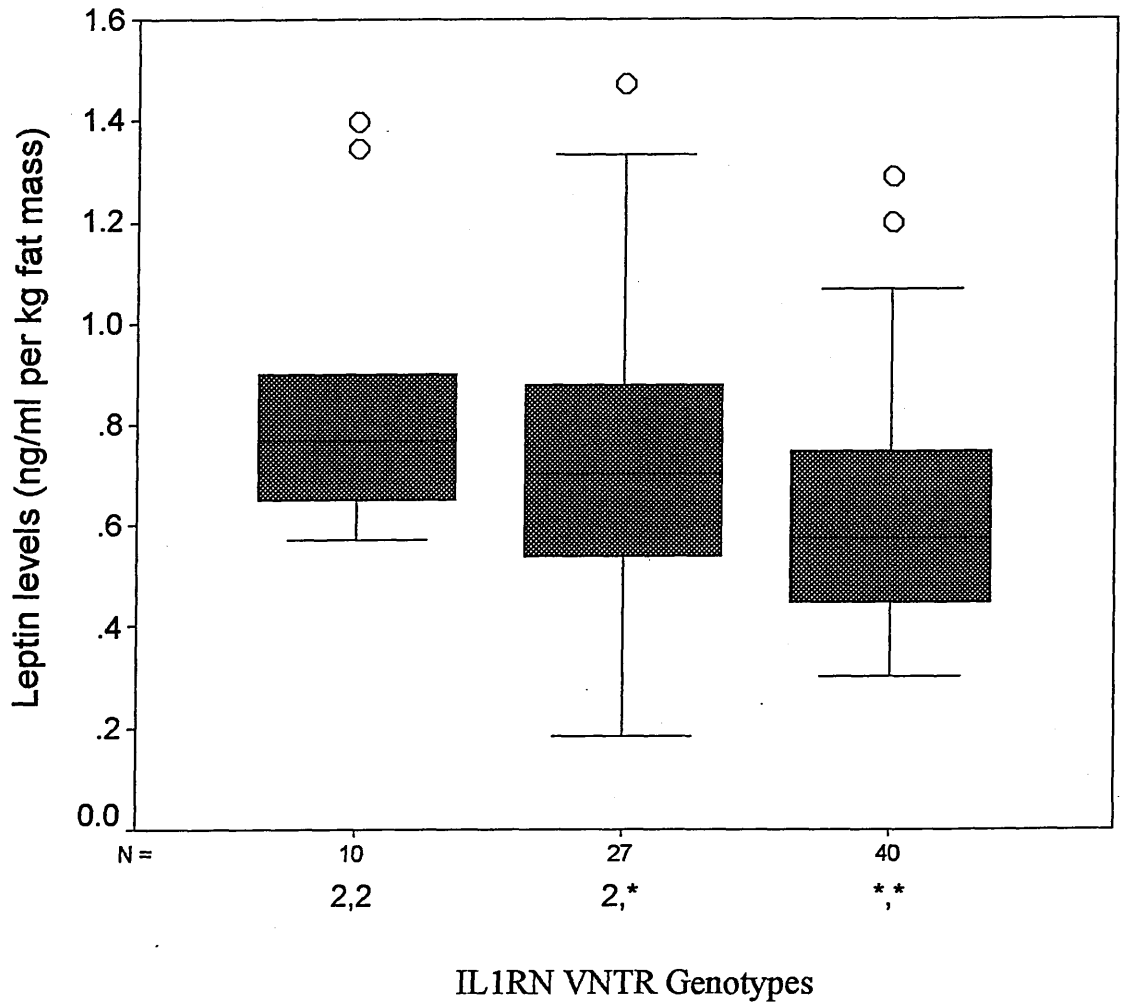


Figure 5.18: Boxplots of normalised serum leptin levels grouped to the ILRN VNTR genotypes. * represents the 4 or 5 repeat allele. Comparison of the mean levels between the *,* and the combined other 2 groups reveals that the mean levels are significantly lower in the *,* group (p=0.02). Also, the mean levels are lower in the *,* group compared to the 2,2 genotype group (p=0.006).

5.4 Discussion

The genetic basis underpinning weight regulation has been gradually teased apart since breakthrough discoveries in the mid-nineties. It has become clear that obesity in humans is most likely polygenic in the majority of cases. With this in mind, the activity of different candidate susceptibility genes, other than those involved in the leptinergic system, are currently being investigated. Cytokines are relevant candidates due to the overlapping, integrated actions of these proteins.

In this study, polymorphisms in the TNF-α, IL1RN and *LEP* genes have been analysed in relation to markers of adiposity. Of the three genes studied, only the *LEP* -2548 promoter SNP showed more than one association, with the A allele associated with lower mean BMI, normalised and actual leptin levels. Although fat mass is generally assumed to be a more accurate indicator of adiposity, individuals with the A allele did not have significantly lower levels although the levels were lower arithmetically. Furthermore, a larger data set were available for BMI than for fat mass perhaps making it more accurate overall.

Associations with this SNP have been observed before. Mammes and colleagues (1998) demonstrated that the A allele was associated with higher leptin levels in a mixed sex cohort.

Subsequent to this, Mammes and colleagues (2000) showed that the G allele was more frequent in overweight individuals in a case-control mixed sex association study. Furthermore, carriage of the G allele was associated with lower leptin levels in men from the cohort. The results from this present study suggest an opposite effect.

It agrees, however, with a study by Le Stunff and colleagues (2000) who observed that obese Caucasian girls of similar adiposity had lower leptin levels associated with

carriage of the A allele. It is tempting to speculate that the agreement between this present study and that carried out by Le Stunff is perhaps partly due to to the limited affect of sex hormones in both studies. A substantial proportion from Le Stunff's study would have been prepubscent due to their age (mean of 11.5 years ± 2.9 years standard deviation). In our study all women were postmenopausal. The menstrual cycle is associated with fluctuations in leptin levels (Hardie *et al.*, 1997, Quinton *et al.*, 1999). For this reason, both studies may not be confounded by this variation unlike the studies carried out by Mammes and colleagues.

No associations were uncovered for the TNF-alpha G –308A SNP. However the SNP was worthy of investigation due to reported associations with obesity (Hoffstedt *et al.*, 2000, Brand *et al.*, 2001) and particularly with insulin resistance in obesity (Dalziel *et al.*, 2002, Wybranska *et al.*, 2003).

Reasons why no associations were discovered in this study could include the relatively small cohort size, and also the possibility that the penetrance of risk alleles depends upon the genetic background present in the study group. Also, the prevalence of an environmental risk factor may differ between different groups being studied, affecting any observations (or lack of). These differences may partly explain conflicting results from association studies.

With the IL1RN VNTR, individuals lacking the two repeat allele had significantly lower mean levels of actual and normalised serum leptin levels. Leptin induces IL-1ra expression and secretion from monocytes (Gabay *et al.*, 2001). Although no studies have been reported of IL-1ra modulating leptin expression and secretion, a cross-talk system may be operating.

As no associations were detected for BMI or adiposity, this association does not appear to extend to adiposity. However, roles for leptin in infection and immunity have

become apparent. Leptin is generally proinflammatory in action inducing *in vitro* secretion of IFNγ and IL-2 from stimulated T-cells in human peripheral or umbilical cord blood, which predictes a shift to a T helper 1 proinflammatory environment (Martin *et al.,* 2000). In monocytes, leptin stimulates TNF-α, IL-6 and IFNγ in resting or endotoxin-stimulated peripheral blood mononuclear cell cultures (Zarkesh *et al.,* 2001). Therefore an intrinsic relationship with IL-1ra (a major anti-inflammatory cytokine) might be expected to balance leptin's proinflammatory effects.

Chapter 6

Case-Control Association study of

LEPR SNPs in

Anorexia Nervosa

6.1 Introduction

Although described since Roman times, the incidence of the eating disorder, anorexia nervosa (AN) has markedly increased over the past 50 years. Its causes include sociocultural influences, individual risks linked to subjection to abuse, as well as biochemical and genetic components.

The DSM-IV diagnostic criteria for AN include a maintained body weight of less than 85% of normal for age and height, intense fear of fatness, disturbed experience of body weight or shape and amenorrhoea for at least 3 consecutive cycles. The disorder can be sub-divided into restriction of food intake or binge/purge endophenotypes.

AN has been shown to have a heritable component, with increased prevalence of eating disorders amongst relatives of probands compared to controls (Gershon *et al.,* 1984 and Walters *et al.*, 1985). Furthermore, concordance rates between monozygotic twins with AN has been estimated to be between 52-56% (Treasure and Holland, 1994). These observations indicate a genetic component.

Although substantial research has been carried out on the effects of the leptin system on obesity, relatively little work has been concerned with leptin alterations in anorexic individuals. As part of the "adipostat", leptin maintains the body's adiposity by reducing feeding and increasing energy expenditure in response to food intake. Any modifications in the leptin system may contribute to anorexia.

Several lines of experimental evidence point to a potential role for the leptin system in AN. Mantzoros *et al.,* (1997) showed that body fat was lower in long term recovered AN individuals suggestive of an underlying genetic factor controlling body fat in these people. Whilst during recovery, leptin levels progressively increase to reach higher than expected levels for the body weight (Hebebrand *et al.,* 1997).

Krizova *et al* (2002) observed that soluble leptin receptor levels in anorexic women were significantly higher compared to healthy female controls and were not affected when measured 6 weeks into partial re-feeding. Such apparent modifications compared to healthy individuals suggest the possibility of an etiological role for the leptin system (For review see Brichard *et al.*, 2003). In a recent genome-wide linkage study using families of European ancestry, Grice *et al* (2002) detected an anorexia nervosa susceptibility locus using marker D1S3721. The marker has been mapped to 1p34.2. The leptin receptor is a plausible candidate gene that has been mapped to 1p31.2, some distance from that marker on the same chromosome arm.

Of particular relevance to this study, Quinton *et al* (2001) demonstrated a decrease in BMI and fat mass associated with the extracellular domain GLN223ARG leptin receptor SNP in a population-based association study. An adenine to guanine DNA base change substitutes the amino acid arginine for glutamine. This suggests the potential for altered ligand binding dependent on which allelic form is expressed. Indeed, preliminary evidence of lowered ligand-binding with the 223ARG SNP has been presented elsewhere in this thesis (chapter 4, figure 4.13).

As this polymorphism is associated with a decrease in BMI, it is a good candidate to test in anorectic populations who, by definition, have lowered BMI and fat mass levels. Furthermore, linkage disequilibrium with nearby polymorphisms in the *LEPR* gene has been established, in a Caucasian cohort elsewhere in this thesis, between the LYS109ARG and GLN223ARG SNPs but neither in LD with LYS656ASN (chapter 3, table 3.10). For completeness, the LYS109ARG and LYS656ASN SNPs have also been investigated to determine whether a nearby SNP is actually having an effect or whether specific haplotypes are associated with AN.

6.1.1 Aims of the study

Three leptin receptor SNPs, LYS109ARG, GLN223ARG and LYS656ASN, were studied. The aims of this study include;

- genotyping of female anorexic subjects and healthy female controls for the three SNPs.
- to determine if allele and / or haplotype frequencies, for the three SNPs, differ between the anorexic and control cohorts, and also between specific anorexic subtypes and the control cohort.

6.2 Study Design

Blood samples were collected from a cohort of British Caucasian patients with AN.

All subjects provided informed consent and ethical approval was granted for the study.

Purified genomic DNA obtained from the blood samples was available for 175 unrelated Caucasian female AN subjects (DSM-IV criteria) who attended the Yorkshire Centre for Eating Disorders. A sub-set of the anorexic patients were classified into restricting (n=64) and binge/purge (n=77) sub-types with the remaining patients unable to be so categorised at the time of assesement. The mean age of diagnosis for the entire cohort was 18.1 years, with a minimum mean body mass index of 13.73 kg/m^2 (BMI data provided is the lowest BMI recorded during treatment).

The control cohort contained 145 unrelated unaffected Caucasian females. The mean age of the females was 30.28 years with an average BMI of 22.02 kg / m^2. The latter were screened for a personal or immediate family history of an eating disorder.

6.2.1 Analysis

Genotyping was carried out using a PCR-RFLP method as described in chapter 2, section 2.2.1 and 2.2.2. Visualisation of alleles for the GLN223ARG SNP is shown in figure 6.1.

Cases and controls were compared to check for associations with susceptibility to AN per se, and binge/purge and restricting sub-types were compared for specific associations with these endophenotypes. The distribution of genotypes was tested for deviation from Hardy-Weinberg equilibrium by χ^2 analysis. Comparison of allele frequencies between groups was also tested by χ^2 analysis. The frequencies of specific haplotypes in the control and patient samples was calculated using the EH software program as was the assignment of haplotypes with unknown phase via a permutation function present in the program. The FASTEH software was employed to obtain a p-value via the χ^2 statistic, comparing the distribution of haplotype frequencies between case and control individuals and between binge/purge and restricting anorexia endophenotypes.

6.3 Results

A total of 175 anorexic samples and 145 control samples were genotyped for the LYS109ARG, GLN223ARG and LYS656ASN SNPs in order to determine allele, genotype and frequencies. The calculated allele frequencies for the anorexic cohort are shown in tables 6.1-3 alongside other published allele frequencies. There is good agreement between the allele frequencies in this study and previous studies. The observed balance of homozygous and heterozygous genotypes for each of the three

SNPs, in the case and control cohorts, did not differ significantly from that expected of a population in Hardy-Weinberg equilibrium (tables 6.4 and 6.5). The allele frequencies and carriage rates of the three SNPs for both the anorexic and control groups are shown in table 6.6. χ^2 analysis of 2x2 contingency tables comparing allele frequencies and carriage rates between the cases and controls for each of the SNPs did not highlight any significant difference in frequencies. Furthermore, subdivision of the case cohort into restricting and binge/purging groups (table 6.5) did not highlight any significant differences either.

The EH computer program calculated haplotype frequencies for case, control, binge/purge and restricting types (table 6.7). Comparison of haplotype frequencies between groups was analysed using a χ^2 statistic by the FASTEH program. A p-value of 0.70 was obtained when comparing case versus control haplotypes. A p-value of 0.45 was obtained comparing binge/purge to restricting types. In both instances, therefore, there was no significant difference in haplotype frequencies.

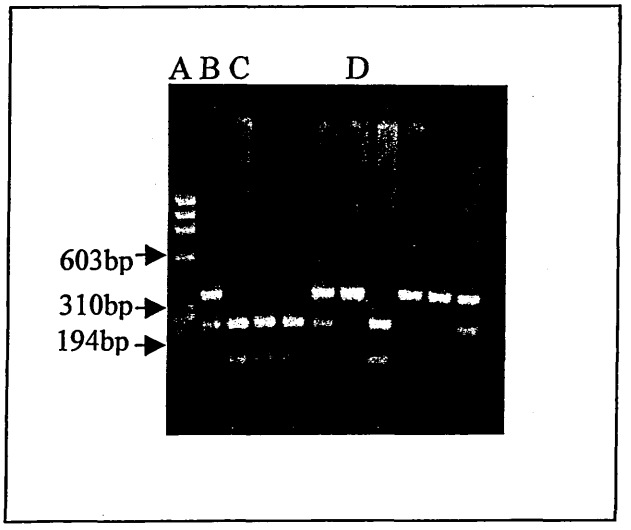


Figure 6.1: Photograph of the GLN223ARG SNP genotypes on a 2.5% agarose gel containing, **A:** ϕX 174 *Hae* III DNA marker, **B:** Heterozygous AG genotype, **C:** Homozygous G genotype (294 and 157bp), and **D:** homozygous A genotype (421bp)

Allele	**Anorexic women (this study)**	Lean British males (n=132) *Gotoda et al.*, 1997	Obese British males (n=190) *Gotoda et al.*, 1997	Caucasian males and females (n=190) *Chagnon et al.*, 2000
A	**0.75**	0.74	0.73	0.73
G	**0.25**	0.26	0.27	0.27

Table 6.1: Comparison of LYS109ARG allele frequencies for the anorexic cohort with published results. The allele frequencies for the control women are given in table 6.6

Allele	**Anorexic women (this study)**	Lean British males (n=132) *Gotoda et al.*, 1997	Obese British males (n=190) *Gotoda et al.*, 1997	Caucasian males and females (n=190) *Chagnon et al.*, 2000
A	**0.6**	0.57	0.56	0.54
G	**0.4**	0.43	0.44	0.46

Table 6.2: Comparison of GLN223ARG allele frequencies for the anorexic cohort with published results. The allele frequencies for the control women are given in table 6.6

Allele	**Anorexic women (this study)**	Lean British males (n=132) *Gotoda et al.*, 1997	Obese British males (n=190) *Gotoda et al.*, 1997	Caucasian males (n=190) *Chagnon et al.*, 2000
G	**0.86**	0.84	0.82	0.82
C	**0.14**	0.16	0.18	0.18

Table 6.3: Comparison of LYS656ASN allele frequencies for the anorexic cohort with published results. The allele frequencies for the control women are given in table 6.6

	Observed genotype frequency	Expected genotype frequency
LYS109ARG	**n**	**n**
Heterozygotes	68	64
Homozygous A	96	98
Homozygous G	9	11
Total	173	173
GLN223ARG	**n**	**n**
Heterozygotes	81	84
Homozygous A	65	63
Homozygous G	29	28
Total	175	175
LYS656ASN	**n**	**n**
Heterozygotes	41	42
Homozygous G	128	128
Homozygous C	4	3
Total	173	173

Table 6.4: Predicted genotype distributions obtained from the Hardy-Weinberg equation for the LYS109ARG, GLN223ARG, and LYS656ASN SNPs in the anorexic cohort using the determined allele frequencies. The p-value for the χ^2 analysis of comparing the expected to the observed genotype frequencies was 0.49, 0.66 and 0.74 for the LYS109ARG, GLN223ARG and LYS656ASN SNPs respectively (calculated to 1 df). Note that the total number of genotypes for each SNP differ due to failure of PCR amplification for some samples.

	Observed genotype frequency	Expected genotype frequency
LYS109ARG	**n**	**n**
Heterozygotes	48	46
Homozygous A	72	73
Homozygous G	6	7
Total	126	126
GLN223ARG	**n**	**n**
Heterozygotes	70	72
Homozygous A	46	45
Homozygous G	29	28
Total	145	145
LYS656ASN	**n**	**n**
Heterozygotes	33	31
Homozygous G	90	91
Homozygous C	2	3
Total	125	125

Table 6.5: Predicted genotype distributions obtained from the Hardy-Weinberg equation for the LYS109ARG, GLN223ARG, and LYS656ASN SNPs in the control cohort using the determined allele frequencies. The p-value for the χ^2 analysis of comparing the expected to the observed genotype frequencies was 0.58, 0.8 and 0.6 for the LYS109ARG, GLN223ARG and LYS656ASN SNPs respectively (calculated to 1 df). Note that the total number of genotypes between SNPs (for each group) may differ due to failure of PCR amplification for some DNA samples.

	Anorexic Cohort					
Codon	**109**	n = 173	**223**	n = 175	**656**	n = 173
Allele	**A**	**G**	**A**	**G**	**G**	**C**
Allele frequency	0.75	0.25	0.60	0.40	0.86	0.14
Carriage Rate	0.95	0.45	0.83	0.63	0.98	0.26
	Bingeing / purging group					
Codon	**109**	n = 76	**223**	n = 75	**656**	n = 77
Allele	**A**	**G**	**A**	**G**	**G**	**C**
Allele frequency	0.76	0.24	0.6	0.44	0.86	0.14
Carriage Rate	0.93	0.41	0.83	0.63	0.97	0.25
	Restricting group					
Codon	**109**	n = 61	**223**	n = 64	**656**	n = 61
Allele	**A**	**G**	**A**	**G**	**G**	**C**
Allele frequency	0.78	0.28	0.58	0.42	0.85	0.15
Carriage Rate	0.95	0.51	0.83	0.67	0.97	0.26
	Control Cohort					
Codon	**109**	n = 126	**223**	n = 145	**656**	n = 129
Allele	**A**	**G**	**A**	**G**	**G**	**C**
Allele frequency	0.76	0.24	0.56	0.44	0.85	0.15
Carriage Rate	0.95	0.43	0.80	0.68	0.98	0.28

Table 6.6: Allele frequencies and carriage rates for the anorexic, restricting, bingeing/purging and control cohorts with three LEPR SNPs. Note that differences in the total number of genotypes between SNPs (for each group) differ due to failure of PCR amplification from some DNA samples. Also, not all anorexic individuals were able to be classified as bingeing or restricting at time of recruitment.

Haplotype			**control cohort**	**anorexic cohort**	**Bingeing/ purging anorexics**	**restricting anorexics**
LYS109ARG	**GLN223ARG**	**LYS656ASN**				
A	**A**	**G**	0.46	0.45	0.46	0.43
A	**A**	**C**	0.10	0.07	0.07	0.06
A	**G**	**G**	0.21	0.20	0.20	0.22
A	**G**	**C**	0.03	0.02	0.05	0
G	**A**	**G**	0.05	0.04	0.05	0.04
G	**A**	**C**	0	0.03	0.03	0.04
G	**G**	**G**	0.13	0.16	0.14	0.18
G	**G**	**C**	0.02	0.02	0	0.03

Table 6.7: Haplotype frequencies estimated for control/anorexia groups and bingeing/purging groups.

6.4 Discussion

To date, there have been no published studies investigating associations between SNPs in the *LEPR* gene and AN. There has been one published study which investigated a polymorphism (G -1387A) in the region upstream of the *LEP* gene by comparing allele frequencies for the SNP between individuals with AN and healthy underweight people. No associations were uncovered (Hinney *et al.*, 1998).

The results from this case-control study suggest that there is no association with the SNPs studied of the *LEPR* gene and the eating disorder, anorexia nervosa, as a whole or with its specific restricting and binge/purge sub-types.

The difference in allele frequencies between the anorexic and control groups is not significant, whilst the frequencies in both groups agree well with published data on Caucasians. Furthermore, there is no significant difference in haplotype frequencies between cases and control or binge/purge and restricting endophenotypes.

Previous studies have shown that polymorphisms in the *LEPR* gene are in linkage disequilibrium (Thompson *et al.*, 1997, Gotoda *et al.*, 1997). If the SNPs studied are in linkage disequilibrium then certain haplotypes may be associated with anorexia and heritable. However the EH and FASTEH computer programs did not detect any particular haplotype which is associated with anorexia nervosa as a whole or more specifically within the binge/purge or restricting sub-types.

Although the anorexic cohort has a mean lower BMI than the control group, there are no associations between anorexia and the GLN223ARG SNP. Unfortunately, individual BMI values were not available to enable BMI analysis per genotype group for the control and test cohorts respectively.

An association was found between BMI and the GLN223ARG SNP in postmenopausal women (Quinton *et al.*, 2001). A specific lowered BMI in older aged individuals (middle-aged men only) has also been previously reported by Chagnon *et al* (2000).

Bearing this in mind, it is possible that the SNPs in the leptin receptor may predispose to lower BMI but that confounding hormonal factors present in young women and not present in postmenopausal women may mask this. This may be the case for both the control and anorexic individuals. Also to consider, is that although the mean BMI data was available for the control and anorexic groups, unfortunately individuals' BMI values were not available for this study, which would have enabled analysis of associations of BMI with a particular genotype in either the cases or control groups.

Several association studies have been conducted testing various candidate genes for AN. Some, including studies on the 5-hydroxytryptophan (serotonin) receptor, have generated associations (Nacmias *et al.*, 1999), whilst others have not (Hinney *et al., 1997* and Campbell *et al.*, 1998). As such, at the biological level, variation in the leptin receptor gene may not predispose to anorexia. Considering the undoubtedly complex gene-gene and gene environment interactions involved in the disorder (probably more heterogeneous in nature than a quantitative trait such as lowered BMI), there is a distinct possibility that more than one particular genetic background may predispose to AN and that several of these will be present in a cohort of anorexic individuals. Because of this, the frequency of any one kind may be lower and less easily detected.

7.1 Discussion

The basic aim of this study was to characterise and expand upon our knowledge of the genetic and functional features of the leptin receptor. Firstly, genetic association studies were undertaken to examine the effect of leptin receptor SNPs on markers of adiposity in a postmenopausal European Caucasian and also an anorexic cohort. To complement this, genetic studies were also carried out using other relevant cytokine gene polymorphisms (including a SNP in the *LEP* gene). Secondly, recombinant protein expression and *in vitro* functional characterisation of the leptin receptor was undertaken.

Leptin is a cytokine predominately secreted from adipose tissue but also by other tissues including the stomach (Bado *et al.*,1998), skeletal muscle (Wang *et al.*,1998) and the brain (Weisner *et al.*,1999). It exerts its effects by crossing the blood-brain barrier and binding to the leptin receptor in the hypothalamus. The leptin receptor is expressed in various tissues of the body, including the placenta (Akerman *et al.*, 2002, Hauguel-de Mouzon, 2003) and skeletal muscle (Wang *et al.*, 1998). However, initially it was located to the hypothalamus in the brain (Tartaglia *et al.*, 1995). Indeed, although the known roles of both proteins has expanded since their discoveries to include involvement in processes as apparently diverse as reproduction and immunity, the majority of work to date has concentrated on elucidating their role in notifying the brain of the body's level of adiposity.

The hypothalamus is the structure in the brain that controls food intake, energy expenditure and, consequently, body mass. An increase in body fat raises serum leptin concentrations, which results in increased signalling of the long form of the receptor in

the hypothalamus leading to downstream events that contribute to a reduction in food intake and increases in thermogenesis and physical activity. BMI and fat mass have a positive linear correlation with leptin levels. In obese individuals, leptin levels are (excessively) high, suggesting that, at the molecular level, leptin resistance may occur, possibly through the saturable nature of leptin transport into the brain (Banks *et al.*, 2000, Maness *et al.*, 2000).

In humans, mutations that significantly disrupt these proteins and cause monogenic obesity have only been discovered in a handful of families (Montague *et al.*, 1997b, Clement *et al.*, 1998). However, multifactorial obesity influenced by a variety of genetic and environmental factors, appears much more prevalent. Several SNPs exist in the leptin and leptin receptor genes that may influence the level of protein expression or alternatively, alter functional characteristics including ligand binding, receptor dimerisation and downstream signalling events.

Using DNA from a cohort of postmenopausal Caucasian women (used to avoid confounding cyclic effects of reproductive hormones on serum leptin levels and LBA), the SNPs, LYS109ARG and LYS656ASN were examined to see whether any associations were observed with indicators of adiposity. Previous work on the GLN223ARG SNP highlighted associations with several indicators of adiposity in this cohort (Quinton *et al.*, 2001). In the present study, The LYS109ARG SNP was associated with fat mass and BMI. Linkage disequilibrium calculations determined that the LYS109ARG and GLN223ARG SNPs were in linkage disequilibrium. As the GLN223ARG SNP is also associated (more strongly) with indicators of adiposity and the two SNPs are in linkage disequilibrium, then it remains uncertain if the two SNPs are having separate effects, perhaps of varying potency, or if only one SNP is having an effect. Furthermore, they could also mark a third functional site for which these

SNPs are acting as LD markers. For example, a functional mutation might exist between the two SNPs in exon 5, although nothing has been found in this region to date. No significant associations were observed with the LYS656ASN SNP.

Another study has suggested that either of these SNPs may affect adiposity, with the GLN223ARG SNP associated with BMI and fat mass and the LYS109ARG SNP linked to BMI and fat mass (Chagnon *et al.*, 2000). Also, several studies have not highlighted an association with any SNPs in the *LEPR* gene and adiposity (Echwald *et al.*, 1997, Matsuoka *et al.*, 1997, Silver *et al.*, 1997). There are several reasons for the conflicting data. If more than one SNP is present over a relatively small genetic distance and linkage disequilibrium exists over that region, then it is difficult to ascertain which allele of which particular SNP may influence susceptibility. Furthermore, it is difficult to recruit genetically isolated populations. This would be beneficial because phenomena such as population sub-structure, whereby unrecognised combinations of distinct genetic populations (each having distinct allele frequencies at a particular loci) coexist in one larger population, are common. As a result, apparently significant differences in allele frequencies at candidate loci may be detected between population strata (e.g. between cases and controls) and a spurious association assigned. However recent investigations suggest that this may be a less relevant factor than has sometimes been suggested (Ardlie *et al.*, 2002).

Linkage studies are an alternative genetic approach to circumvent this problem. Co-segregation studies are carried out in families and consequently limit genetic heterogeneity. However, these studies have classically been used for monogenic disorders and may be more difficult to apply in multifactorial disorders where, for example, a proportion of the susceptibility is due to a frequent allele variant of small

effect. Also, it is possible that different markers may be linked to the same condition in different families.

In the approach described in this thesis, association was sought in a normal population (not an obese cohort). To transform the study into a case-control study, allowing comparison of allele frequencies between cases and controls, individuals would need to be classified by, for example, BMI. Due to the limited overall numbers present in the cohort, the power would be reduced if they were split into case and control groups. Such classifications are, to some extent, arbitrary whereby certain individuals can be classified as case or control depending on which variable is chosen for classification purposes.

Associations may reflect linkage disequilibrium with nearby markers (e.g. other SNPs in LEPR or a nearby gene) or may indicate a direct functional effect on the protein product. Functional analysis of variant leptin receptor proteins including different combinations of amino acid variants could clarify the situation of the relative effects of SNPs in the LEPR gene. To our knowledge, no such studies have been published.

A set of soluble leptin receptor protein variants were constructed, expressed and purified, each comprising different combinations of the GLN223ARG and LYS109ARG alleles. These may allow comparative analysis of ligand binding (by the leptin-binding assay) that could measure potential functional differences between variants. The actual basis for any differences may stem from an altered conformation of the receptor that would change its ligand-binding characteristics or affect the ability of receptors to dimerise with each other (which might subsequently be represented in the ability to bind leptin). The soluble form of the receptor may have a role in the sequestration of circulating leptin (reducing the amount of available leptin and so

reducing activity) and / or a role in protecting leptin from degradating enzymes whilst in the circulation (a positive role in prolonging activity). Consequently, differential leptin binding by the variant soluble receptors may influence either of these processes. It is not clear, however which of these roles the soluble receptor is involved in and as such any effects of the variant receptors should be considered in light of either scenario. Future studies could involve incubating leptin with different degradating enzymes in the presence (or absence) of soluble leptin receptor to determine if leptin protection is occurring.

It is also possible that the SNPs may alter the intrinsic signalling properties of the membrane bound receptor, as the extracellular domain is identical for these isoforms and the soluble form. An example of this is the GLN269PRO mutation in the *fa* rat (White *et al.*, 1997). In this rodent model of obesity, the amino acid substitution in the extracellular domain of the receptor appears to induce a conformational change that mimics that achieved upon ligand binding and imparts constitutive activation of STAT1 and –3 specifically. Contrastingly, significant impairment of ligand-induced activation of STAT5B is also observed in the *fa* rat. Which of these defects is responsible for the *fa* rat phenotype remains to be resolved.

One possible strategy to be considered for future work would be to heterologously express the full-length receptor expressing different combinations of the SNPs. Then, upon leptin administration to the cells expressing the different membrane-bound variants, the cell supernatants could be harvested and levels of specific phosphorylated STATs and JAKs (known to be activated by leptin signalling) could be quantitatively compared between the different cell cultures by western blotting and / or flow cytometry to assess any possible signalling differences (measured by differences in the level of phosphorylated STATs and JAKs).

The preliminary results presented in this thesis suggest reduced binding to the 223ARG variant compared with the 223GLN variant. This is surprising as the ARG223 variant is associated with lower BMI. It might be expected that individuals who express the 223ARG variant would have greater binding, relative to the GLN variant, suggestive of an improved signalling response to leptin leading to lower BMI. However, it should be noted that the leptin binding data are presented in this thesis as only preliminary results, which would need to be repeated for verification. As the binding data does not readily explain the observed lower BMI in individuals expressing the 223ARG variant, then one explanation is that affinity of leptin for the leptin receptor is not a contributory factor towards obesity and that leptin resistance lies in downstream events such as the JAK-STAT pathway. However, the indication of altered binding between variants would be worth following up especially when obesity is likely to be multifactorial in the majority of cases involving a combination of various genes (with the added complexity that different combined gene effects may be causal in different obese people) each contributing modestly towards the obese state. In such a complex scenario, a simplistic hypothesis that greater binding to certain variants of the leptin receptor leads to lowered BMI may not hold true.

Considering the effects of the SNPs on the soluble receptor, reduced binding to the 223ARG variant could cause a reduction in sequestering of leptin (allowing more "bio-active" leptin to be available, potentially increasing activity), and may possibly explain the lower BMI associated with the 223ARG variant. However, if the same binding affinity is seen with the membrane-bound form then this might off-set the potential for increased activity from less sequestration, due to less active signalling.

Alternatively, reduced binding to the 223ARG variant would lead to a reduction in its ability to protect leptin from degradation. This negative effect would be mirrored with

the membrane-bound form, where reduced binding would be assumed to lead to less activity. As a result of these combined effects, a higher BMI would be expected which is not what is observed from the results obtained in this thesis.

Regardless of the ambiguous results regarding leptin binding and measures of adiposity, it should be noted that in the obese human state, leptin is present in excess and so variation in leptin sequestration and / or degradation may be of moderate importance when leptin is present in excess. Alternatively, if leptin is binding the membrane-bound receptor at saturable levels, but with poorer avidity with certain variants, then variants that bind with greater avidity may have a more prominent biological effect compared to differences in sequestration or protection from degradation. However, there are a proportion of obese individuals who have relatively low leptin levels for their fat mass. In these situations, the effects may become more prominent (Maffei *et al.,* 1996)

Future studies of these engineered proteins should involve quantitative kinetic analysis requiring incubating each variant with increasing concentrations of radioactive leptin and measuring the binding counts for each leptin concentration. This would enable dissociation constants to be calculated for each variant providing a more accurate comparison of binding between variants. If leptin receptor variants do indeed have functional differences, then this work has the potential to direct therapeutic regimes against body weight dysfunction via the leptinergic system. Therapeutic drugs may be tailored towards a particular receptor variant providing a targeted approach depending upon an individuals SNP genotype at the *LEPR* locus. Whilst obese people have an excess of leptin, it is arguable that increasing affinity of the leptin receptor for leptin, in individuals with high circulating levels of leptin would have little benefit. In which case, drugs aimed at improving leptin binding may be more beneficial for individuals

who are modestly overweight or in the case of obese individuals who have been shown to have relatively low leptin levels for their BMI (Maffei *et al.*, 1996).

Unfortunately due to time constraints towards the end of these studies, the ARG109ARG223 construct was not tested for its leptin binding, which could have been used to determine if there was any additive effect of the 109ARG on 223ARG compared with the 109LYS223GLN variant. Also due to lack of time, the 109ARG223ARG construct was not created (this could have determined the effect of the 109ARG variant on the GLN223 background). Both of these issues would need to be addressed in future studies.

The leptin receptor expressed in this study lacked the putative signal sequence of the receptor (Tartaglia *et al.*, 1995). The protein product was only found in the cell lysate. However, the receptor was extensively glycosylated, which is surprising for a protein lacking the signal sequence. Cell biology dogma indicates that signal binding protein (SBP) binds to the signal sequence of the nascent protein and locates it to the ER and Golgi apparatus where post-translational modifications are incorporated. No obvious signalling or sorting sequences were located in the remaining protein sequence. A possible explanation is that novel sequences are present. Alternatively, as the overall protein yield was substantially lower than that expected for expression in COS-7 cells, it is possible that the majority of the nascent protein was degraded in proteosome organelles. Any residual protein (perhaps in excess to that which could effectively be degraded by proteosomes) might be forced down alternative trafficking pathways, including those which incorporate post-translational modifications. To explore the cellular activity of the variant proteins, specific antibodies could be concurrently

directed towards the receptor and different sub-cellular structures of the transfected COS cells to determine the cellular locations and trafficking of the protein.

Cytokines are a group of proteins that have overlapping and redundant modes of activity as demonstrated by the same signalling pathways being activated by different cytokines and also the ability of certain cytokines to signal via more than one receptor complex (see Paul, 1989 and Leonard, 1994 for review). In such a balanced, interconnected system, any cytokines that may be affected by, or affect leptin may in turn influence adiposity. For these reasons and the unique qualities of our post-menopausal study cohort, investigation of SNPs in genes apart from *LEPR* was conducted. The obvious candidate to complement studies of the leptin receptor is the *LEP* gene. Of several SNPs that have been located in the leptin gene, the -2548 *LEP* promoter SNP has been associated with obesity in more than one study (Mammes *et al.*, 1998, Li *et al.*, 1999, Mammes *et al.*, 2000). In this thesis, the results indicate that individuals homozygous for the A allele have lower mean leptin levels and BMI. This contradicts results from studies that suggest that the G allele is associated with lower leptin levels and BMI (Mammes *et al.*, 1998, Mammes *et al.*, 2000). However it agrees with data from a study by Le Stunff and colleagues (2000) who observed that Caucasian obese girls of similar adiposity had lower leptin levels if they carried the A allele. Postmenopausal women and (a substantial proportion) of the young girls do not undergo menstruation. It is possible that the lack of hormonal variation associated with absence of menses present in these two groups could explain their similar outcomes.

TNF-alpha is a pro-inflammatory cytokine. No significant associations were found between the -308 TNF alpha SNP and indicators of adiposity. However, associations have previously been reported with this SNP and obesity (Fernandez-Real *et al.*, 1997,

Hofstedt *et al.*, 2000, Brand *et al.*, 2001), as has lack of association (Waltson *et al.*, 1999, da Silva *et al.*, 2000). The SNP may be exerting a biological effect that is clinically evident in some populations and not others due to additional factors present or absent in each population (e.g. differences in diet). Also to consider is the small size of the cohort that is being analysed and what affect this has on highlighting any associations. Clearly, the larger the study group, the more powerful the statistical analyses, and some associations that are seen as significant with smaller cohorts become non-significant with larger groups.

IL-1ra is an important anti-inflammatory molecule that functions by competitively inhibiting IL-1 binding to receptors. IL-1ra is produced in excess in many human chronic inflammatory and autoimmune diseases (Tarlow *et al.*, 1994, Blakemore *et al.*, 1994, Mansfield *et al.*, 1994). This activity may not immediately suggest a role in obesity, via the leptinergic system. However, Luheshi and colleagues (1997) demonstrated that IL-1 mediates leptin's actions on food intake and that injection of IL-1ra into the cerebral ventricles of mice inhibited leptin-induced reduction in food intake. Also, IL-1ra serum levels are increased in human obesity (Meier *et al.*, 2002) and positively correlated with leptin levels.

A two repeat allele of a VNTR polymorphism located within intron 2 of the IL1RN gene is associated with a variety of diseases (Blakemore *et al.*, 1994, Mansfield *et al.*, 1994). This polymorphism was studied in the cohort of postmenopausal women, and although no associations were observed with markers of adiposity, the polymorphism was highly significantly associated with leptin levels.

It is tempting to speculate from these results that the response of IL-1ra to leptin is modulated depending upon which VNTR alleles are present and that feedback mechanisms may subsequently alter leptin levels accordingly. Unfortunately, serum

measurements for IL-1ra were not available from this study to determine whether they correlated with the VNTR alleles. As no associations were seen with indicators such as BMI and fat mass, the association with leptin levels may be related to leptin's activity, in peripheral tissues, in inflammation and immunity processes (Heymsfield *et al.*, 1999, Zarkesh *et al.*, 2001, Lord *et al.*, 2002). As IL-1ra is implicitly involved with inflammatory responses and autoimmune activities then suggested processes stand out and the observed association may well be recording the interaction between IL-1ra and leptin in these activities.

Clearly physiological homeostasis is affected by a lack of nutritional intake as well as an excess. At the opposite end of the body weight spectrum to obesity are the eating disorders, in particular anorexia nervosa, where in the worst cases the effects of starvation can lead to death. Characterised by emotional and physiological disturbances based around obsessionality and an altered objectivity of ones self, there is debate concerning the degree to which genetics predisposes to AN. Twin studies have provided conflicting results with one study indicating concordance, for anorexia, of about 67% between monozygotic twins (Holland and Treasure, 1989) and yet a higher concordance rate amongst dizygotic twins in another study (Walters and Kendler, 1995). Clearly, a higher concordance rate would be expected amongst monozygotic twins if a genetic predisposition were present. However, an equal shared environment (e.g. co-socialisation in adolescence) for twins, is an assumption in heritability estimates and may confound the results depending upon how accurate the assumption is. Nevertheless, AN clusters in families (Strober *et al.*, 1999) and although research is at an earlier stage than that devoted towards obesity, it is expanding rapidly.

Whilst fewer animal models of anorexia exist than models of obesity, the *anx* mouse model is the primary example, which suffers from starvation and motor disturbances and has an altered distribution of NPY in the arcuate nucleus of the hypothalamus (Broberger *et al.*, 1997).

Several association studies in humans have looked at candidate genes which may be involved in anorexia, such as the 5-hydroxy tryptophan (serotonin) transporter gene and the estrogen receptor 2 gene, and have had varying results. For example, studies of the 5-hydroxy tryptophan transporter gene have reported both positive (Nacmias *et al.*, 1999) and negative associations (Hinney *et al.*, 1997 and Campbell *et al.*, 1998). Groups have recorded lower leptin levels (Hebebrand *et al.*, 1997) and increased leptin receptor levels (Krizova *et al.*, 2002) in anorexic individuals, compared to controls, which is maintained even after weight restoration suggesting an underlying biological dysregulation of the leptinergic system.

As recognised in this and other studies, alleles of SNPs in the leptin receptor are associated with variations in adiposity. When a particular allele is associated with lowered levels of fat mass or BMI, then a hypothesis is that anorexic populations, who by definition have very low measurements of adiposity, may have an increased prevalence of that allele. To our knowledge, no published studies have tested this hypothesis. A case-control association study was therefore undertaken to test the hypothesis. No statistically significant differences were observed between anorexics and controls, in allele frequencies for the LYS109ARG, GLN223ARG and LYS656ASN SNPs or a particular haplotype composed of alleles from these SNPs. With the potentially more pronounced emotional disturbances that afflict sufferers with eating disorders like AN, compared to obesity, the lack of an association with SNPs in the leptin receptor, which are perhaps too simplistically associated with measurements

of adiposity alone, may be obvious. However, it should be acknowledged that the prevalence of an environmental risk factor for the disorder might differ between populations, leaving the door open to investigate other anorexic populations.

Future studies may employ a transmission disequilibrium test (TDT) whereby the frequency at which a candidate susceptibility gene is transmitted from parents to an affected sibling, is compared to transmission to non-affected offspring. This procedure circumvents the problem of population admixture or the need to provide control samples that are practically impossible to match perfectly for all criteria wished for. The TDT approach was recently applied to polymorphisms in the 5-HT2A receptor with results suggesting no association with AN (Gorwood *et al.*, 2002).

If a genotype-phenotype correlation were detected from complementary genetic and functional studies as discussed in this thesis, it would be a significant achievement to tailor therapeutic regimes based on an individual's SNP profile. This approach could be directed towards a whole range of conditions with a genetic component. As institutions grasp the exciting possibilities that should emanate from this area of research they are setting up large scale 'biobank' sample facilities (such as the Wellcome trust/ UK government initiative to collect DNA samples from 500,000 people) that might give sufficient power to elucidate the genetic basis of complex diseases. The potential to unravel complex disease and develop new drugs as a result, leading to an improvement in the welfare of the sufferers, and the reduction in the health cost burden would be considerable.

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