REFERENCE

Leptin and the Leptin Receptor: Molecular and Genetic Studies

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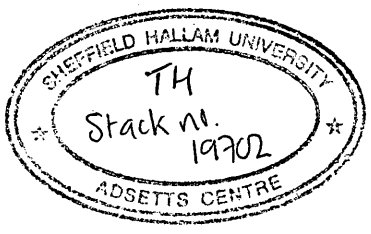
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Leptin and the Leptin Receptor: Molecular and Genetic Studies

Leptin, a 16kDa protein, is secreted primarily by adipose tissue. Its effects are pleiotropic, with known roles in body mass regulation, haematopoiesis, immune function and reproduction.

Studies of the leptin system in females were undertaken to complement existing animal and human studies. Serum leptin levels were found to increase in the luteal phase of the menstrual cycle raising the possibility of a role for leptin at the time of implantation. RT-PCR studies showed that leptin receptors were expressed in the human endometrium throughout the menstrual cycle. In these preliminary studies, these receptors appeared to be functional in response to leptin; increasing proliferation and decreasing TNF-α production in cultured endometrial cells. This implies that leptin has a local effect at the level of the endometrium.

Many cytokines are bound to proteins in serum; a proportion of this binding can be attributed to a soluble cytokine receptor. Soluble receptors and binding proteins have a variety of roles, acting as scavengers, carriers, agonists and antagonists. In order to investigate this phenomenon in the leptin system, a radio-receptor assay was developed to measure leptin-binding activity (LBA). The leptin receptor has an extracellular domain that is common to all isoforms and therefore, LBA may also reflect the binding parameters of cell surface receptors. Serum leptin levels of LBA were found to be low at birth, high in early childhood, to fall steadily through puberty and remain at the post-pubertal levels throughout adult life. This suggests that leptin and LBA has an important role in the initiation of puberty. There was no significant variation in LBA during the menstrual cycle.

Several single nucleotide polymorphisms (SNP) exist in the leptin receptor gene. Studies of two of these SNPs, GLN223ARG and LYS656ASN, present in the extracellular domain of the receptor were undertaken to assess if genetic changes are associated with differences in phenotype or effect ligand binding. Homozygosity of the G allele of GLN223ARG is associated with lower fat mass, BMI and leptin levels in postmenopausal Caucasian females. This polymorphism changes the binding characteristics of the receptor, with a higher LBA being associated with homozygosity of the G allele. This suggests that the actual binding of leptin to its receptor may be an important factor in the regulation of body weight and adiposity.

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Abbreviations

α-MSH	α-melanocyte stimulating hormone
AIDS	Acquired immune deficiency syndrome
ALA	Alanine
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APS	Ammonium persulphate
ARG	Arginine
ASN	Asparagine
BBB	Blood brain barrier
BMI	Body mass index
C/EBPα	CCAAT/enhancer binding protein
cAMP	cyclic AMP
CART	Cocaine and amphetamine related transcript
CDMEM	Complete Dulbecco's modified eagle medium
cDNA	complementary DNA
CNS	Central nervous system
CPM	Counts per minute
CRF	Corticotrophin releasing factor
CV	Coefficient of variation
db	*diabetes*
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EASIA	Enzyme amplified sensitivity immunoassay
EDTA	Ethylenediamine tetra???
ELISA	Enzyme linked immunosorbent assay
ESLD	End stage liver disease
ESRD	End stage renal disease
fa	*fatty*
FISH	Fluorescent *in situ* hybridisation
FSH	Follicle stimulating hormone
G-CSF	Granulocyte-colony stimulating factor
GH	Growth hormone
GH-BP	Growth hormone binding protein
GLN	Glutamine
GLUT4	Glucose transporter 4
GnRH	Gonadotrophin releasing hormone
HBSS	Hank's balanced salt solution
HEC-1B	Human endometrial carcinoma 1B cell line
HEK293	Human embryonic kidney 293 cells
HPLC	High performance liquid chromatography
I	Iodine
IFNγ	Interferon γ
IGF-BP	Insulin like growth factor binding protein
IL	Interleukin
IVF	*In vitro* fertilisation
Jak	Janus kinase
kb	Kilobase
kDa	Kilodalton
LBA	Leptin binding activity
LH	Leutinising hormone

LIF	Leukaemia inhibitory factor
LYS	Lysine
MAPK	Mitogen activated kinase pathway
$MgCl_2$	Magnesium chloride
mRNA	messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NHGRI	National Human Genome Research Institute
NIDDM	Non insulin dependent diabetes mellitus
nm	nanometres
NPY	Neuropeptide Y
NSB	Non-specific binding
ob	*obese*
Ob-R	Leptin receptor
PA-1	Plasminogen activator 1
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PRO	Proline
RACE	Rapid amplification of cDNA ends
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassay
RNA	ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
sCNTF-R	Soluble ciliary neurotrophic factor receptor
SDS	Sodium dodecyl sulphate
SER	Serine
SHBG	Sex hormone binding globulin
sIL-1RII	Soluble interleukin1 receptor-II
sIL-2R	Soluble interleukin 2 receptor
sIL-6R	Soluble interleukin 6 receptor
sLIF-R	Soluble LIF receptor
SNP	Single nucleotide polymorphism
SOCS	Suppressors of cytokine signalling
Sp1	Stimulatory protein 1
SPSS	Statistical package of the social sciences
SSCP	Single stranded conformational polymorphism
STAT	Signal transducer and activator of transcription
sTNF-RII	Soluble tumour necrosis factor –receptor II
TAE	Tris-acetate EDTA buffer
TAG	Tri-acyl glycerol
TBE	Tris-borate EDTA buffer
TEMED	N,N,N',N'- Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TSH	Thyroid stimulating hormone
US	United States
YAC	Yeast artificial chromosome

Publications relevant to this thesis

Papers

Serum leptin levels during the menstrual cycle of healthy fertile women
Quinton, ND, Laird, SM, Okon, MA, Li, TC, Smith, RF, Ross, RJM, Blakemore, AIF
1999 *British Journal of Biomedical Science*, **56** 16-19

Leptin binding activity changes with age: the link between leptin and puberty?
Quinton, ND, Smith, RF, Clayton, PE, Gill, MS, Shalet, S, Justice, S, Simon, SA, Walters, S, Postel-Vinay, MC, Blakemore, AIF, Ross, RJM.
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Oral Communications

Association of the GLN223ARG polymorphism in the leptin receptor gene with serum leptin levels and BMI in postmenopausal Caucasian women
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1999, Journal of Endocrinology, **160** P147

Presented at the 18th British Endocrine Society Meeting, April 1999, Bournemouth.

Association of GLN223ARG polymorphism in the leptin receptor gene with serum leptin levels and BMI in postmenopausal Caucasian women

Blakemore, AIF, Lee, AJ, Quinton, ND, Ross, RJM, Eastell, R

European Journal Of Human Genetics, 1998 **6** P3035

Presented at the European Society for Human Genetics meeting, June 1998, Lisbon.

Serum leptin increases in the luteal phase of the menstrual cycle

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1998 Journal of Endocrinology, **156** S1-P41

Presented at the 17th British Endocrine Society meeting, March 1998, Edinburgh

1.1 Introduction

Leptin is a 16KDa protein, secreted primarily by the adipose tissue and also by the stomach, brain and placenta. It is pleiotropic: having known roles in body mass regulation, satiety, reproduction, and the immune response. Leptin acts via the leptin receptor, a class I cytokine receptor of which there are at least 5 isoforms present in humans. This thesis aims to further investigate the role of leptin in human reproductive processes and to assess the effect of genetic variation within the leptin receptor on body weight and adiposity.

1.2 Adipose tissue

Adipose tissue is increasingly being recognised as an important organ. In recent years, the belief that this tissue existed only as a special storage depot for nutrients in the form of fat have been largely dismissed. The adipose tissue is a highly metabolic organ able to synthesise many proteins (Figure 1.1), including cytokines, such as tumour necrosis factor-α (TNF-α) (Hotamisligil *et al.*, 1993), interleukin 6 (IL-6) (Mohamed *et al.*, 1997), and other proteins including adipsin, a serine protease which is identical to complement factor D (Napolitano *et al.*, 1994), plasminogen activator-1 (PA-1) (Halleux *et al.*, 1999) and proteins associated with the renin-angiotensin system (Engeli *et al.*, 1999). The full function of the adipose tissue is still being elucidated, although investigations into its primary roles in reproduction and the development of diabetes and obesity are underway. The distribution and amount of both brown and white fat, present in both males and females is vitally important for good health. Females have more fat mass than males and this fat tends to be positioned on the lower body. In males, fat appears to accumulate around the abdomen. Central obesity, white fat tissue that accumulates at the abdomen, is associated with the development of cardiovascular disease and non-insulin dependent diabetes mellitus (NIDDM). In females, a certain level of fat mass is essential for reproduction.

1.3 Obesity

Obesity can be defined as at least 20% over ideal weight (Lonqvist *et al.*, 1996) and is an increasing concern for many Western and developing societies. Obesity can be accompanied by complications such as hypertension, atherosclerosis and NIDDM. These disorders, in turn, can lead to heart disease, stroke and premature death. Obesity is also related to an increased risk in developing certain cancers, for example, cancer of

the colon and of the endometrium (Garfinkel, 1995). Treatment costs for the indirect consequences of obesity exceeded $98 million in the United States (US) in 1997 (American Diabetes Association, 1998). A study in the US of more than 11 000 women, showed body mass index (BMI) to be the dominant predictor of developing type 2 diabetes mellitus (Colditz *et al.*, 1995). Until recently obesity has been considered a lifestyle disorder, with the direct cause being overeating and decreased physical activity. However, there is increasing awareness that metabolic factors are also important factors in obesity (Grossman, 1995).

1.4 The identification of leptin

The study of body mass regulation began in the 1950s. Kennedy (1953) postulated that circulating signals were produced, that were in proportion to body weight, and would provide information to the brain in order to regulate body mass. It had been observed that a number of inbred strains of mice were grossly obese, and infertile. Coleman (1973) postulated that a blood-borne factor was responsible for regulating body mass in normal weight mice and that in these obese mice, *ob/ob* (*obese*) mice, the blood-borne factor was absent or mutated. Parabiosis studies were performed. When the blood systems of a normal mouse and an *ob/ob* mouse were joined, the obese mouse began to lose weight. This satisfied the hypothesis that a blood-borne factor was responsible for the mice losing weight. Another obese strain of mice, which developed diabetes, *db/db* (*diabetes*) mice, were also used in the parabiosis experiments. When their blood systems were joined with a normal mouse, they did not lose any weight. This allowed researchers to postulate that in this strain, the murine obesity was not caused by the absence or damage to this blood borne factor, but by another mechanism. Both *ob/ob* mice and normal mice died of starvation after being joined to *db/db* mice: this was most likely to be due to increased amounts of the effective blood borne factor being present.

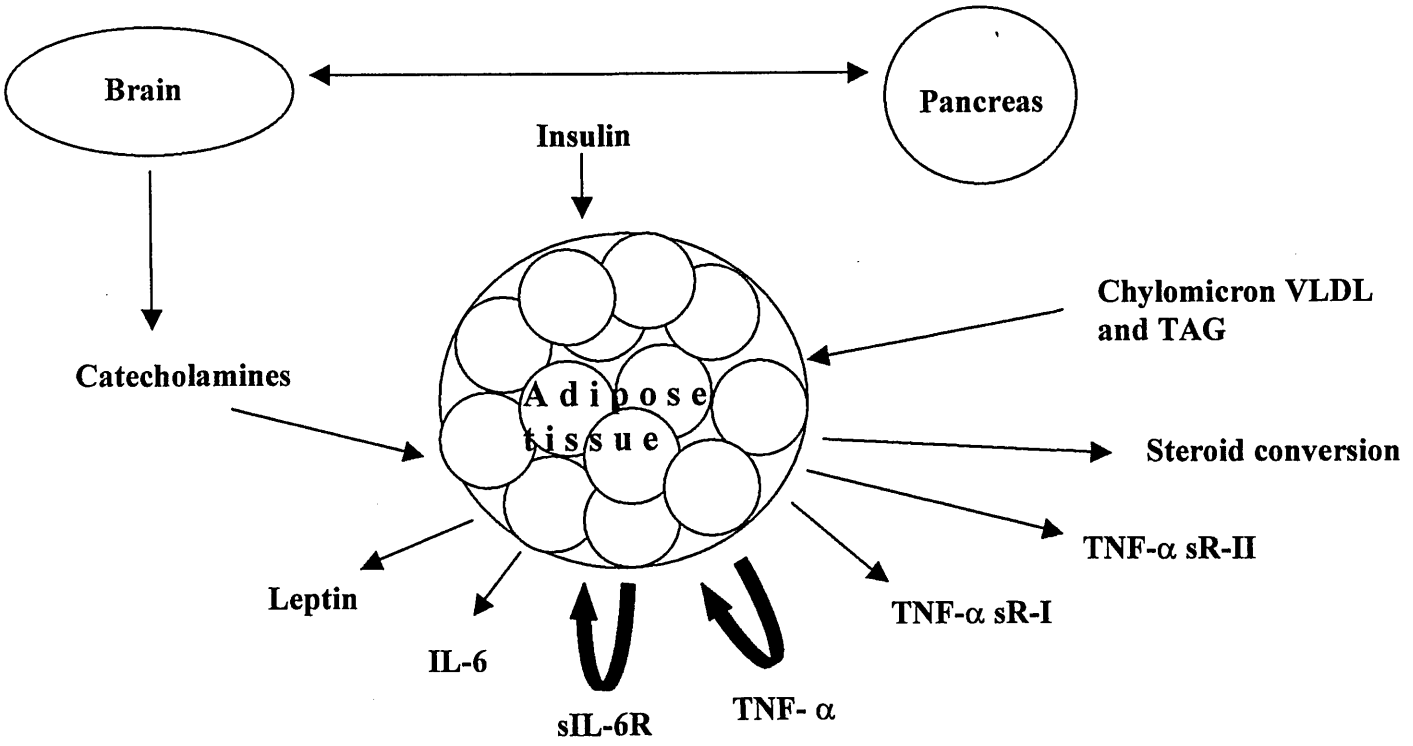


Figure 1.1 Adipose tissue: principal afferent and efferent signals (Adapted from Mohamed-Ali *et al.*, 1998).

In 1994, Zhang *et al* (1994) used a positional cloning strategy to identify the *ob* gene in mice. RFLP analysis defined the relevant region on chromosome 6. The DNA between two markers was cloned into yeast artificial chromosomes (YAC), using 24 clones to construct a vector series over the *ob* region. Exon trapping, a relatively new technique for the time, was employed. This techniques allows exons to be isolated from genomic clones using a special vector which has splice donor and acceptor sites either side of the insert. Digests from the clones, containing the *ob* gene, were sequenced and the sequences matched to a genomic library. RNA from different tissues was analysed by northern blotting analysis and RT-PCR for any possible exons of the *ob* gene being expressed.

One exon was found to be expressed in adipose tissue, this provided a candidate for the *ob* gene. An identical product was found in 20-fold lower concentrations in non-obese mice. Screening of a mouse adipose tissue cDNA library enabled sequencing of the *ob* gene itself. The *ob/ob* mouse has a nonsense mutation in the *ob* gene, resulting in a premature stop codon. The human *ob* gene was shown to be 84% homologous to the mouse gene. Examination of the sequence suggested that the *ob* gene product, leptin, was an 18 kDa protein with a signal peptide sequence to direct secretion, which in turn is cleaved to leave a 16 kDa native peptide. The presence of cysteine residues in the structure of this protein indicated a secreted globular protein (Zhang *et al.*, 1994). Using fluorescent *in situ* hybridisation (FISH), the *ob* gene was mapped to human chromosome 7q31.3 (Isse *et al.*, 1995). The human *ob* gene spans 20 kb, encoding 167 amino acids. The open reading frame reveals three exons and two introns (Niki *et al.*, 1996). The first intron is approximately 10.6 kb in length, and occurs in the 5' untranslated region (Isse *et al.*, 1995). Madej *et al.* (1995) suggested that leptin belonged to the family of helical cytokines, on the basis that the leptin gene sequence is very similar to the IL-2 gene sequence when using threading analysis. This family includes interleukin-2 (IL-2) and growth hormone (GH).

He *et al.* (1995) described the genomic organisation of the murine *obese* gene, including its promoter. The promoter contains several consensus sequences for transcription factors, notably, a stimulatory protein-1 (Sp1) motif and a CCAAT/enhancer binding protein (C/EBPα) motif. Similar studies have been performed on the human leptin gene. Isse *et al.* (1995) performed analysis of the human leptin gene and identified the promoter region using rapid amplification of cDNA ends (RACE). They demonstrated

the presence of *cis*-regulatory elements including GC boxes, an AP-2 binding site and a C/EBPα motif. Gong *et al.* (1996) investigated a large area (3.5 Kb) of the 5' region of the *obese* gene including the area containing the promoter. They identified a number of putative binding sites for known transcription factors such as Sp-1 sites, cAMP response element, a glucocorticoid response element and C/EBPα sites. These are of interest as other workers have reported leptin gene expression changes in response to both insulin (Saladin *et al.*, 1995) and dexamethasone (Murakami *et al.*, 1995). Both of these studies, performed in rats, caused increases in leptin mRNA expression. These effects may be transmitted through one or more of the transcription factor binding sites present in the *obese* gene promoter.

1.5 Expression of leptin protein in human tissues

Leptin is primarily expressed and secreted by the adipose tissue in rodents and humans. Circulating levels of leptin correlate well with adiposity (Takahashi *et al.*, 1996). Leptin expression has also been described in several other tissues. Bado *et al.* (1998) state that the stomach is a source of leptin. There experiments found that both leptin mRNA and leptin protein were present in rat gastric epithelium. They suggest that the presence of leptin in the stomach may be involved in the cholecystokinin- mediated effects after food intake and possibly in invoking satiety. Breidert *et al.* (1999) confirm these results in humans, showing leptin expression in the gastric epithelial glands and leptin receptor isoforms in the epithelium and the mucosa.

Skeletal muscle is also a tissue that expresses leptin. Wang *et al.*, (1998) found *ob* gene expression to be induced by glucosamine in skeletal muscle. This suggests an important biochemical link between availability of nutrients and leptin gene expression. Rat hepatic stellate cells have also been identified as a source of leptin (Potter *et al.*, 1998). The cells were only found to express leptin after 14 days in culture.

In humans, the placenta and the breast epithelial cells are sites of leptin expression. Several studies have shown the leptin production by the placenta (Hassink *et al.*, 1997, Masuzaki *et al.*, 1997, Senaris *et al.*, 1997). Placental leptin may serve to regulate foetal growth and development. Smith-Kirwin *et al* (1998) used RT-PCR analysis to identify leptin transcript in total breast tissue, pinpointing the leptin expression to the ductal epithelial cells using immunocytochemistry. They also state that leptin is carried in the breast milk to the infant to continue the regulation of growth and development.

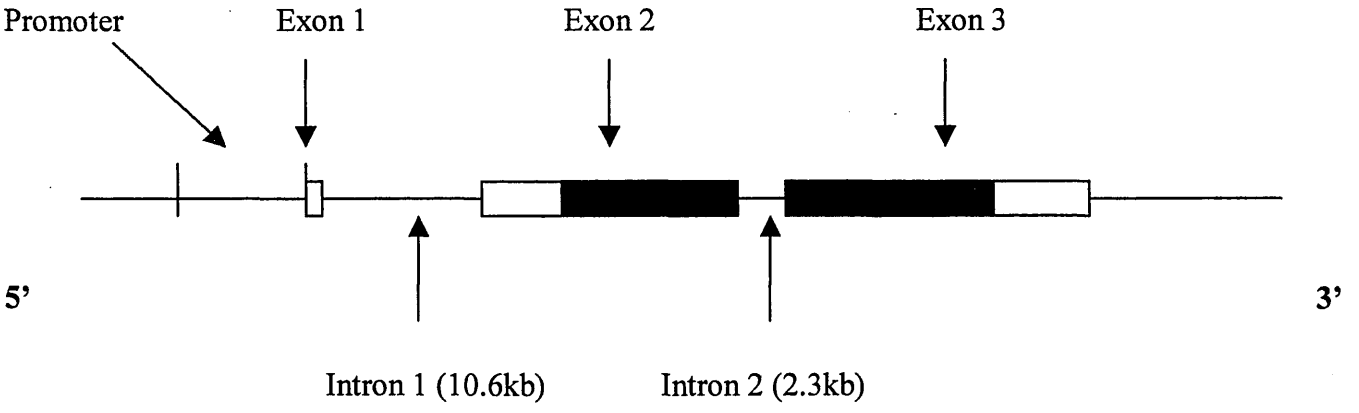


Figure 1.2 The genomic structure of the human *obese* gene, leptin (LEP) (Adapted from Karvonen *et al.*, 1998).

Another source of leptin that has recently been proposed is the brain. Wesiner *et al.*, (1999) measured leptin levels in blood taken from the jugular vein and an artery in the forearm: they report that leptin levels are higher in the blood sampled from the jugular vein and this may suggest release or production of leptin by the brain. Further to this, they have calculated the cerebral contribution to the circulating leptin level. Their assessment was that the brain contributed 13% and 41% of the circulating leptin levels in lean men and women respectively. At present, there are no reports that leptin mRNA is present in the brain. This may be due to many of the studies performed to identify leptin mRNA in the brain have used whole brain tissue and not a restricted neuronal area or section of brain. The tissue closest to the brain where leptin has been found to be expressed is the pituitary gland (Jin *et al.*, 1999). These workers used human normal and neoplastic pituitaries to detect leptin mRNA expression by RT-PCR. Their studies provide evidence for an auto/paracrine loop in the production of leptin in the pituitary gland.

1.6 Identification and cloning of the leptin receptor

Tartaglia *et al.* (1995) identified the leptin receptor (Ob-R) in mouse choroid plexus. They used [^{125}I]-leptin and leptin-alkaline phosphatase fusion proteins to identify the receptor. The leptin receptor is a single membrane spanning receptor, of the class I cytokine receptor family. The leptin receptor is similar in structure to the receptors for interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF), although Ob-R does not utilise the gp130 signalling accessory chain. Initial experiments predicted that the extracellular domain of the receptor was large and that the intracellular domain was short, suggesting that this receptor may not have signalling capabilities. Screening of cDNA libraries revealed that there are multiple forms of the leptin receptor. Lee *et al.* (1996) furthered this work by identifying six alternatively spliced forms of the leptin receptor in mice and pinpointed a mutation that causes the *db* (*diabetes*) phenotype. Sequencing of RT-PCR products of Ob-Rb from the *db/db* mouse showed a 106bp insertion between the splice donor site at codon 889 and the splice acceptor site at codon 890. The sequence inserted here was identical to the first 106bp of the c-terminal end of Ob-Ra exon. This mutant protein then has a termination codon five amino acids down after the splice, resulting in a receptor lacking most of the cytoplasmic domain, including the essential "box 2" motif which is essential for Jak/STAT signalling.

All forms of the leptin receptor share common N-terminal exons that code for the extracellular and transmembrane domains. The C-terminal exon generates mRNA that can be alternatively spliced to make the various intracellular domains of the leptin receptor. Several of the isoforms are identical until a lysine residue at position 889, beyond this point the receptors have differing cytoplasmic portions and have been designated Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re. In mice, the isoform Ob-Re differed after a histidine residue at position 796: this is likely to encode a soluble form of the leptin receptor. Murine and human leptin receptors are very similar in amino acid sequence: the extracellular sequence having 74% homology and the intracellular sequence 71% homology. In humans four major membrane-bound isoforms of the receptor have been reported (Figure 1.3). There is a long form, Ob-Rb or hOB-R/B219.2, which has Jak/STAT signalling capabilities, and three short forms, which have various length intracellular chains, some of which may be able to signal using MAPK (Barr *et al.*, 1999). There is also evidence for a soluble form of the receptor, which consists of only the extracellular domain (Liu *et al.*, 1997).

1.7 Signalling by the leptin receptor

The binding of leptin to the long form of the leptin receptor initiates a complex cascade of signalling molecules similar to those resulting from stimulation of the gp130 cytokine receptor family, which includes LIF and IL-6. This family of receptors activates gene transcription using the Janus tyrosine kinases (Jak) and signal transducers and activator of transcription (STAT) pathway. On ligand binding, the Jak proteins associated with the receptor are auto- and trans-phosphorylated. This induces kinase activity which in turn, causes phosphorylation of a tyrosine residue on the receptor itself. This area of phosphorylation attracts STAT proteins. These are then phosphorylated by the kinase activity. STAT proteins form homo- or heterodimers and undergo nuclear translocation. In the nucleus, the STAT complex can bind to DNA and alter transcriptional activity (Schindler and Darnell 1995) (Figure 1.4).

Two Jak binding boxes have been identified in the long form of the leptin receptor. Jaks are associated with intracellular domain binding sites and phosphorylate the intracellular domain on binding (Tartaglia, 1997). Transfections of cell lines with the long form of the leptin receptor have identified that STAT 1, 3, 5, and 6 respond to ligand binding. Two reports show that STAT 3 and 5 are stimulated in COS cells on ligand binding. Only STAT 3 has been identified as a response to ligand binding *in vivo* (Vaisee *et al.*, 1996).

The signalling function of the leptin receptor is similar to that of the IL-6 family of receptors, which requires the gp130 chain to signal. The gp130 chain is a signal transducing receptor component that is required to allow signalling. Baumann *et al.* (1996) carried out an investigation using anti-gp130 antibodies. These antibodies are capable of blocking IL-6 receptor signalling, which requires the gp130 component. The antibodies did not inhibit the leptin receptor signalling, thus, it can be concluded that the leptin receptor does not utilise the gp130 component in its signalling. The leptin receptor may utilise a second transmembrane component in its signalling. Growth hormone receptors and the G-CSF receptors both undergo homo-oligomerization in order to signal. Chimeric receptors were constructed using the intracellular domain of the G-CSF receptor and the leptin receptor and vice versa. Both of these constructs were able to signal (White *et al.*, 1997). The leptin receptor may not need an accessory binding chain and it is postulated that intracellular homodimerization may provide the key to leptin signalling (White *et al.*, 1997). Barr *et al.* (1999) investigated leptin receptor internalisation after lignand binding. All forms of the leptin receptor were internalised using a clathrin-mediated process, however the rate of internalisation varied; the long form of the receptor was internalised in the shortest time. Ghilardi *et al.* (1996) has shown that the long form of the leptin receptor (Ob-Rb) is able to activate STAT 3, STAT 5 and STAT 6, although no other leptin receptor isoform has been identified to signal using this pathway. At least two isoforms of the leptin receptor (Ob-Ra and Ob-Rb) are believed to be able to activate the mitogen activated protein kinase (MAPK) pathway. Yamashita *et al.* (1998) have demonstrated that the short form of the leptin receptor is able to induce the MAPK pathway whereas the long form of the leptin receptor was able to induce both MAPK and Jak/STAT pathways. A summary of leptin rceptor signalling is shown in figure 1.4.

Other workers have suggested the induction of other signalling cascades by leptin. Bouloumie *et al.* (1999) have shown that leptin activated the terminal c-Jun kinase of the stress activated protein kinase pathway whilst Zhao *et al* (1998) report a novel leptin signalling mechanism using phosphoinositide-3-kinase dependent activation of cyclic nucleotide phosphodiesterase 3B in the decrease of insulin secretion.

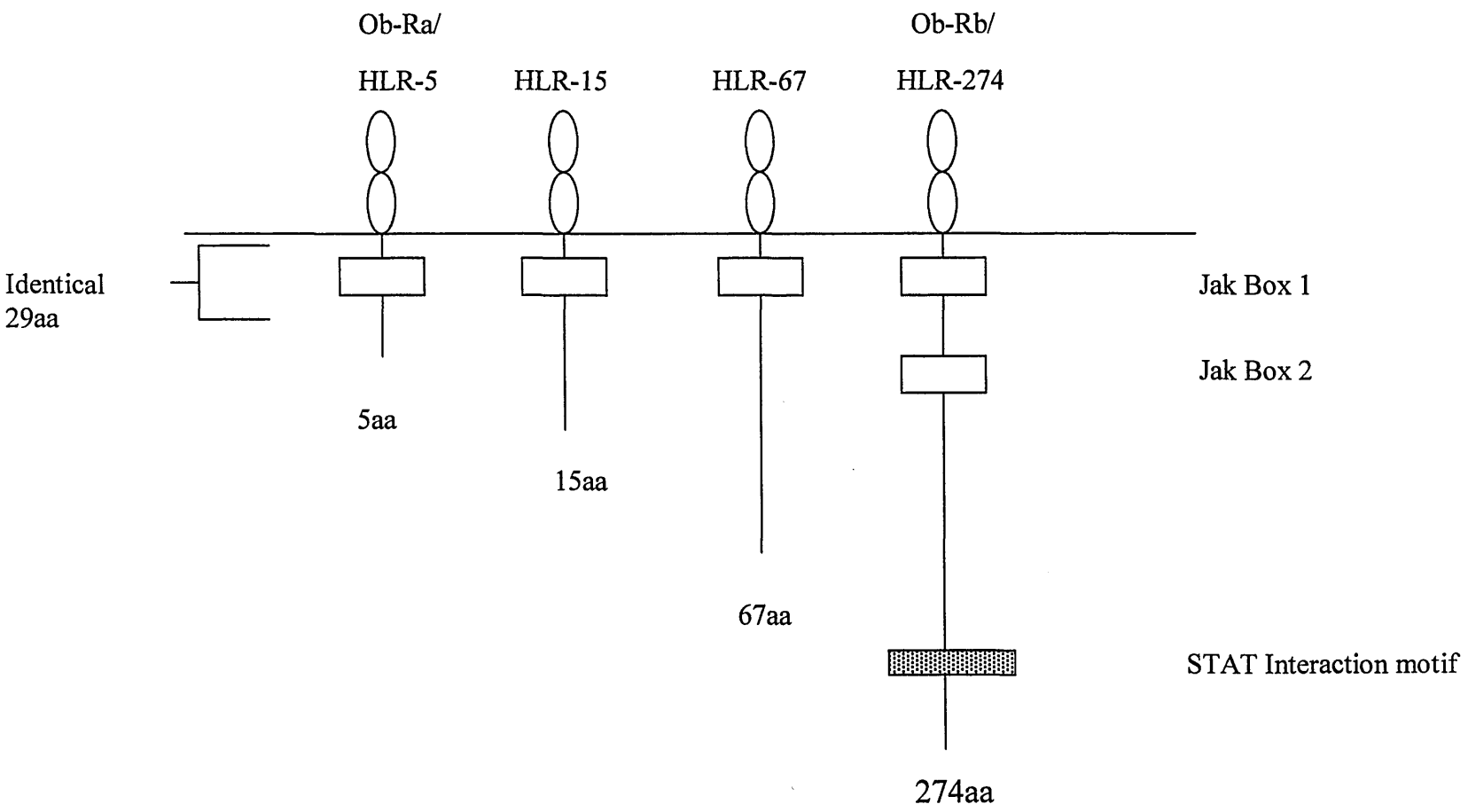


Figure 1.3 Illustration of membrane-bound human leptin receptor isoforms. (Adapted from Barr *et al.*, 1999).

Although much is known about cytokine signalling, receptor aggregation, activation of the Jak/STAT and MAPK pathways and alteration of gene transcription, the mechanism by which cytokine signal transduction is switched off is only now being investigated. Starr *et al.* (1997) described 3 proteins involved in the inhibition of cytokine signalling. These proteins are known as suppressors of cytokine signalling (SOCS). Leptin is known to activate SOCS-3 in the human hypothalamus (Bjorbaek *et al.*, 1999). As SOCS-3 is able to inhibit Jak 2 phosphorylation, it is postulated that excessive SOCS-3 expression may be a potential mechanism for leptin resistance (Bjorbaek *et al.*, 1999, Emilsson *et al.*, 1999).

1.8 Expression of the leptin receptor isoforms in human tissues

Leptin receptor expression varies with isoform. The short isoform of the leptin receptor is expressed almost ubiquitously in all tissues, being found in the brain, specifically the hypothalamus and choroid plexus, kidney, lung, liver, spleen, stomach, testes and ovary. The long form of the leptin receptor is preferentially expressed in the hypothalamus, but also in the lung, liver, testes, ovary and adrenal gland. Jin *et al.* (1999) report the presence of both the long and the short forms of the leptin receptor to be present in normal and neoplastic pituitaries. In the human adrenal gland, once again, all three membrane-bound isoforms of the leptin receptor were found (Glasow *et al.*, 1999). The placenta is a site of leptin receptor expression and is a known site of expression of the soluble form of the leptin receptor in mice (Gavrilova *et al.*, 1997). Leptin receptor isoforms have been identified in murine foetal tissues, including bone, cartilage, brain, lung and kidney (Hoggard *et al.*, 1998). There was no identifiable expression of the receptor in foetal heart, liver adrenal gland or pancreas. More recently, the long form of the leptin receptor has been identified in human platelets (Nakata *et al.*, 1999). This receptor is functional; the effect on the platelets in the presence of leptin is to induce platelet aggregation. The workers hypothesise that this may be a linking factor between cardiovascular disease and obesity. The long form of the receptor identified in the human lung also has a functional effect, this time on cell proliferation (Tsuchiya *et al.*, 1999). Using RT-PCR, the presence of Ob-Rb was confirmed in both human lung tissue and a lung cell line; the proliferative response was observed in the cell line. To complement the presence of leptin gene expression in the stomach, Breidert *et al.* (1999) reported the presence of the leptin receptor in the stomach. Specifically the long form and other short isoforms were pinpointed to the lower gastric epithelium and throughout the mucosa.

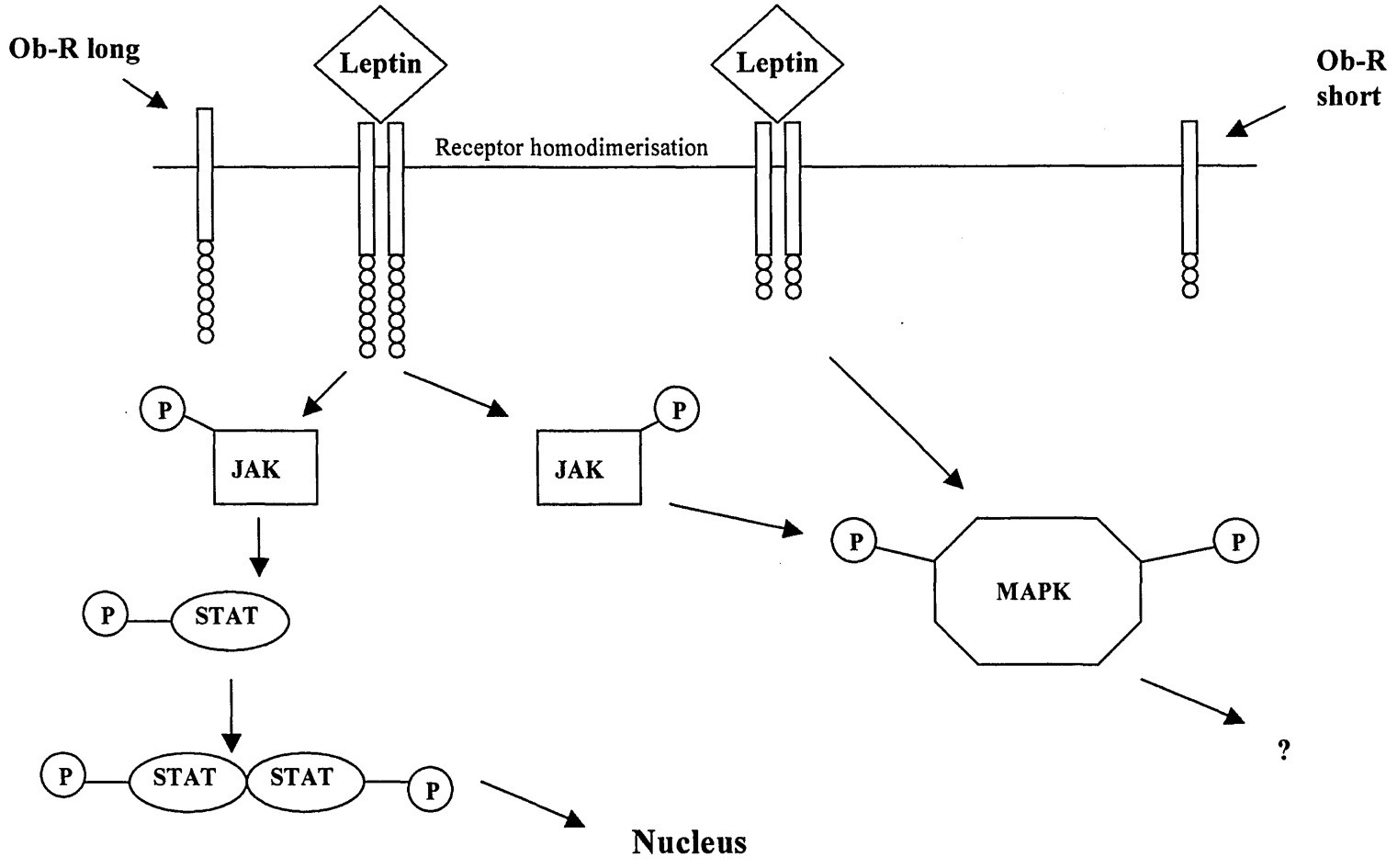


Figure 1.4 Schematic diagram of leptin receptor signalling pathways. After leptin binding and receptor homodimerisation, the long form of the leptin receptor activates the Jak/STAT and MAPK pathway. On leptin binding the short form of the receptor is reported to activate the MAPK pathway.

1.9The action of leptin

1.9.1 Leptin, satiety and weight regulation

Long-term body weight, in healthy subjects, is well regulated throughout each individual's lifetime (Horn *et al*, 1996). Kennedy *et al.* (1953) suggested the lipostatic theory; here the size of the body fat store is sensed by the central nervous system (CNS) via a factor produced by the adipose tissue itself. This is turn affects energy balance by interactions with the hypothalamus. The main action of leptin, with respect to body mass regulation, is in the hypothalamus (Hamann &Matthaei, 1996). Stephens *et al.* (1995) noted high affinity binding of leptin to rat hypothalamus. Hypothalamic neuropeptide Y (NPY), released from the arcuate nucleus (Lonnqvist, 1996) stimulates appetite and food intake, increases insulin and glucocorticoid concentrations and decreases thermogenesis. Leptin binds to receptors in the ventromedial nucleus of the hypothalamus and acts to directly inhibit NPY. *Ob/ob* and *db/db* mice and *fa/fa* rats have high neuropeptide Y expression due to their lack of a functional leptin protein or receptor (Wilding *et al.*, 1993, Schwartz *et al.*, 1996). Neuropeptide Y is 36 amino acid protein, synthesised throughout the brain and at especially high levels in the neurones of hypothalamus.

Neurotransmitters that affect food intake

Stimulatory	*Inhibitory*
Neuropeptide Y	5-hydroxytryptamine
Orexin	CRF
Galanin	α-MSH
CART	Glucagon-like peptide-1
Melanin-concentrating hormone	Interleukin 1

Table 1.1 Neuropeptides that affect feeding behaviours.

Neuropeptide Y is not the only neuropeptide involved with leptin in the maintenance of body weight and food intake, the melanocortin system is also of direct importance. This system involves proteins that are processed from a precursor, pro-opiomelanocortin (POMC) which is produced by neurones within the hypothalamus. Many products of the POMC gene, including α-melanocyte stimulating hormone (α-MSH), are involved in the regulation of feeding. It is believed that the melanocortin system is responsible for

mediating responses to leptin in the brain. Leptin receptor isoforms have been co-localised with POMC neurones in the arcuate nucleus (Inui, 1999). Treatment of rats and *ob/ob* with leptin increased the expression of POMC mRNA in the hypothalamic area of the brain (Schwartz *et al.*, 1997). Neuropeptides that influence feeding behaviours are listed in Table 1.

Treatment of obese mice with recombinant leptin induces a dramatic weight loss. *Ob/ob* mice increased their physical activity and ate 60% less food, after four days treatment with leptin (Haalas *et al.*, 1995). After four weeks, their fat mass had been reduced by 75%. Normal mice treated in the same manner showed a reduction in their fat mass of 12 % over the same period. Pellymounter *et al.* (1995) suggest a role for leptin in controlling energy expenditure. When *ob/ob* mice are treated with leptin, their energy expenditure increased. The group initiated a low calorie diet in two groups of *ob/ob* mice. One group was also treated with leptin; the other followed the diet only. The leptin-treated mice lost more weight; thus suggesting that leptin has a role in controlling body weight. These workers also investigated the effect of long-term leptin administration on insulin secretion in *ob/ob* and wild type mice. After 28 days of intraperitoneal injections of leptin, a dose-dependent decrease in insulin secretion was observed in the *ob/ob* mice, but not in the wild type mice (Pellymounter *et al.*, 1995). Emilsson *et al.*, (1997) performed *in vivo* and *in vitro* experiments on *ob/ob* mice to investigate the action of leptin on insulin secretion. RT-PCR studies showed the presence of the long form of the leptin receptor in the pancreatic islets of both the wild-type and the *ob/ob* mouse. Administration of leptin produced a dose-dependent inhibition of glucose-stimulated insulin secretion in *ob/ob*. They postulate that leptin overproduction from abdominal adipose tissue, may modify basal and glucose-stimulated insulin secretion. They suggest that it is by this mechanism that leptin is involved in the development of diabetic syndrome. Ceddia *et al* (1999) suggest that leptin modulates insulin secretion, in the rat, by changing the flux of potassium ions through channels in the pancreatic islets. More recent work by Ahren *et al.* (1999) was carried out to investigate the effect of leptin on insulin secretion in a rat insulinoma cell line. They found that leptin inhibits insulin secretion by the cell line only when levels of intracellular cyclic AMP (cAMP) are high. This provides evidence for a role for the cAMP-protein kinase A signal transduction pathway as a target for leptin to inhibit insulin secretion in insulin-producing cells.

1.9.2 Leptin and its relationship with hypothalamic-pituitary-gonadal function

Yu *et al.* (1997) provided evidence for a role for leptin in hypothalamic-pituitary function. These workers performed *in vitro* studies on rat pituitary glands and on median eminence-arcuate nuclear explants form the same animals. Incubation of the tissue with various concentrations of leptin caused a dose-related increase in follicle stimulating hormone (FSH) and luteinising hormone (LH) in the pituitary and an increase in LH-releasing hormone in the arcuate nuclear explants. These data indicated that leptin was important in controlling gonadotropin secretion by stimulatory hypothalamic and pituitary actions. In both human and rodent anterior pituitaries, leptin and leptin receptor isoforms, both the long and short forms, have been identified (Jin *et al., 2000*). This provides further evidence that leptin may have an effect on the hormones produced by the anterior pituitary. Both male and female *ob/ob* mice are infertile. Administration of leptin restores the fertility of female *ob/ob* mice and increases luteinising hormone in these mice (Chehab *et al.*, 1997, Ahima *et al.*, 1997). Leptin has been shown to induce the onset of puberty in normal female mice (Ahima *et al.*, 1997), with leptin initiating earlier opening of the vaginal tract, earlier onset of first oestrus cycle and an increase in the weight of the uterus and ovaries when compared to the organs of untreated animals. In male *ob/ob* mice, administration of leptin caused an increase in testicular weight when compared to untreated *ob/ob* controls. Leptin appeared to increase cellular activity in the seminiferous tubules, associated with an increase in testosterone levels (Barash *et al.*, 1996). This provides evidence that leptin has an effect at the hypothalamo-pituitary-gonadal level. Leptin receptors have also been identified in reproductive tract tissues including the ovaries and testes. These receptors may be responsible for transmitting a signal directly to the organ, rather than via the hypothalamo-pituitary-gonadal axis. All of these studies provide strong evidence for a role for leptin and its receptor in primary reproductive processes, and not only as a secondary effect of energy balance.

1.9.3 Leptin interaction in the cytokine network

Leptin has been reported to interact with the cytokine network in a number of different situations. The presence of leptin is a necessary factor for the proinflammatory immune response (Matarese, 2000). The *db/db* mouse has reduced levels of T and B lymphocytes (Bennett *et al.*, 1996), this suggest that leptin may have an important role in lymphopoiesis. Santos-Alvarez *et al.* (1999) report the activation of TNF-α and IL-6 production from human monocytes. They further report that leptin enhances the

activation and proliferation of human circulating T lymphocytes, specifically by increasing the production of IL-2 and IFN-γ. (Martin-Romero *et al.*, 2000). Leptin is believed to be important in the neuroendocrine response to starvation (Ahima *et al.*, 1996. This corresponds well with murine studies carried out by Lord *et al.* (1998). These workers found that leptin modulates cell-mediated immunity in mice; starvation-induced immunosuppression is reversed in mice after treatment with leptin. Evidence now exists for the effects of leptin to be mediated by IL-1. Studies have shown that both IL-1 and TNF-α increase *ob* mRNA expression and serum leptin levels at least in rodents. In IL-1β knockout mice, LPS (lipopolysaccharide) did not induce an increase in leptin levels, suggesting that IL-1 has an important role in mediating the effects of leptin (Faggioni *et al.*, 1998). Luheshi *et al.* (1999) furthered this work in rats. They found that injection of leptin into the brain of normal rats increased the amount of IL-1β in the hypothalamus, and that central injection of IL-1 receptor antagonist (IL-1ra) inhibited the reduction in food intake caused by leptin. Furthermore, mice lacking the IL-1RI gene showed no response to leptin in the reduction of food intake. All these factors suggest that IL-1 mediates the action of leptin in the CNS.

TNF-α is a proinflammatory cytokine that is produced by macrophages and the adipose tissue. The effect of TNF-α on leptin production is complex. There have been contradictory results from adipocyte cell culture studies treated under identical conditions; both reporting the stimulation or inhibition of leptin release in response to TNF-α (Finck *et al.*, 1997, Yamaguchi *et al.*, 1998). In cultured adipose tissue from obese subjects, TNF-α has been shown to decrease the release of leptin (Fawcett *et al.*, 2000). Zhang *et al.* (2000) report a dual effect of TNF-α on leptin in adipose tissue. Firstly, TNF-α stimulates the release of leptin that is stored in mature adipocytes and differentiated preadipocytes. Secondly, TNF-α inhibits the synthesis of leptin in preadipocyte differentiation and induction of adipocyte dedifferentiation. Other workers have found that short term exposure does not have an effect on leptin production. Prolonged exposure (24 hours) results in a concentration dependent inhibition of leptin expression and secretion by the adipocyte, at least in the obese subject (Medina *et al.*, 1999). At present the mechanism by which TNF-α evokes its effect on leptin is not known although it has been reported that TNF-α acts directly on the adipocyte via the receptor p55 TNFR to increase leptin release (Finck and Johnson, 2000). There is now

some speculation as to the contribution of TNF-α and leptin to the development of insulin resistance and diabetes (Sethi and Hotamisligil, 1999)

1.10 Physiology of leptin concentrations in humans

1.10.1 Leptin concentrations in the normal, healthy human

Leptin is expressed and secreted as a 16KDa protein primarily by the by adipose tissue (Hassink *et al.*, 1997, Considine *et al.*, 1996) and also by the stomach (Bado *et al.*, 1998), skeletal muscle (Wang *et al.*, 1998), brain (Weisner *et al.*, 1999), the mammary gland (Smith-Kirwin *et al.*, 1998), and by the placenta during pregnancy (Hassink, *et al*, 1997). Leptin shows characteristics of both hormones and cytokines, having a circadian rhythm (Sinha *et al.*, 1996a) and a pulsatile secretion (Sinha *et al.*, 1996b). Sinha *et al.* (1996a) noted a nocturnal increase in serum leptin, with the peak being midnight and the early hours, the nadir being in the early afternoon. It has been postulated that this increase in leptin may suppress appetite during the night. The nocturnal increase in leptin mirrors those of prolactin and thyroid stimulating hormone (TSH). This circadian rhythm is now thought to be associated with the timing of meals as opposed to a true pattern of secretion (Sinha, 1997). Basal levels of leptin are markedly increased in obese subjects. As the primary source of leptin is the adipose tissue, leptin levels correlate well with markers of adiposity and weight such as body mass index (BMI) (Considine *et al.*, 1996b) and percentage body fat. Hickey *et al.* (1996) provide evidence of a gender dichotomy of the leptin-adiposity relationship. Leptin levels are higher in women than men at any level of fat mass. Schwartz *et al* (1996) also reported a similar increase in serum leptin in females as opposed to males. The sex difference in levels of leptin is likely to influence and be influenced by the levels of sex hormones (Elbers *et al.*, 1997). Weisner *et al.* (1999) suggest that expression of leptin by the brain may also contribute to the gender difference observed. There is a decrease in levels of leptin after the menopause (Rosenbaum *et al.*, 1996) this is likely to reflect a reduction of female sex hormones and age (Ostland *et al.*, 1996, Elbers *et al.*, 1997). Subjects with anorexia nervosa have also been studied. Grinspoon *et al.* (1996) measured serum leptin in women with BMI of 16.3 ± 1.3 kg/m^2. They found that serum leptin is still correlated with BMI even at low body weight, but, more recent studies have suggested that the reduction in serum leptin levels in women with anorexia nervosa is greater than can be accounted for by the subjects BMI (Herpetz *et al.*, 1997). Successful refeeding of

anorectic subjects results in an increase in leptin levels that does correlate with BMI, thus restoring the normal leptin/BMI relationship (Eckert *et al.*, 1998).

1.10.2 Leptin in early childhood

The role of leptin during childhood has been investigated. It is postulated, that in children, leptin does not only regulate adiposity but is also an important growth factor. Leptin is detectable in cord blood and the levels of leptin are often extremely high just after birth. These then decrease dramatically in the early neonatal period. This is believed to be an important part of the adjustment to the extrauterine environment (Hytinantti *et al.*, 1999) and in the stimulation of feeding behaviour (Schubring *et al.*, 1999). The sexual dimorphism of leptin concentrations seen in adults has also been reported in neonates: females have significantly higher leptin levels than males (Maffeis *et al.*, 1999, Ong *et al.*, 1999). In studies of healthy neonates, those who were large for gestational age and those who were small for gestational age, leptin levels were found to correlate with birthweight, placental weight and insulin levels (Varvarigou *et al.*, 1999).

1.10.3 Leptin during pregnancy

Leptin levels are increased throughout pregnancy in humans, mice and rats, suggesting a significant role for leptin during pregnancy. This increase in circulating leptin concentration may be due to either: an increased production by the adipose tissue; the addition of placental leptin production or an increased level of binding proteins present in the circulation causing an increase in leptin production. Kawai *et al* (1999) investigated the distribution of leptin receptors within the placenta. From their studies in pregnant rats, they found that the various isoforms of the leptin receptor co-localised with placental lactogen-II. The expression of the receptors increased throughout the pregnancy and it is this fact they suggest proves an important role for leptin during the whole pregnancy. Mounzih *et al.* (1999) studied the role of leptin during the pregnancies of mice. Their studies indicated that leptin was not critical for implantation and growth in the normal mouse, but, instead was a necessary factor for a leptin resistance state from mid-pregnancy, in order to stimulate food intake and have sustained energy reserves available for the duration of the pregnancy. It is possible that leptin is increased in human pregnancy as a result of a leptin resistance state that has occurred in order to increase food intake, so that adequate reserves are available.

1.11 Leptin in disease

Leptin levels have been studied in a variety of disease states including cancer-induced cachexia, AIDS, renal disease and NIDDM.

1.11.1 Leptin, obesity and NIDDM

In humans, obesity appears to be a result of leptin resistance, as many obese subjects have high circulating leptin levels. Banks *et al.* (1999) have demonstrated a reduction in the capacity of the blood brain barrier to transport leptin in obese mice. This reduction is not secondary to higher leptin levels being present in the serum, but to a physical reduction in the capacity of the BBB to transport leptin. Obesity is also associated with insulin resistance. It is possible that impaired or reduced leptin secretion is important in the development of diabetes. Hathout *et al.* (1999) investigated the hypothesis that insulin is involved in the regulation of leptin by measuring serum leptin before and during diabetic ketoacidosis in human subjects. During ketoacidosis, the researchers observed a decrease in serum leptin levels. When the patients were treated with insulin, the levels of leptin increased, leading these workers to postulate that insulin is the link between leptin and caloric intake. Several reports have suggested that leptin reduces insulin secretion from pancreatic beta cells (Emilsson *et al.*, 1997, Seufert *et al.*, 1999). One mechanism that has been proposed to explain the reduction in the secretion is through activation of a potassium channel. Harvey *et al.* (2000) demonstrate that activation of the K-ATP channels in the pancreatic islets by leptin, requires phosphoinositide 3-kinase (PI-3 kinase). These workers postulate that the leptin receptor can interact directly with PI-3 kinase (similar to the p55 TNF receptor) to activate K-ATP channels in the pancreatic islets. Liu *et al.* (1999) studied diabetic and normal subjects hypothesising that the leptin secretory response was an important factor in the development of obesity and diabetes. Their diabetic subjects showed normal leptin levels under basal conditions, but a poor leptin response to glucocorticoid levels. This may be due to the accompanying insulin secretory deficiency. Diabetes is a multifactoral disorder, which is unlikely to be easily explained by impaired or abnormal leptin secretion alone. Das (1999) proposes that it is a combination of factors including, TNF-α, glucose transporter-4 (GLUT-4) and leptin interact in the development of insulin resistance, obesity and NIDDM.

1.11.2 Leptin and cachexia

There is debate as to whether leptin has a role in cachexia and wasting in response to infection. As leptin is intrinsically involved in the regulation of energy intake and weight control, it is possible that it may also have a role in cachexia. Other cytokines, such as IL-1, TNF-α and IL-6 (Mantovani *et al.*, 1998), have an important function in the development of cachexia. As TNF-α stimulates secretion of leptin, Inui (1999) postulates a role for leptin in cancer-associated cachexia. Grunfeld *et al.* (1996) investigated serum leptin levels in patients with AIDS. They found that leptin levels were not increased, relative to body fat, in subjects with cachexia, therefore they suggest that leptin is not associated with infections in AIDS patients. Simons *et al.* (1998) studied leptin levels in patients with lung cancer-associated cachexia. They hypothesised that underlying abnormalities in the leptin feedback system in cancer may be the cause of cachexic wasting. The results of their studies showed that leptin was not associated with increased appetite or decreased energy expenditure, and therefore the hypotheses of a defective hypothalamic feedback loops are confirmed. Roberts *et al.*, (1998) hypothesised that decreased serum leptin levels would be associated with end-stage liver disease (ESLD). These workers showed that serum leptin levels were lower than control levels in their cohort of children with ESLD; the levels in the subjects with ESLD did not correlate with fat mass. Moreover, after the children had undergone a liver transplant, serum leptin levels decreased further yet measures of body fat increased. The correlation observed between fat mass and serum leptin was restored. Roberts *et al.* (1998) suggest that the relative increase in serum leptin levels during paediatric ESLD suggests that leptin in this case, is not just a passive marker of body fat but, provides a link for leptin to cachexia.

1.11.3 Leptin in renal disease

Leptin receptors are abundantly expressed in the kidney. Studies performed on glomerular epithelial cells from rats showed that leptin induced TGF-β production and proliferation of renal cells. This provides evidence for a role for leptin in accelerating kidney damage. The kidney has a vital role in removing leptin from the circulation (Cumin *et al.*, 1997, Meyer *et al.*, 1997). Serum leptin concentrations are known to be elevated in renal disease (Koo *et al.*, 1999). Landt *et al.* (1998) report that the hyperleptinaemia associated with end-stage renal disease (ESRD) is reversed after organ transplant. Further to this Fouque *et al.*, (1998) showed that both growth hormone and

IGF-1 were important in controlling leptin secretion in the dialysis patient this, however, may be due to a variation in insulin secretion in these subjects. Leptin is known to be increased above the levels expected for body fat mass in patients with renal disease. Subjects with renal disease often are nutritionally insufficient and their diets are heavily supplemented (Johansen *et al.*, 1998). These workers showed that the increased leptin levels in these patients were associated with low serum albumin levels and with a decrease in protein catabolism. A combination of insufficient removal of leptin by the kidney and the concomitant damage that leptin may cause to renal tissue add to the hypothesis that leptin may act as a cachectic factor in renal disease.

1.11.4 Leptin and polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is associated with insulin resistance, obesity and infertility (Baranowska *et al.*, 1999). The central abnormality of polycystic ovary syndrome (PCOS) is hyperinsulinaemia, which, in turn, leads to ovarian overproduction of testosterone. These changes affect the pituitary-ovarian axis, leading to abnormal production of LH and FSH. The result of the LH and FSH abnormalities is ovarian underproduction of oestrogen, along with abnormal production of progesterone, overproduction of testosterone, and amenorrhoea and infertility. There is an increased risk of miscarriage in women with PCOS; this is attributed to increased testosterone, insulin, or to low levels of progesterone and oestrogens. Baranowska *et al.* (1999) investigated the role of leptin in women with PCOS. They found that plasma leptin was increased in obese women with PCOS when compared with their control lean women. As expected, serum insulin concentration was also raised in their cohort of obese women with PCOS. As positive correlations exist between leptin and BMI, and leptin and testosterone in women with PCOS, they suggest that a disruption to the leptin feedback system exists in women with PCOS. Chapman *et al.*, (1997) investigated the role of leptin in 85 women with PCOS. They used specific criteria to define their cohort of women with PCOS. All the subjects had abnormal ovarian morphology on ultrasound, and at least two; increased serum testosterone; increased serum androstenedione and decreased sex hormone binding globulin (SHBG). Their results indicated that circulating leptin concentrations in women with PCOS, were strongly related to BMI and were not independently affected by circulating levels of insulin, gonadotrophins or sex hormones. Rouru *et al.* (1997) agree with these data, as they found no evidence for a difference in leptin levels between women with polycystic ovary syndrome and regularly cycling women. A recent study by Maliqueo *et al.* (1999)

showed no difference between serum leptin levels between normal women and in women with PCOS. They suggest that although the two groups are metabolically different, in the women with PCOS, the effects of insulin resistance and increased androgens counteract each other. This area of research is still controversial and more work to elucidate if leptin has a primary role in the development and continuation of PCOS, needs to be undertaken.

1.12 Genetic changes within the leptin and leptin receptor genes

1.12.1 Mutations in the human leptin gene

Defects in the *ob* gene are rare in human obesity. Carlsson *et al* (1997) attempted to detect *ob* gene mutations in 100 obese patients, but did not find any such mutations. Changes that lead to mild abnormalities in *ob* gene expression may, however, reflect an altered set point for body mass and therefore play a smaller role in human obesity. Montague *et al* (1997) provide the first genetic evidence that leptin is an important regulator of energy balance in humans. Their studies focus upon 2 children, from a highly consanguineous family of Pakistani origin who, although extremely obese, have very low leptin levels. These children, a male and a female, are not only obese, but have an extreme hyperphagia. The children are homozygous for a frame-shift mutation resulting in the deletion of a single guanine nucleotide in codon 133. The four parents and one of four siblings were heterozygous for this mutation, but were of normal body weight and had expected leptin levels. This is the first evidence of a mutation in the human *ob* gene and is an important step in the search for a genetic cause of obesity. In the three years since the discovery of this mutation, no other individuals have been identified with this mutation of the leptin gene.

Another mutation in the leptin gene that causes extreme obesity and hyperphagia has been identified. This mutation was observed in consanguineous family of Turkish origin. The subjects also have low level of serum leptin, but the mutation in the leptin gene is different to that described by Montague *et al.* (1997). The mutation that gives rise to the extreme obesity and hypogonadism in this family is a missense mutation at codon 105, the same codon that is mutated in the *ob/ob* mouse. In this family, the mutation does not give rise to a premature stop codon, but leads to an amino acid substitution of an arginine for a tryptophan at position 84 of the mature protein. The protein itself is synthesised correctly, but is not secreted. In total, three affected

members of this family have been identified. All are homozygous for the mutation. Again heterozygotes have normal levels of leptin and are not obese. Ozata *et al.*, (1999) have performed a detailed assessment of the endocrine, immune and sympathetic systems in this affected family. All of the affected adults had hypogonadism and had not entered puberty. This defect appears to be at the level of the hypothalamus as all patients showed a normal response to gonadotrophin releasing hormone (GnRH). The heterozygotes, both males and females, had normal pubertal development and most have had children. In total 30 children were observed in the pedigree, of these, 11 were severely obese and homozygous for the leptin gene mutation and of these, 7 have died from infections. The remaining 19 non-obese children heterozygotes and homozygous for the functional leptin gene, are alive and healthy. This family has an odds ratio of death due to an obese phenotype of 25.4. This indicates that the mutation present in the leptin gene severely impairs the ability to survive past childhood. The authors postulate that this is due to due to diminished immune function which is also seen in the *ob/ob* mouse and demonstrated *in vitro* by Lord *et al.* (1998) who showed that leptin promotes T cell activity.

1.12.2 Polymorphisms within the leptin gene

Several polymorphisms are known to exist within the leptin gene itself. Oksanen *et al.* (1997) describes a novel single nucleotide variation in the *obese* gene promoter in cohorts of lean and obese Finnish subjects. The substitution of an A for a C occurs at position –188, upstream of the start site. They did not find any significant association between this polymorphism and levels of leptin, BMI or other obesity markers such as insulin or lipids. However, this polymorphism is in close proximity to several putative regulatory elements and they suggest that it may be important in the regulation of these elements. Karvonen *et al.*, (1998) describe four gene variants in their study of Finnish subjects:

1. an A to G change at position +19,
2. a C to T substitution 33bp downstream of the termination codon.
3. a third base change at codon 48 which changes a G to an A,
4. a substitution at codon 110, which changes an alanine to methionine.

The latter two variants are rare polymorphisms, but are associated with low leptin levels in two obese subjects, who were both heterozygous for the variation. They found no

other association between leptin levels, BMI or other markers of obesity and these polymorphisms.

Hager *et al.* (1998) documented the presence of a novel polymorphism in the 5' untranslated region of the human *obese* gene. The A to G substitution occurs at position 19 of untranslated exon 1. They report that a variant of this polymorphism is associated with lower leptin levels in a cohort of morbidly obese subjects and also in a cohort of randomly chosen lean and obese Caucasian women. Oksanen *et al.* (1998) analysed the effect of the C–188A polymorphism on the activity of the leptin promoter. They demonstrated that reporter gene constructs driven by the wild-type (-188C) or variant (-188A) promoters did not reveal any genotype-associated difference in the binding of cellular proteins and therefore, there was no direct discernible difference in promoter activity due to this polymorphism.

1.12.3 Mutations in the human leptin receptor gene

Recently, a single mutation in the human leptin receptor gene has been identified. Clement *et al.*, (1998) investigated a consanguineous family of Kabilian origin with a prevalence of morbid obesity occurring in early life. These subjects have extremely elevated leptin levels, even when taking their obesity into account; they also have a complete absence of pubertal development. Using single stranded conformational polymorphism (SSCP) analysis, the workers identified a mutation in exon 16 of the human leptin receptor. This genetic change was not present in a large cohort of lean and obese controls indicating that it is a rare mutation. Sequencing of exon 16 in an affected subject revealed a G to A substitution in the splice donor site. Amplification of cDNA from the region around exon 16 (exons 15-17) was performed; this region is common to all transmembrane domain-containing isoforms of the leptin receptor. In those subjects homozygous for the mutant allele, a truncated form of the leptin receptor is produced; this lacks the transmembrane and intracellular domains and, as such, results in production of a large amount of soluble leptin receptor. Heterozygotes for this mutation are obese, but have normal pubertal development. This mutation shows that the leptin receptor is not only important in obesity, but also in pubertal development.

1.10.4 Polymorphisms within the leptin receptor gene

Considine *et al.* (1996) were the first to identify single nucleotide polymorphisms in the coding region of the leptin receptor gene. The main objective of these studies was to

find a mutation, similar to that of the *db/db* mouse or the *fa/fa* rat, in the human leptin receptor gene. In this small study of 15 African-American male subjects, using RT-PCR analysis of hypothalamic tissue, Considine *et al.* (1996) did not find any similar mutation. They did, however, identify the single nucleotide polymorphism at codon 223 (GLN223ARG). This polymorphism results in the substitution of a glutamine with an arginine. Further studies of this and other polymorphisms have been performed by other workers in several populations. Echwald *et al.*, (1997) used SSCP analysis to identify eight sequence variations within the long form of the leptin receptor in young Danish males including both lean and obese subjects. Four of these variants were present in the coding region, LYS109ARG, LYS204ARG, GLN223ARG, and LYS656ASN, all of these SNPs result in an amino acid change and, therefore, potentially result in changes in receptor function. No significant difference in allele frequency between the lean and obese subjects for any polymorphism was observed. Matsouka *et al.* (1997) studied polymorphic variation in a population of lean and obese Japanese subjects. Using automated sequencing, they identified 7 variants in the long form of the leptin receptor. Two of these, SER492THR and ALA976ASP, were novel polymorphisms in the coding region of the leptin receptor gene. These workers did not observe any significant differences in allele frequency between the lean and obese groups for any polymorphism.

Gotoda *et al.* (1997) identified five coding sequence variants, LYS109ARG, GLN223ARG and LYS656ASN as previously reported and two novel polymorphisms at codons SER343SER and PRO1019PRO, in a case control study of white British males aged 40-64 years. These novel variants do not result in an amino acid change. They demonstrated that all of these five variants were in strong linkage disequilibrium, as out of thirty-two possible haplotypes, just six accounted for more than 95% of all haplotypes in all subjects. There was no significant association between any of the genotypes and BMI, skinfold thickness, plasma insulin, triglycerides and glucose in the obese subjects. Mean BMI in the lean subjects was significantly lower in the subjects homozygous for the common allele of the SNP LYS656ASN. They postulate that this single observation was identified as it may portend to maintenance of low body weight rather than obesity. Thompson *et al.* (1997a) observed an association between homozygosity of the G allele and lower leptin levels in a cohort of Pima Indians. They also found an association between this polymorphism and insulin secretion in NIDDM Pima Indians. Earlier, they reported that a gene governing the acute insulin response

was in close proximity to the microsatellite marker, D1S198 (Thompson *et al.*, 1995), on chromosome 1. The leptin receptor gene, they now postulate, may be this element (Thompson *et al.*, 1997b). Recently, Chagnon *et al.* (2000) have identified associations between polymorphisms in the leptin receptor gene and adiposity in middle-aged male subjects. In their study, carriers of the A allele of the SNP GLN223ARG, had higher BMI, fat mass, percent fat mass and leptin than non-carriers. They postulate that the leptin receptor gene variation has a significant effect on adiposity, at least in Caucasian male subjects aged 44-64 years. Figure 1.5 shows the position of the polymorphisms in the coding region of the leptin receptor.

Another polymorphism in the 3' untranslated region of the leptin receptor is associated with serum insulin levels in obese subjects (Oksanen *et al.*, 1998). Serum insulin is higher in subjects homozygous for the absence of the insertion. This insertion of CTTTA occurs 60 nucleotides after the stop codon. They postulate that its presence generates an A + U rich mRNA sequence, this is also known as the A+U-destabilising element, that may be able to form a stem-loop structure. This feature may affect mRNA stability within the cell. Stem-loop structures are known to exist in other cytokine transcripts, for example, granulocyte-colony stimulating factor (Brown *et al.*, 1996). It is suggested that these sequences are important in binding regulatory proteins, which may affect the translation rate or degradation of mRNA.
It is likely that leptin receptor gene variants have an important role in the maintenance of body weight and adiposity.

1.11 Aims of the study

Since leptin is known to be an important factor in the regulation and maintenance of body mass and in the maintenance and facilitation of reproductive function, studies to further elucidate the role of leptin in these processes were performed.
There were three main aims of the studies described in this thesis:

1. to develop and validate an assay to measure serum levels of leptin-binding activity and use this to examine the effect of leptin binding in serum.
2. to investigate the role of leptin and the leptin receptor in human reproductive processes such as pregnancy, the normal menstrual cycle and puberty
3. to identify any associations between leptin receptor polymorphisms and measures of body weight and adiposity, to assess the effect of genetic variation on receptor function and to examine the effect of linkage disequilibrium in this population.

These studies should enable the further elucidation of leptin's role in reproductive function and to assist in understanding the genetic influence of leptin in body mass regulation.

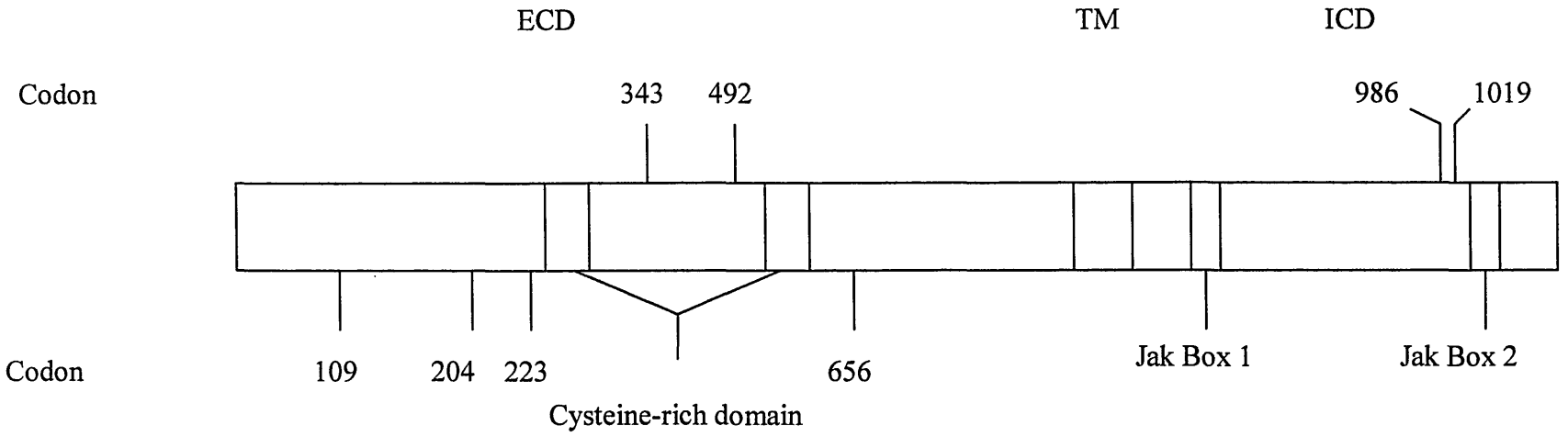


Figure 1.5 Schematic diagram of the position of leptin receptor polymorphisms.

ECD - extracellular domain, TM - transmembrane domain, ICD - intracellular domain

2.1 Nucleic acid purification techniques

2.1.1 Genomic DNA Extraction

2.1.1.1 Buffers required for DNA extraction

Buffer A

0.32M sucrose (Sigma),

0.01M Tris base (Sigma),

0.15M $MgCl_2$ (Sigma),

The pH was altered to 8 with concentrated hydrochloric acid. The buffer was then autoclaved and once cool, 1% Triton (Sigma) was added.

Buffer B

0.4M Tris base,

0.06M EDTA (Sigma),

0.005M NaCl (BDH),

The pH was altered to 8 with concentrated hydrochloric acid. The buffer was autoclaved, and once cool, made-up to 1L. SDS (Sigma) was added to a final concentration of 1%.

2.1.1.2 DNA extraction protocol

20 ml of venous blood was collected into tubes anticoagulated with EDTA. To this, 30ml of Buffer A was added to provide hypotonic lysis of the cells and thus, remove the haemoglobin and other cellular proteins. This was then mixed end-over-end for 4 minutes at room temperature (RT), and centrifuged (Sorvall RT 6000D) at RT, 1000g for 15 minutes. The supernatant was removed and discarded and the pellet (containing the white cell nuclei) resuspended in 20ml of buffer A. This was centrifuged again, at RT, 1000g for 15 minutes. Again the supernatant was discarded and 1ml of buffer B was added to resuspend the pellet. The suspension was then transferred to an eppendorf tube; 300µl of 5M sodium perchlorate was added, to precipitate the proteins, and the sample rotated end-over-end for 10 minutes followed by high speed microcentrifugation,

10000g, for 10 minutes. In a clean eppendorf tube, 600µl of supernatant was added to 700µl of ice-cold chloroform. The sample was then mixed end-over-end for 3 minutes and centrifuged at high-speed (10 000g) for 10 minutes, in a microcentrifuge. The upper layer was then transferred to a clean eppendorf tube and twice the volume of ice-cold ethanol was added to precipitate the DNA. This was gently mixed and centrifuged (Sorvall Super T21) at 4°C, 10000g for 15 minutes. The supernatant was removed and the DNA pellet allowed to air-dry for 10 minutes. The pellet was resuspended in water (200-500µl of water depending on the pellet size) and rotated en-over-end for 1 hour at 4°C.

2.1.1.3 Assessment of DNA purity and concentration

The concentration of human genomic DNA was estimated by measurement of the absorbance at 260nm (GeneQuant spectrophotometer). In a 1cm-pathlength quartz cuvette, 1 absorbance unit at 260nm is equal to 50mg/ml of DNA. DNA samples were diluted (5µl of stock DNA solution in 995µl of water) and the absorbance measured at 260nm. The stock DNA was diluted in water to a final concentration of 50ng/µl. Samples were stored at -20°C until required.

The purity of each of the DNA samples was assessed as the concentration was estimated. The absorbance was measured at 260nm and 280nm in a 1cm-pathlength quartz cuvette and a ratio calculated. A ratio of 1.8 was considered a pure sample. The quality of the DNA samples was assessed by agarose gel electrophoresis (Figure 2.1) An example of pure, good quality DNA is shown in Figure 2.1.

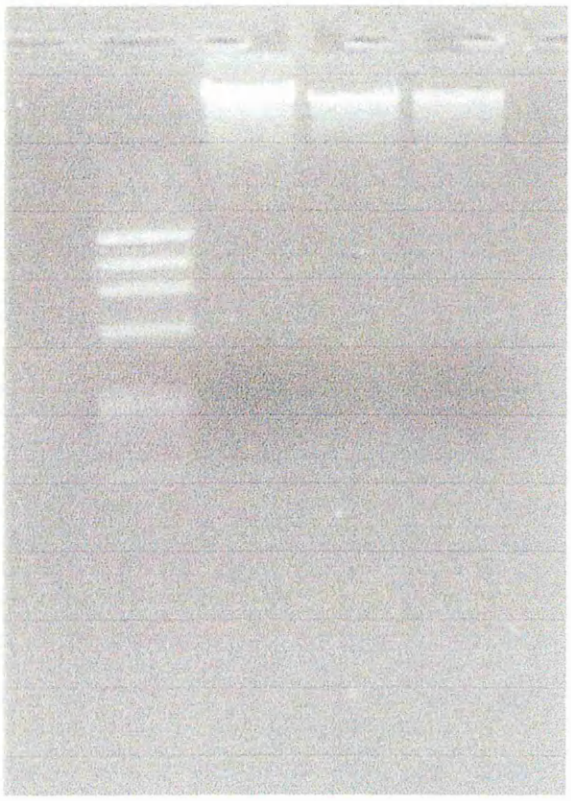


Figure 2.1 Genomic DNA electrophoresed on a 1% agarose gel and stained with ethidium bromide. The first lane contains DNA ladder (ϕ174 DNA HaeIII), the next three lanes (2-4) Contain genomic DNA.

2.1.2 RNA extraction from lymphocytes, tissue and cultured cells.

Extraction of RNA from lymphocytes, cultured cells and tissues uses a modified method first described by Chomczynski and Sacchi (1987). Homogenisation or lysis of the cells or tissue in the RNAzol (Biogenesis, Poole, Dorset) or Tri Reagent (Sigma) allows the integrity of the RNA to be retained whilst the cells are disrupted and the cell components are dissolved.

2.1.2.1 Extraction of RNA from Lymphocytes

Whole blood was collected into Vacutainer™ tubes anticoagulated with EDTA. 15ml of lymphoprep (Nycomed, Oslo, Norway) was placed into a sterile universal tube (Falcon). An equal volume of blood was carefully layered over the lymphoprep. This was centrifuged (Sorvall RT 6000D) at RT, 400g, for 30 minutes. The mononuclear cells from the buffy coat were harvested into a sterile 50ml tube. The tube was topped up to 50ml with 1x phosphate buffered saline (PBS) and centrifuged, as before, for 5 minutes. The supernatant was discarded and the cell pellet washed and resuspended in 50ml of 1x PBS. Again this was centrifuged for 5 minutes. The supernatant was discarded and the cell pellet was vigorously resuspended in 1ml of RNAzol (Biogenesis, Poole, Dorset) or Tri Reagent (Sigma). At this stage the samples could be stored frozen at -80°C until the RNA extraction procedure was carried out.

2.1.2.2 Extraction of RNA from cultured cells

All cultured cells used were adherent cells that grew in monolayers. The volume of the plastic flask was measured. The cell culture media was removed and the cells washed twice in 10ml of 1x PBS to remove any cell debris. To the flask, 1ml of RNAzol or Tri Reagent was added per $10cm^3$ of flask area. For example, if the area of the flask was $75cm^3$, 7.5ml of RNAzol or Tri Reagent was added. The liquid was pipetted vigorously until the cells had lysed. At this stage the samples could be stored frozen at -80°C until the RNA extraction procedure was carried out.

2.1.1.3 Extraction of RNA from tissue samples

Small pieces of tissue, approximately 1g in weight, were frozen in liquid nitrogen and crushed with a sterile pestle and mortar. The freezing made the tissue extremely brittle and therefore, very easy to break into small fragments. It was extremely important to keep the tissue frozen, this had a dual purpose, to stop the RNA from degrading and also to allow the samples to be easily fragmented. Once the samples had been adequately crushed, 1ml of RNAzol or Tri Reagent was added. The tissue was pipetted vigorously until in suspension. At this stage the samples could be stored frozen at -80°C until the RNA extraction procedure was carried out.

2.1.1.4 RNA Extraction Protocol

Frozen samples were allowed to thaw on ice. The extraction procedure was the same for all sample types whether frozen or not. The samples were mixed and then incubated on ice for 5 minutes. To this, 100µl of ice-cold chloroform (per ml of RNAzol) was added and incubated on ice for 10 minutes. The samples were then centrifuged (Sorvall Super T21) for 15 minutes, 10 000g at 4°C. The upper aqueous layer (approximately 500µl) was removed and an equal amount of ice-cold isopropanol was added. This was inverted gently and allowed to incubate on ice for 1 hour, this process allowed precipitation of the RNA. At the end of this time, the sample was centrifuged as before for 15 minutes. The supernatant was discarded and the pellet gently resuspended in 250µl of 80% ethanol. This was centrifuged at 4°C, 10 000g for 5 minutes, the ethanol removed and the pellet allowed to air-dry for 10 minutes. The pellet was then resuspended in 20-50µl of sterile (suitable for injection) water.

2.1.1.5 Assessment of RNA purity and concentration.

The concentration of RNA in each sample was assessed by measurement of the absorbance at 260nm. In a 1cm-pathlength quartz cuvette, 1.0 absorbance unit at 260nm is equal to 40µg of RNA. The samples were adjusted to a concentration of 1µg/µl with sterile water. Samples were stored at -80°C until required. As the concentration was assessed, the purity of the RNA sample was calculated by measuring the ratio of absorbancies at 260nm and 280nm. A ratio of 2.0 was considered pure.

2.2 Polymorphism Analysis

2.2.1 Polymerase Chain Reaction (PCR) screening for polymorphisms in the leptin receptor

PCR was performed using either purified DNA or dried blood spot as a template. Three polymorphisms in the leptin receptor were investigated; GLN223ARG, LYS656ASN, LYS204ARG.

Primers to amplify the polymorphic regions of the leptin receptor were taken from Echwald *et al.*, (1997). The primer sequences for the PCR analyses are listed in appendix I.

2.2.1.1 GLN223ARG and LYS204ARG

2.2.1.1a PCR from purified genomic DNA

PCR reactions were out carried in a 50µl volume containing; PCR buffer (a final concentration of 20mM Tris-HCl, 50mM KCl) (Gibco), 2.5mM magnesium chloride (Gibco), 0.6µM of the forward primer, 0.6µM of the reverse primer, 0.2mM deoxynucleotide triphosphates (dNTPs) (Gibco), 100ng of DNA template. A negative control was included in each batch of PCR, here the DNA template was replaced with 2µl of water. Reaction mixes were overlaid with 40µl of mineral oil (Sigma). The tubes were placed onto a thermocycler (Biometra) and heated to 95°C for 10 minutes. The samples were cooled to 4°C and 2.5 units of Taq DNA polymerase (recombinant from *Thermus aquaticus*) (Gibco) were added to each reaction. The PCR was carried out on a thermocycler using the following conditions: heating to 95°C for 3 minutes, 35 cycles of heating to 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension period of 72°C for 5 minutes. The PCR products were stored at 4°C until required for electrophoresis and restriction endonuclease digestion.

2.2.1.1b PCR from dried blood spots

PCR reactions were out carried in a 50µl volume containing; PCR buffer (a final concentration of 20mM Tris-HCl, 50mM KCl) (Gibco), 2.5mM magnesium chloride (Gibco), 0.4µM of the forward primer, 0.4µM of the reverse primer, 0.2mM deoxynucleotide triphosphates (dNTPs) (Gibco). A very small piece of filter paper covered with dried blood, approximately $1mm^2$ was added to the tube containing the mix. A negative control was included in each batch of PCR, here the dried blood spot was replaced with 2µl of water. All the reactions were overlaid with 40µl of mineral oil (Sigma). The tubes were placed onto a thermocycler (Biometra) and heated to 95°C for 10 minutes. The samples were cooled to 4°C and 2.5 units of Taq DNA polymerase (recombinant from *Thermus aquaticus*) (Gibco) were added to each reaction. PCR cycles were then performed as for the genomic DNA templates.

2.2.1.1c Genotyping GLN223ARG by restriction digest with MspI and LYS204ARG by restriction digest with HinfI

The leptin receptor polymorphisms GLN223ARG and LYS204ARG are both in exon 4 (Echwald *et al.*, 1997) and both polymorphisms are contained within the 416bp PCR product. Therefore, the same PCR product can be used to identify both of these polymorphisms. Digestion of the PCR product with MspI (Promega) allows detection of the alleles for GLN223ARG and digestion of the PCR product with HinfI (Promega) allows detection of the alleles for LYS204ARG. Digestion reactions for both polymorphisms were identical, and were carried out in a total volume of 30µl, containing 20µl of PCR product, 3µl of buffer (final concentrations of 6mM Tris-HCl, 50mM NaCl, 6mM $MgCl_2$, and 1mM DTT), 6µl of water and 1µl (10 units) of restriction enzyme. The tubes were incubated in a water bath at 37°C for 2 hours. After digestion, the samples underwent 2% agarose gel electrophoresis in order to visualise the different genotypes (Figure 6.1, 6.2 respectively In chapter 6).

2.2.1.2 LYS656ASN

2.2.1.2a PCR from genomic DNA and dried blood spots

PCR reactions were out carried in a 50µl volume containing; PCR buffer (final concentrations of 20mM Tris-HCl, 50mM KCl) (Gibco), 2mM magnesium chloride (Gibco), 0.2µM of the forward primer, 0.2µM of the reverse primer, 0.2mM deoxynucleotide triphosphates (dNTPs) (Gibco), 100ng of DNA template or a small piece of filter paper covered in dried blood. A negative control was included in each batch of PCR, here the DNA template was replaced with 2µl of water. All the reactions were overlaid with 40µl of mineral oil (Sigma). The tubes were placed onto a thermocycler (Biometra) and heated to 95°C for 10 minutes. The samples were cooled to 4°C and 2.5 units of Taq DNA polymerase (recombinant from *Thermus aquaticus*) (Gibco) were added to each reaction. The PCR was carried out on a thermocycler using the following conditions: heating to 95°C for 3 minutes, 35 cycles of heating to 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension period of 72°C for 5 minutes. The samples were stored at 4°C until analysed by SSCP (Shown in Figure 6.3 in Chapter 6).

2.3 Gene Expression Analysis

2.3.1 Reverse Transcriptase – PCR (RT-PCR)

2.3.1.1 First strand cDNA synthesis

First strand cDNA synthesis was carried out in 40µl reaction mix containing: 5x AMV Reverse Transcriptase Buffer (with final concentrations of 100mM Tris/HCl, 10mM magnesium chloride, 10mM DTT) (Gibco); 50mM potassium chloride (Gibco), 1mM dNTPs (Gibco); 0.6µg of Oligo d(T) (Gibco). For each RNA sample, triplicate tubes were prepared, to two tubes 4µl of 1µg/µl total RNA was added. 3 units of AMV (avian myeloblastosis virus) reverse transcriptase (RT) (Gibco) was added to one reaction containing RNA, to the other RNA tube, 1.2µl of pure water, this tube acted as a control reaction. To the third tube, 4µl of pure water and 3 units of RT were added, this acted as a negative control. Each tube was overlaid with 40µl of mineral oil (Sigma) to prevent

evaporation whilst on the thermocycler. The samples were incubated on the thermocycler (Biometra) at 37.5°C for 1 hour to allow cDNA synthesis, then heated for 5 minutes at 99°C to denature the RT enzyme. The samples were either used directly in PCR or were stored at -20°C until required.

2.3.1.2 Polymerase chain reaction of leptin receptor isoforms

PCR of all leptin receptor isoforms from cDNA was carried out using identical conditions. PCR from cDNA was carried out in a 20µl reaction containing: 16.15µl sterile (suitable for injection) water, 10x PCR Buffer (with final concentrations of 20mM Tris-HCl, 50mM KCl) (Gibco, Life Technologies, Paisley Scotland); 1.25mM magnesium chloride (Gibco); 0.12µM forward primer, 0.12µM of reverse primer, 1.25 units of Taq DNA polymerase and 5µl of appropriate cDNA sample. The samples were overlaid with mineral oil (Sigma) to prevent the reaction mixture evaporating.

PCR was carried out on a thermocycler (Biometra) using the following conditions: denaturation at 96°C for 1 minute, 40 cycles of; denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and a final extension of 5 minutes at 72°C. The samples were then electrophoresed on 2% agarose gels.

2.3.1.3 Polymerase chain reaction of 7B6 gene

PCR from cDNA was carried out in a 20µl reaction containing: 15.25µl pure water, 10x PCR Buffer (with final concentrations of 20mM Tris-HCl, 50mM KCl) (Gibco, Life Technologies, Paisley Scotland); 1.25mM Magnesium chloride (Gibco); 0.3µM forward primer, 0.3µM of reverse primer, 1.25 units of Taq DNA polymerase and 5µl of appropriate cDNA sample. The samples were overlaid with mineral oil (Sigma) to prevent the reaction mixture evaporating.

PCR was carried out on a thermocycler (Biometra) using the following conditions: denaturation at 96°C for 1 minute, 35 cycles of; denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute, extension at 72°C for 1 minute, and a final extension of 5 minutes at 72°C. The samples were then electrophoresed on 2% agarose gels.

2.3.1.4Primers used for RT-PCR of leptin receptor isoforms and 7B6

Primers used to detect three isoforms of the leptin receptor (Ob-R) were used in these studies. A method by Bennett *et al.*, (1996) was modified, the primer sequences remained unchanged. A gene that is expressed ubiquitously, 7B6, identified by Francis and Duff (1993) was used as a positive control to ensure the RNA and the RT-PCR system was working. Appendix 2 lists the primer sequences of all RT-PCR primers.

2.4 Electrophoretic Techniques

2.4.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise genomic DNA, PCR and RT-PCR products. 2% agarose gels were made by adding 1g of agarose to 50ml of 1x Tris Borate EDTA (TBE) buffer. TBE buffer was made up at 10x concentration (0.89M Tris, 0.89M Boric acid, 0.025M EDTA in distilled water) for storage and diluted to 1x TBE in water for use. The gel was dissolved by heating, either in a microwave oven or on a heated stirrer. The gel was allowed to cool and 2µl of ethidium bromide (stock solution 10mg/ml in water) (Sigma) was added. The molten agarose was allowed to set in the gel apparatus (Biometra, Luton, Beds.). For PCR 10µl of product and for RT-PCR 15µl of product was diluted with 5µl of loading dye (1% bromophenol blue in 10% sucrose in 50ml 1x TBE). For each row of electrophoresis, a DNA marker was electrophoresed alongside the samples to allow sizing of the products. In each case either PhiX174 DNA/HaeIII (Promega) or PhiX174 DNA/Hinf I (Promega) was used. Preparation of the marker was as follows; 0.5µl of marker (1µg/µl) was diluted with 15µl of water and 5µl of loading dye. A total of 20µl was added to the gel. The gels were electrophoresed for 1 hour at 80-100V. Visualisation of the products was achieved by UV transillumination. Photographs were taken on a Polaroid camera or the images captured on a digital camera (Kodak electrophoresis documentation and analysis system, Rochester, NY).

2.4.2 Single Stranded Conformational Polymorphism (SSCP) Analysis

For detection of the alleles of the polymorphism LYS656ASN, SSCP was used.

2.4.2.1 Sample preparation

Denaturation of the PCR products was achieved by incubating 2µl of PCR product with 10µl of water, 5µl of loading dye and 8µl of SSCP buffer (95% Formamide (Sigma) in 10mM EDTA/NaOH with 0.2% Blue Orange loading dye (Promega)). The samples were heated to 95°C on a thermocycler block for 10 minutes. Immediately after heating the samples were placed on ice and loaded onto a previously prepared polyacrylamide gel for electrophoresis.

2.4.2.2 PAGE gel preparation

For detection of the alleles in the samples PAGE separation was employed. Two PAGE gel systems from Bio-Rad Laboratories (Hemel Hempstead, Herts.) were used; the PROTEAN II xi Cell system and the Mini-PROTEAN II Cell system. For each system the same gel mix was used but the different gel systems required different amounts. A 40% Acrylamide-Bisacrylamide solution (Sigma) at a ratio of 49:1 was utilised to allow clear definition of the single stranded products. A gel of final concentration 9% acrylamide-bisacrylamide with 10% glycerol (Sigma) was used. 25% ammonium persulphate (APS) (Sigma) was made-up fresh, each day PAGE gels were prepared. The addition of APS and TEMED (Sigma) was to crosslink the acrylamide/bisacryamide and therefore, allow polymerisation. These two components were added last.

The glass plates had been washed in distilled water and wiped with alcohol. The gel apparatus was set-up according to the manufacturer's instructions and the gels allowed to polymerise for 40 minutes. The gels were transferred to the cold room where they were chilled for a minimum of one hour. Samples were loaded onto the gel in the cold room and the electrophoresis carried out at 4°C. The Mini-PROTEAN II gels were electrophoresed for 2 hours at 160V. The PROTEAN II gels were electrophoresed at 200V for 6 hours. Visualisation of the DNA bands was achieved using silver staining.

	Mini-PROTEAN II	PROTEAN II
10x TBE	1ml	4ml
Acrylamide/Bisacrylamide	2.25ml	9ml
Water	5.75ml	23ml
Glycerol	1ml	4ml
TEMED	12.5µl	50µl
Ammonium Persulphate (25%)	40µl	160µl
Total gel volume	10ml	40ml

Table 2 PAGE gel components for SSCP of LYS656ASN polymorphism in the leptin receptor.

2.4.3 Silver Staining

The PAGE gel was fixed for 10 minutes in Gel Fixer (10% ethanol, 5% glacial acetic acid in water) with gentle mixing. The fixer was poured off and the 1% Silver nitrate in water added. The gel was mixed continuously for 15 minutes. The silver nitrate solution was poured off and the gel washed twice in distilled water. Developer (0.38M NaOH, 3.75ml Formaldehyde in 1L of distilled water) was added to the gel and quickly mixed to avoid patchy development. The gel was allowed to develop until the bands were clearly visible or until 15 minutes had elapsed. The developer was poured away and the gel washed twice in distilled water. The gel was fixed in final fixer (7.5% Sodium carbonate in distilled water) for 15 minutes. Photographs were taken on a Polaroid camera or the images captured on a digital camera (Kodak electrophoresis documentation and analysis system, Rochester, NY).

2.4.5 Preparation of PCR product DNA for sequencing

In order to ascertain if the genotypes of the LYS656ASN polymorphism were being correctly assessed, 3 samples were prepared and sent for automatic sequencing. One of the two samples was believed to be homozygous for the G allele, one was believed to be homozygous for the C allele and the other a heterozygote. Sequencing of the PCR product used in the SSCP analysis would be sufficient to identify the 3 allelic states.

2.4.5.1 Isolation of specific DNA product

A 50ml 1% agarose gel was made as before but, using 1x Tris acetate EDTA buffer (TAE) (10x TAE buffer 0.4M Tris-acetate, 0.01M EDTA made up to 1L with distilled water, diluted as required to 1x TAE with distilled water). The gel was cooled and 2µl of ethidium bromide (stock solution 10mg/ml) was added. The gel was poured into the gel forming apparatus and a comb with large teeth positioned at one end. Each well of this comb could be filled with 60µl of DNA. A 50µl PCR reaction was required for sequencing. This total volume was mixed with 10µl of loading dye (20% sucrose solution in 1x TAE containing bromophenol blue), the total of 60µl was loaded onto the gel. Alongside this a DNA marker was also added as before to allow for product sizing. The gel was electrophoresed for 1 hour at 50v. After 1 hour the bands on the gel were visualised by UV transillumination. A clean transparency was placed underneath the gel to avoid any foreign DNA contaminating the gel. A sterile blade was used to cut out the gel surrounding the band(s) of interest. A small hole was pierced through the bottom of a 0.5ml-eppendorf tube and a small amount of siliconised glass wool was placed in the bottom of the tube. This small eppendorf was placed in a large 1.5ml eppendorf tube. The lid was removed from this tube. Each slab of gel was placed into the pre-prepared eppendorf apparatus as described and the tubes centrifuged at 10 000g for 15 minutes. This forces the liquid containing the DNA of interest out of the gel and into the large eppendorf. Approximately 100µl of liquid was recovered from each gel slab.

2.4.5.2 Removal of Ethidium Bromide

To each sample an equal volume of water-saturated butanol was added. This was mixed vigorously and centrifuged at RT, 10 000g, for 30 seconds. The upper butanol layer was removed and the process repeated.

2.4.5.3 Precipitation of DNA

To the DNA sample, 2.5 times the volume of ice-cold ethanol was added (for 100µl of sample 250µl of ethanol) and one tenth of the original total volume (in this case 10µl) of 3M sodium acetate was added. The sample was mixed and the nucleic acids precipitated by incubation at -70°C for 30 minutes. The sample was then centrifuged at 10 000g for 15 minutes at 4°C. The supernatant was removed and the pellet washed in 70% ethanol and allowed to air-dry for 15 minutes. The sample was then ready for automatic sequencing.

2.4.5.4 Automatic Sequencing

Miss Hazel Holden at the Department of Molecular and Genetic Medicine, University of Sheffield carried out the automatic sequencing. Forward and reverse primers (at a concentration of 1µM) were supplied with the DNA pellets. A total of 3.2µl of each primer was required for each sequencing reaction. The primers were the same as used in the PCR reaction. Sequencing reactions are listed in Appendix 3.

2.5 Measurement of leptin and leptin-binding activity (LBA)

2.5.1 Measurement of leptin in serum, plasma and cell culture supernatants.

Serum, plasma and cell culture supernatant samples were analysed using one of two commercial kits: a human leptin radioimmunoassay (Linco Research Inc., St Louis Mo.) and Quantikine™ human leptin ELISA (R&D Systems, Abingdon, Oxon.). In both cases, the assays were carried out according to the manufacturers' instructions.

For the RIA, samples were assayed in duplicate and did not require dilution unless the sample contained more than 100ng/ml of leptin. For the ELISA method, serum and plasma samples were diluted 100-fold in the supplied calibrator diluent, cell culture supernatants were assayed neat, diluted 2-fold, 5-fold and 10-fold. Samples were assayed in duplicate. Single samples were assayed when the total number of subjects in the study was greater than 150.

The RIA samples were counted on an automatic gamma counter (Wallac, Turku, Finland), the ELISA plates were measured on an automatic plate reader (Wallac Victor^2 1420 multilabel counter.) at 450nm.

Both the RIA and the ELISA methods measured free and bound leptin.

2.5.2 Assay for the measurement of Leptin-Binding Activity (LBA)

2.5.2.1 Preparation of serum from whole blood

Venous blood was collected into a sterile tube (without anticoagulant) and allowed to clot for 20 minutes at room temperature. The blood was centrifuged at 1000g (Sorvall RT 6000D) for 10 minutes. The upper serum layer was carefully removed and aliquotted into 1.5ml eppendorf tubes. The serum was frozen at -20°C until required for analysis.

For the LBA assay, two individuals (one obese and one of normal BMI) were identified to use as controls each time the assay was performed. The subjects donated 100ml of venous blood. Serum was prepared from these samples as described above. Approximately 50ml of serum from each of the two subjects was obtained, this was divided into 250µl aliquots and stored at -20°C until required for analysis.

2.5.2.2 Stripping of leptin from serum sample

To 200µl of serum, 500µl of prechilled 2% Norit A charcoal (Norit) 0.2% Dextran T70 (Pharmacia) in assay buffer was added at room temperature, vortex mixed and immediately placed on ice for 5 minutes. The sample was centrifuged at 4°C for 12 minutes at 10 000g. The supernatant (stripped of free leptin) was removed by pipette and placed in a new tube.

2.5.2.3 Measurement of LBA

50µl of stripped serum was incubated with 150µl of assay buffer (0.01M phosphate buffer pH 7.4, 0.18M $MgCl_2$, 1% BSA), 100µl of [^{125}I] Leptin 135µCi/µg (Linco Research Co, Research Park Drive, St Louis, USA), in the presence (to measure non-specific binding) or absence (to measure total binding) of 1µg unlabelled leptin (R&D Systems, Abingdon, Oxford, UK). For separation of the bound from the unbound fractions, 1ml of prechilled 2% charcoal-0.2% Dextran T70 slurry in assay buffer was added to the overnight incubation, vortexed, placed on ice for 10 minutes, and centrifuged at 4°C for 12 minutes at 10 000g. 1ml of the supernatant was aspirated by pipette and counted in an automatic γ-counter. The specific binding (total binding minus non-specific binding) obtained was expressed as a percentage of the total [^{125}I]-Leptin counts per minute incubated in 50ul of serum. All samples were measured in duplicate.

As mentioned above, serum samples with previously measured high and low levels of LBA were used as control serum and were run in each assay at the beginning and end of the assay.

2.5.2.4 HPLC analysis of LBA

The HPLC method was developed from a method used to measure GHBP by Tar *et al* (1990). HPLC separations were performed using a liquid chromatograph (model 600, Waters, Milford, MA) equipped with a sample injector (model U6K) fitted with a 1ml loop and an analytical Protein Pak 300sw column (Waters; 0.75 x 30cm). Absorbance at 280nm was monitored with an LC spectrophotometer (Waters), and radioactivity was

recorded on-line using a Bertold LB 504 γ-detector (Bertold, Elancourt, France) connected to an Apple IIe computer.

Serum (150μl) was incubated overnight at 4°C with 30μl of assay buffer and 20μl of ^{125}I-Leptin. A parallel incubation was carried out in the presence of an excess of unlabelled leptin (2μg). After filtration through a 0.45μm Millipore minifilter, the entire incubation was placed on to an HPLC Protein Pak 300sw column. Elution was performed autocratically using a degassed buffer (0.1M Na_2SO_4 and 0.1M potassium phosphate, pH7.0) pumped at a rate of 0.5ml/minute. The column was calibrated with blue dextran, BSA, phosphorylase-b, aldolase, and [^{125}I]-Leptin

This analysis was carried out in its entirety by Miss Sylvie Simon and Dr Marie-Catherine Postel-Vinay at the Faculte de Medicine Necker in Paris, France.

2.6 Measurement of cotinine in serum

Serum cotinine levels were measured using an in-house RIA modified from the original method of Knight *et al.* (1989). Assignment of smoking status in the serum samples from pregnant women was carried out in collaboration with Dr Robert Smith in the Division of Biomedical Sciences.

2.6.1 Iodination of cotinine

In a reaction tube, 100µl of trans-3-carboxy-cotinine (2µg in 0.5M phosphate buffer) (a kind gift from Dr George Knight), was added to 10µl of ^{125}I (1.0 mCi) and mixed. To this 10 µl chloramine T (Sigma) (1.0 mg/ml in 0.1M phosphate buffer) was added and the reaction allowed to proceed for 10 seconds. To quench the reaction, 20µl of sodium metabisulphate (Sigma) (1.0mg/ml in 0.1M phosphate buffer) was added. The entire reaction mixture was applied to a diethylaminoethyl (DEAE) column equilibrated to pH3.0. The fractions were eluted from the column using 0.1M phosphate buffer (pH3.0). In total 10 fractions with a volume of 0.5ml were collected into tubes containing 1.0ml phosphate buffer (pH 3). A small amount (10µl) of each fraction was diluted in 1ml of 0.1M phosphate buffer and counted on a γ-counter. A peak at fractions two and three was present, this was the iodinated cotinine. These fractions were pooled together and stored at 4°C in 0.1M phosphate buffer (pH 3) until required.

2.6.2 Measurement of cotinine in serum

Cotinine (Sigma) standards, with concentrations of 0, 12.5, 5, 25, 50, 100, 200, 400 and 800ng/ml were prepared and stored at –20°C until required. To a polycarbonate tube, 10µl of sample or standard was added. Samples and standards were assayed in duplicate. To each tube, 750µl of assay buffer (5% horse serum in 0.01M phosphate buffer, 0.15M sodium chloride pH7.5) 100µl of iodinated cotinine label and 100µl of rabbit anti-cotinine antibody (a kind gift from Dr G Knight) was added. Alongside the samples and standards, duplicate tubes containing 100µl of iodinated cotinine label only (total tubes) and duplicate tubes not containing any sample or standard (non-specific binding, NSB)

were prepared. All tubes were vortex mixed and incubated overnight at 4°C. To the overnight incubation, 100µl of anti-rabbit IgG antiserum was added to each tube (except the total tubes). All tubes were mixed and allowed to incubate at room temperature for 2 hours. To separate the bound and free fractions, 1ml of PEG buffer (8% polyethylene glycol (Sigma), XM sodium chloride (Sigma) made up in water) was added to each tube (except the total tubes), mixed and centrifuged at 4°C, 5000g for 40 minutes. The supernatant was removed by aspiration and the pellet counted on a γ-counter. A batch of samples consisted of the zero tubes, 8 standards, total counts tubes, NSB tubes, 2 quality control samples with known cotinine concentration and up to 40 samples. Levels of cotinine were calculated using RIAcalc data analysis package.

2.7 Endometrial biopsy collection and cell culture

2.7.1 Endometrial Cell Culture

Endometrial biopsies were collected by Mr T.C. Li at the Jessop Hospital for Women, from women undergoing gynaecological surgery for reasons other than the endometrium. The women were all aged between 24 and 40 years, with regular menstrual cycles of 25-35. All subjects gave informed consent. The biopsies were prepared and the cells cultured by Miss Beverley Cork or Mrs Elizabeth Tuckerman at the Jessop Hospital for Women.

2.7.1.1 Epithelial and Stromal Cell separation

To separate the epithelial and stromal cells of the endometrial biopsies an established digestion and centrifugation method has been adopted (Laird *et al.*, 1993, Laird *et al.*, 1997). The endometrial biopsies were collected into Hanks' balanced salt solution (HBSS) (Sigma) containing penicillin and streptomycin (each at a concentration of 100µg/ml). The tissue was washed twice in HBSS and chopped finely with scissors. The tissue was then incubated in Dulbecco's modified Eagle medium (DMEM) (Sigma) containing 0.2% collagenase (type1a) (Sigma) for 45 minutes at 37°C. During, and at the end of, the incubation the tissue was pipetted to disperse the cells. The epithelial cells and stromal cells were separated by centrifugation at 100g. The supernatant containing

the stromal cells was removed and the pellet containing mainly the epithelial cells was incubated in 5ml of DMEM with 0.2% collagenase (type 1a) for a further 45 minutes at 37°C. The cells were dispersed again by gentle pipetting and the epithelial cells pelleted by centrifugation at 100g. The stromal cells present in the supernatant were pooled with the first supernatant and pelleted by centrifugation at 300g.

The epithelial and stromal cells were purified further by unit density sedimentation. Each cell type was resuspended in 2ml of DMEM containing 10% fetal calf serum (FCS) (Sigma), glutamine (4mmol/l) (Sigma), penicillin and streptomycin (100µg/ml) (CDMEM) and gently pipetted onto 8ml of CDMEM in a test tube and left for 30 minutes at RT. Stromal cells from the top 8ml were used for cell culture. Epithelial cells from the bottom 2ml were used for cell culture. Cells were seeded into 96-well plates or into flasks and grown in an incubator at 37°C, 5% carbon dioxide and 95 % air to confluency.

2.7.1.2 Proliferation of endometrial epithelial and stromal cell cultures.

Cells had previously been seeded into 96-well plates at a density of 0.8 x 10^5 cells per well. The cells were grown to confluency for 48-72 hours in an incubator at 37°C, 5% carbon dioxide and 95 % air. To assess if the cells proliferated in response to leptin a thymidine uptake assay was performed. 60µl (60µCi) tritiated thymidine ([^{3}H] thymidine) (1mCi/ml, specific activity 60-90 Ci/mmol) (ICN, Basingstoke, Hants.) was added to 16ml CDMEM, mixed and divided into 4ml aliquots. Leptin were added to the medium to give final concentrations of 0, 0.1, 1.0 and 10.0 ng/ml. To each well, 250µl of media was added. For each concentration of leptin, 10-15 wells of replicates were carried out. The plates were then incubated for 48 hours in an incubator at 37°C, 5% carbon dioxide and 95 % air. After incubation, the supernatants were collected and frozen at -20°C until required for further analysis. The cells were washed twice in 1x PBS and 100µl trypsin/EDTA (Gibco BRL) was added to each well. The plates were incubated for 30 minutes at 37°C. The cells were harvested using a semi-automatic cell harvester (Skatron, Newmarket, Suffolk). Each filter paper was added to 2ml of scintillation fluid (Wallac Optiphase HiSafe, Wallac, Turku, Finland.) in a scintillation vial. Samples were counted for 3 minutes each on an automatic β-counter (Tri-Carb 1900CA, Packard, Pangbourne, Berks.).

2.8 Cytokine Assays

2.8.1 Measurement of Leukaemia Inhibitory Factor (LIF) in endometrial cell culture supernatants

LIF was measured using a specific kit, an enzyme amplified sensitivity immunoassay (EASIA) from Biosource Europe. The measurement of the cytokines in the endometrial cell culture supernatants was carried out according to the manufacturer's instructions. The sensitivity of the LIF assay was 20pg/ml with an intra and inter-assay CVs of 4.5% and 6% respectively. The final absorbance of the plates was measured on an automatic plate reader (Wallac, Turku, Finland.) at 450nm. The assay used to measure LIF did not cross-react with other cytokines or its soluble receptor. Dr Susan Laird carried out this analysis.

2.8.2 Cytotoxic Bioassay for the measurement of Tumour Necrosis Factor-α (TNF-α) in endometrial cell culture supernatants

This bioassay uses the cytotoxic effect of TNF-α present in the samples, in this case in the cell culture supernatants, on a pre-sensitised murine aneuploid fibrosarcoma cell line (L929). The cells were seeded into a 96-well plate at a density of 0.2×10^5 cells/well in 100µl of RPMI (Sigma) containing 10% FCS, penicillin and streptomycin (100µg/ml). The plate was placed in an incubator at 37°C, 5% carbon dioxide, 95% air for 24 hours. TNF-α (R&D Systems) standards were prepared from a stock solution (10µg/µl, 40 000 IU/ml) to give solutions of 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.19 IU/ml. 100µl of standard or samples was added to the plate in duplicate. 4µl of actinomycin D (0.1 mg/ml) (Sigma) was added to each well to give a final concentration of 2µg/ml in each well, in order to sensitise the cells to TNF-α. The plate was incubated for 24 hours as described. The cells were washed in 200µl of PBS and fixed for 15 minutes in 200µl of methanol. The methanol was removed and the plate air-dried in a fume hood. To each

well, 200µl of crystal violet (0.1% solution in 200mM boric acid) was added and the plates allowed to incubate for 20 minutes. The plate was washed thoroughly three times with water. To solubilise the cell layer, 50µl of 10% glacial acetic acid was added and the plate incubated for 30 minutes. The absorbance at 570nm was then read on an automatic plate reader (Wallac). The higher the absorbance the less TNF-α was present in the samples. The amount of TNF-α in the samples can be calculated from the standard curve. This analysis was carried out with the assistance of Dr Susan Laird.

2.9 Anthropometric Measures

2.91 Calculation of Body Mass Index (BMI)

BMI was calculated from the equation;

$$BMI = \text{Weight in kg} / \text{Height in m}^2$$

Weights and heights in imperial measurements were converted to metric as required.

2.92 Assessment of body composition

Body composition (lean and fat masses) were assessed using dual energy X-ray adsorpiometry (DEXA) scanning, by qualified personnel at the Northern General Hospital, Sheffield, UK.

2.93 Assessment of testicular volume

Testicular volume was assessed by an experienced paediatrician using an orchidometer.

2.10 Statistical and Other Data Analyses

Statistical analyses were performed using either Microsoft Excel™ or SPSS™ software packages.

2.10.1 Hardy-Weinberg equilibrium

To calculate whether the observed genotypes are those expected from the observed allele frequencies the Hardy-Weinberg equation was used. The equation:

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

(Where p = frequency of common allele and q = frequency of rare allele.) The equation gives the expected frequency of homozygotes (p^2 and q^2) and heterozygotes (2pq). If the observed genotype distribution is not significantly different from the expected genotype distribution, then the genotypes are said to be in Hardy-Weinberg equilibrium.

2.10.2 Chi- squared (χ^2) analysis of contingency tables and Yates' correction

The χ^2 analysis allows a measure of the extent to which observed numbers in a contingency table depart from the expected values. Tables of the χ^2 distribution then show if the observed value is larger than would be expected by chance on a Null hypothesis that postulated no difference between observed and expected distributions. The value of χ^2 is given by the equation:

$$\chi^2 = \Sigma (o-e)^2/e$$

where o = observed number

and e = expected number

The number of degrees of freedom are calculated as the product of one less than the number of rows and one less the number of columns. χ^2 tables were then used to generate a p-value.

A simple method to calculate χ^2 can be employed using a 2x2 contingency table (Table 4).

		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 2.4 An example of a 2x2 contingency table.

χ^2 can then be calculated from this equation:

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

When the values in a 2x2 contingency table are small, a correction for continuity, known as Yates' correction, can be applied. It is common to use Yates' correction when the total numbers in the contingency table are less than 100 or when one cell value is less than 10.

The modified χ^2 analysis is thus;

$$\chi^2 = [(| ad - bc |) - 0.5 (a + b + c + d)]^2 (a + b + c + d) / (a + b) (c + d) (b + d) (a + c)$$

χ^2 tables are then used to determine the level of significance.

2.10.3 ANOVA and the F-test

The F test allows the variance between two populations to be compared. The f test was used to compare the variance between different populations. Analysis of variance (ANOVA) is a method of testing the null hypothesis that several group means are equal in the population by comparing the sample variance estimated from the group means to that estimated within the groups. ANOVA is an extension of the two-sample t test. Once the difference between means has been established, post hoc tests can be performed in order to find exactly where the differences lie. Bonferroni correction is a multiple comparison post hoc test, it allows the observed significance to be adjusted for the fact that multiple comparisons are being made. ANOVA was used to test for differences in group where a population contained more than two groups.

2.10.3 t test and the Mann-Whitney U test

The t test was used to test the difference in means between two populations. Different types of t test are used, depending on the sample populations; a paired t test is used to test paired data, a t test assuming equal variance was used when the sample data had an equal variance. The Mann-Whitney U test is the non-parametric equivalent to the t test and it is able to test whether two independent samples are from the same population. This test was used when the data was not normally distributed.

2.10.4 Pearson and Spearman Rank Correlations

The degree of association is measured by Pearson's correlation coefficient; *r,* which is a measure of linear association. Pearson's correlation was used on normally distributed data, to determine linear relationships between variables. When the data set was either not normally distributed or when outlying points of data away from the main data set were present, Spearman Rank correlations were utilised. This correlation is a non-parametric test and replaces the observations with their ranks in the calculation of the correlation coefficient.

2.10.5 Regression Analysis

Linear regression estimates the coefficients of the linear equation involving one or more independent variables that best predict the value of the dependent variable. Linear regression was used to analyse how a single dependent variable was affected by the value of one or more independent variables. Logistic regression was used to predict the presence or absence of an outcome based on several predictor variables. Logistic regression is suited to data sets where the dependent variable is dichotomous. Stepwise multiple regression analysis was used to determine which of the possible explanatory variables could be used to predict an outcome.

2.10.6 Scatchard Analysis of Receptor-Ligand Interaction

Estimation of binding characteristics from a curve is often not very meaningful, therefore manipulation of the data to provide a linear relationship is preferred, one method by which this can be achieved is Scatchard Analysis. The Scatchard plot is drawn by dividing the concentration of bound ligand by free ligand and plotting this against the concentration of bound ligand eg.

$$\frac{[\text{Bound Ligand}]}{[\text{Free Ligand}]} \text{ Vs } [\text{Bound Ligand}]$$

From this plot the dissociation constant (K_d) can be obtained as the slope of the line will be:

$$\frac{-1}{K_d}.$$

Figure 2.2 illustrates a typical Scatchard plot, showing extrapolation of the slope to the x-axis. In this thesis, the Scatchard analysis was performed using LIGAND-EBDA software version 2.0 (Elsevier-Biosoft, Cambridge, UK.).

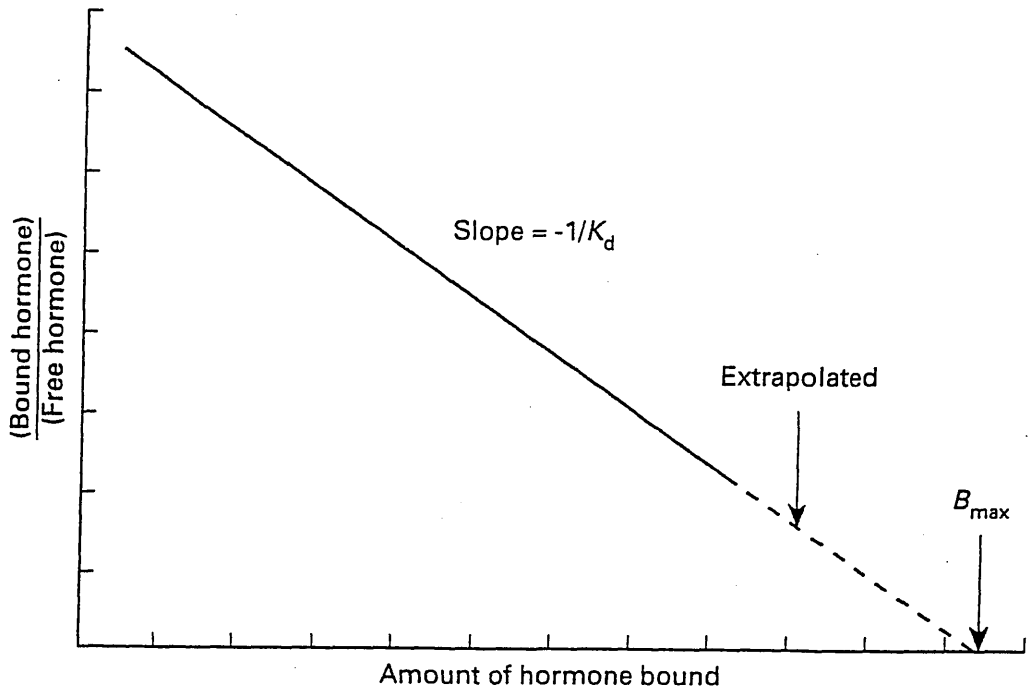


Figure 2.2 A typical Scatchard plot (taken from Hancock, 1997).

2.11 Equipment and reagent suppliers

Alexis Biochemicals

Alexis Corporation (UK) Ltd.

3 Moorbridge Court,

Moorbridge Road East,

Bingham,

Nottingham,

NG13 8QG,

Bio-Rad Laboratories Ltd.,

Bio-Rad House,

Marylands Avenue,

Hemel Hempstead,

Hertfordshire, HP2 7TD.

Biosource Europe,

Lifescreen Ltd.

Unit 15, The Metro Centre,

Tolpits Lane,

Watford,

Herts, WD1 8SS.

Gibco BRL,

Life Technologies Ltd,

3 Fountain Drive

Inchinnan Business Park,

Paisley, PA4 9RF.

ICN Pharmaceuticals Ltd,

Biomedical Research Products,

1 Elmwood,

Chineham Business Park,

Crockford Lane,

Basingstoke,

Hants, RG2 8WG.

Linco Research Co.,

St Louis,

Mo.

USA.

NEN Life Science Products,

Hounslow,

London,

TW5 9RT.

New England Biolabs (UK) Ltd,

67 Knowl Piece,

Wilbury Way,

Hitchin,

Hertfordshire, SG4 0TY.

Packard,

Brook House,

14 Station Road,

Pangbourne,

Berkshire,

RG8 7AN.

Pharmacia Biotech,

Pharmacia LKB Biotech Ltd,

Science Park,

Milton Road,

Cambridge,

CB4 4FJ.

Promega UK,

Enterprise Road,

Chillworth Research Centre,

Southampton,

SO16 7NS.

R&D Systems,

R&D Systems Europe, Ltd.

4-10 The Quadrant,

Barton Lane,

Abingdon,

Oxon, OX14 3YS.

Sigma,

Sigma Aldrich Company Ltd.

Fancy Road,

Poole,

Dorset, BH12 4QH.

Sorvall,

Du Pont (UK) Ltd.

Srvall Products,

Wedgwood Lane,

Stevenage,

Herts, SG1 4QN.

Chapter 3

Leptin Binding Activity: Assay Development and Validation

3.1 Introduction

Circulating soluble receptors are a feature of a number of members of the cytokine family (Muller-Newen *et al.*, 1996), including IL-1, IL-6, TNFα and growth hormone (GH). Soluble receptors have a wide range of functions. These functions can include actions as a scavenger, a carrier, as an agonist, as an antagonist and for protection against cell degradation. Soluble receptors can be generated in two ways; firstly the extracellular domain of the receptor can be proteolytically cleaved off the cell surface and transported in the blood or around the interstitial fluid, this is known as shedding. Secondly, the receptor can be expressed as an alternately spliced transcript of the gene. The most widely studied with respect to the endocrine system is the growth hormone binding protein (GHBP). GHBP was first described by Ymer and Herington (1985) and has since been well characterised. In rats and mice, the generation of GHBP involves an alternatively spliced mRNA, whereas in rabbits, it is derived by proteolytic cleavage, from the extracellular domain of the growth hormone receptor. It is likely that this is the same mechanism in humans (Dastot *et al.*, 1996, Ross *et al.*, 1997) as no mRNA expression of the GHBP has been found in humans.

The receptor serves as a carrier protein in the case of soluble interleukin-4 receptor (sIL-4R) (Vitetta, 1991). For TNF-α bound to its receptor, the complex retains its bioactivity for a longer length of time. This complex protects against proteolysis and degradation.
Soluble receptors can also act as antagonists. Soluble leukaemia inhibitory factor receptor (sLIF-R) has been shown to antagonise the action of LIF (Layton, 1992) and the soluble receptor for interferon γ (IFNγ) neutralises the antiviral activity of IFNγ (Gentz *et al.*, 1992).
Soluble receptors can also have agonistic effects. This is the case for the IL-6 and soluble IL-6 receptor (sIL-6R) complex. This complex can activate the Jak/STAT signalling cascade as it interacts with the gp130 accessory chain, even in the presence of membrane bound receptors (Hibiet *et al*, 1991)
In disease situations, levels of soluble receptors can fluctuate. Soluble ciliary neurotrophic receptor (sCNTF-R) is increased in the CSF after nerve injury from 10 ng/ml to 500 ng/ml. Soluble TNF-Receptor II (sTNF-RII) is increased 2-3 fold on normal levels with malignancies. Soluble interleukin 1 receptor II (sIL-1-RII) is increased in the synovial fluid from rheumatoid arthritis patients. It has been reported

that sIL-6R levels are increased after infection with human immunodeficiency virus (HIV) (Handa *et al*, 1991). The most work has been performed on soluble IL-2 receptor (sIL-2R), where levels have been demonstrated to be increased after infection by virus (HIV), bacteria (tuberculosis) or parasite (malaria). In many autoimmune diseases, levels of sIL-2R are also increased.

It has been postulated that there may be similar binding proteins present for leptin. Recent reports have identified binding proteins for leptin in human serum (Houseknecht *et al*, 1996, Sinha *et al*, 1996, Diamond *et al*, 1997) including a soluble form of the leptin receptor, Ob-Re (Diamond *et al*, 1997). The leptin-binding proteins are not yet well characterised and the full role of the proteins, including the soluble receptor has yet to be elucidated. A possible role for the soluble leptin receptor is as a mechanism for carrying leptin to the blood-brain barrier (Golden *et al.*, 1997) although there is potential for this soluble receptor to act as an antagonist or agonist.

Amit *et al.,* (1990) developed a method for the measurement of GH-BP. This method involved the incubation of serum, in a phosphate buffer with magnesium chloride and ^{125}I-GH, then separation of the bound radioactivity from the free using a 2% charcoal, 0.2% dextran T-70 slurry. This method was the basis for the development of the leptin-binding assay.

Radioligand binding experiments are based on a simple model, the law of mass action. For ligand receptor interactions the law can expressed as:

Ligand + Receptor ⇔ Ligand.Receptor

The law of mass action is based on four simple assumptions:

1. Binding occurs when ligand and receptor (or binding proteins) collide due to diffusion, this collision must be in the correct orientation and have enough energy.
2. Once bound, the ligand and receptor remain bound together for a time influenced by the affinity for the ligand and receptor for one another.
3. After dissociation, the ligand and receptor are the same as before binding, therefore there is no irreversible conformational change on binding.
4. Equilibrium is reached when the association rate equals the dissociation rate.

When the ligand-receptor system is at equilibrium, complexes form at the same rate they dissociate. Chilling of all reagents used in the binding assay will prevent this binding equilibrium being disrupted.

3.2 Development of the assay

Modification of the basic method from measuring GHBP to leptin binding activity required a number of factors to be taken into consideration. The full method for the measurement of LBA can be found in Chapter 2, but in outline, involves incubation of the samples with a radioactive leptin in the presence or absence of cold (unlabelled) leptin. After incubation, the bound and free fractions are separated by the addition and incubation with charcoal dextran slurry, followed by chilled centrifugation. The supernatant contains the bound leptin and it is this fraction that is counted on a γ-counter. After determination of incubation conditions and suitable sample types, the most important consideration in the development of this assay is the possible interference of the endogenous levels of leptin. It is known that most obese subjects have high levels of leptin, reflecting their increased fat mass, and this excess could cause interference in the assay. In addition, it is possible that levels of leptin from normal weight subjects would interfere with the assay, by exerting some effect on the binding equilibrium. Removal of the endogenous levels of leptin from all samples allowed standardisation of the samples.

The approach taken to remove the endogenous leptin, was the same as to separate the bound from the free fractions. Incubation on ice with dextran-coated charcoal followed by centrifugation was employed. We believed this to be the most effective method of removing the leptin. The charcoal used in the assay was Norit A charcoal, these are steam activated carbon particles that are micronized so the final charcoal is in the form of extremely fine particles. Dextran-T70 is added to the charcoal slurry for two reasons:

1. It renders the charcoal stickier and therefore easier to pellet by centrifugation.
2. It acts as a barrier preventing large molecules (in this case the binding proteins) from being adsorbed to the charcoal, yet allows the small molecules to be removed.

In order to minimise loss of the binding proteins during this stripping step, the samples were incubated on ice, buffers were chilled, and the separation by centrifugation was performed at 4°C. It is possible that if the solutions were not chilled, the charcoal would absorb the binding proteins themselves.

Experiments were carried out in order to validate that the results of the assay reflected the physiological amount of serum leptin binding activity. This was achieved by:

- Homologous competitive binding curves and Scatchard analysis.

- Confirmation of the assay's specificity for leptin-binding activity.
- Confirmation of the reproducibility of the assay.
- Comparison of the LBA assay with HPLC analysis.

3.3 Selection of Assay conditions

3.3.1 Investigation of the incubation time of sample with radioactive ligand

3.3.1.1 Experimental Design

It was important that binding equilibrium had been reached when the samples were separated and the binding proteins measured. In order to ascertain the optimum time for equilibrium to occur at 4°C, the length of incubation time with radioactive ligand was investigated.

3.3.1.2 Study Design

A sample was thawed and the assay set-up as described in Chapter 2.

The incubation time with the radioactive ligand was varied. Incubation times of 10 minutes, 30 minutes, 1, 2, 4, 24 and 48 hours were used. Samples were assayed in duplicate.

3.3.1.3 Results

There was no significant binding of the radioactive ligand to the binding protein until an incubation of time of 4 hours was reached. At 24 hours, maximal binding was reached. Samples were incubated for 48 hours, but there was no further significant increase in LBA level, as binding equilibrium had been reached.

Time of Incubation	LBA (%)
10 minutes	0.0
30 mintes	0.4
1 hour	0
2 hours	0
4 hours	9.5
24 hours	10.8
48 hours	10.7

Table 3.1 Effect of variation of incubation time on ligand binding (n = 4 for each time point)

3.3.1.4 Conclusion

Binding equilibrium was reached by 24 hours incubation at 4°C and, this time point was chosen for the assay. Binding equilibrium would be reached more quickly at 20°C and 37°C, but these temperatures were considered unsuitable, as the separation technique requires the samples and buffers to be chilled. At these higher temperatures, there may have been changes in the binding equilibrium causing the equilibrium to be less stable. At 37°C, increases in proteolytic enzyme activity would have needed to be addressed. Therefore, an incubation time of 24 hours at 4°C was chosen as the optimum incubation time.

3.3.2 Selection of sample type

3.3.2.1 Experimental Design:

In this study, serum samples were used. It would be useful, however, to use samples that had been treated with anti-coagulants. This would allow samples collected for other purposes than for LBA measurement to be utilised. Comparison of LBA measurements in blood with and without anticoagulants was carried out. Whole blood was collected and the samples taken from the same venepuncture were placed into sterile tubes and a portion of the sample was treated with two different anti-coagulants. All samples were subjected to the same conditions. The serum samples were allowed to clot for 30 minutes at room temperature, the plasma samples remained on the benchtop. The serum and plasma were separated from the cellular components by centrifugation at 1000g. The samples were aliquotted and frozen at -20°C until required. The samples were

stripped of their endogenous leptin and LBA measured as described in Chapter 2. The samples were assayed in duplicate and the measures repeated twice. Means of these repeated measures are shown.

3.3.2.2 Results

	Serum Sample	Plasma Samples	
Subject		**EDTA as anti-coagulant**	**Lithium Heparin as anti-coagulant**
A	10.0	9.4	10.1
B	11.5	11.9	11.6
C	7.8	7.2	7.6
D	7.6	8.2	7.6
E	9.6	9.5	8.9

Table 3.2 The effect of sampling conditions on LBA (%).

3.3.2.3 Conclusions

There was no significant difference in levels of LBA measured in samples that had been treated with anti-coagulants and samples that had not been treated with anticoagulant. Serum and plasma samples appear to both be suitable for the measurement of LBA.

3.4 Stripping the serum of endogenous leptin for the measurement of LBA

3.4.1 The effect of incubation time with dextran-coated charcoal on stripping the serum of leptin

3.4.1.1 Assay Design

The same sample was subjected to variation of incubation time with 2 % charcoal-0.2 % dextran T-70 on ice. Seven time points were used: 1, 2, 5, 10, 15, 30 and 60 minutes. A single sample was incubated with the charcoal dextran slurry for 24 hours. LBA and leptin levels (assessed by the RIA method) were measured in these samples. Samples were assayed in duplicate.

3.4.1.2 Results

Time of Incubation (minutes)	LBA (%)	Leptin (ng/ml)
1	7.3	2.6
2	8.5	1.8
5	11.5	<1
10	10.5	<1
15	10.5	<1
30	10.3	<1
60	8.3	<1
24 hours	3.4	<1

Table 3.3 Effect of prolonged stripping on leptin and LBA (n = 4).

3.4.1.3 Conclusions

The results of this experiment show that for the maximum LBA and the minimum leptin level (undetectable) an incubation time of 5 minutes was sufficient. After 15 minutes incubation, there was a reduction in the level of LBA. After 24 hours, the levels of LBA were greatly reduced, it is likely that the binding proteins in the serum sample had been adsorbed to the charcoal. An incubation time of 5 minutes was chosen as the optimal time for incubation to retain the most binding protein and to minimise the level of residual leptin.

3.4.2 The effect of stripping serum of endogenous leptin in a cohort of men and women

3.4.2.1 Subjects

Subjects were recruited from the Division of Clinical Sciences laboratory at the Northern General Hospital. Basic clinical information was collected; age, height, weight and sex. The subjects included both men and women, aged between 20 and 50 years with a range of BMIs.

3.4.2.2 Sample Preparation

Venous blood was collected without anti-coagulant and was allowed to clot for 30 minutes at room temperature. Following centrifugation for 10 minutes at 1000g (Sorvall RT 6000D); the serum was removed and frozen in 500µl aliquots until required.

3.4.2.3 Experimental Design

Two aliquots of serum from each subject were thawed. One aliquot was stripped of its endogenous leptin as described in Chapter 2. The other aliquot was used without pre-

treatment in the assay. Both the stripped and unstripped serum for each subject was assayed for leptin by RIA.

Subject	BMI (Kg/m^2)	LBA (%)		Leptin (ng/ml)	
		Neat Serum	Stripped Serum	Neat Serum	Stripped Serum
1	26.3	11.2	11	6.1	<1
2	19.4	14.3	10	1.9	<1
3	22.2	10.2	6	12.6	<1
4	22.4	8.3	7.8	2.9	<1
5	23.1	9.8	11.5	<1	<1
6	21.4	9.8	8.5	3.4	<1
7	23.9	11.7	4.5	4.3	<1
8	21.6	10.9	8.3	1.9	<1
9	26.3	9.5	7.5	3.7	<1
10	23.0	5.3	5	2.1	<1
11	27.7	5.7	4.8	4.6	<1
12	24.0	5.2	4	2.6	<1
Mean levels	***23.4***	***9.3***	***7.4***	***3.8***	***<1***

Table 3.4 Levels of LBA and leptin, in stripped and unstripped serum, in male subjects

Subject	BMI (Kg/m^2)	LBA (%)		Leptin (ng/ml)	
		Neat Serum	Stripped Serum	Neat Serum	Stripped Serum
1	21.5	12.8	10.5	16.1	<1
2	22.6	5.5	10	18.6	1.3
3	22.6	12.5	12.5	11.5	1.5
4	24.3	9.4	16.5	39.2	<1
5	20.9	8.8	6.5	8.8	1.0
6	23.4	12.9	15.3	8.7	1.0
7	28.0	8.2	9.3	16.7	<1
8	22.2	17.9	16.5	8.3	<1
9	20.2	9.8	11.3	6.1	<1
10	33.0	5.3	12.8	40	<1
11	25.6	8.9	8.3	10.2	<1
12	45	4.4	10.8	106	1.2
Mean levels	***25.8***	***9.7***	***11.7***	***24.2***	***<1***

Table 3.5 Levels of LBA and leptin, in stripped and unstripped serum, in female subjects.

The mean serum leptin level was significantly lower in the male subjects than in the female subjects (t test, $p = 0.02$). After stripping the serum, leptin was undetectable in 19 of the 24 samples. A paired t test was used to assess the difference between LBA in the stripped and unstripped samples. The difference in LBA was not significant in the male subjects. In some subjects, the levels of LBA were reduced. In females, 8 of the 12 subjects LBA increased after stripping the serum of its endogenous leptin. The increase in the stripped serum was most pronounced in subjects with high levels of leptin. The hypothesis that endogenous leptin might interfere with the LBA assay seems true in the case of women with high levels of leptin. Male subjects with increased levels of leptin were not available and, therefore, this observation was not made in male subjects.

3.4.2.4 Conclusion

Stripping the serum of the endogenous level of leptin was adopted in the method for LBA. All samples from both males and females were stripped of their endogenous leptin to normalise the variation in leptin level.

3.5 Validation of the assay

3.5.1 Homologous competitive binding curves and Scatchard Analysis

3.5.1.1 Study design

In order to investigate the LBA assay and assess its validity, a competitive binding assay was performed. This assay used the same compound for the hot and cold ligand and therefore, is known as a homologous competitive binding experiment. Serum was stripped as described in chapter 2 and was used either undiluted or diluted 1:1, 1:2, and 1:3 with sterile water. These samples were incubated with radioactive ligand and increasing amounts of cold (unlabelled) leptin (0-10μg/tube). This analysis was performed, to answer two questions about the actual binding that occurred in the assay. Firstly, could the radioactive leptin be displaced from the binding site by increasing amounts of unlabelled leptin? Secondly, would the amount of binding activity be reduced when the sample had been diluted?

Scatchard analysis is a method of analysing ligand-receptor interactions. A Scatchard plot is a graph of the amount of radioligand bound divided by the amount of radioligand free in the solution (B/F) (on the y-axis) versus the amount of radioligand bound (Bound) (on the x-axis). For the Scatchard analysis, samples were stripped of their endogenous leptin and incubated with increasing amounts of cold leptin (0-10μg). The amounts of leptin were calculated in pMolar (0-600pM) and Scatchard analysis performed using LIGAND-EBDA software version 2.0 (Elsevier-Biosoft, Cambridge, UK).

3.5.1.2 Results

The addition of increasing amounts of cold leptin resulted in more radioactive ligand being displaced (Figure 3.1). The diluted samples followed a parallel pattern each with successively lower counts, reflecting a differing amount of binding protein.

Scatchard analysis revealed a single binding species, with high affinity typical of a hormone or cytokine receptor (Gammeltoft and Kahn). The binding affinity was 1.0 – 1.4 x 10^9 M^{-1}. (Figure 3.2)

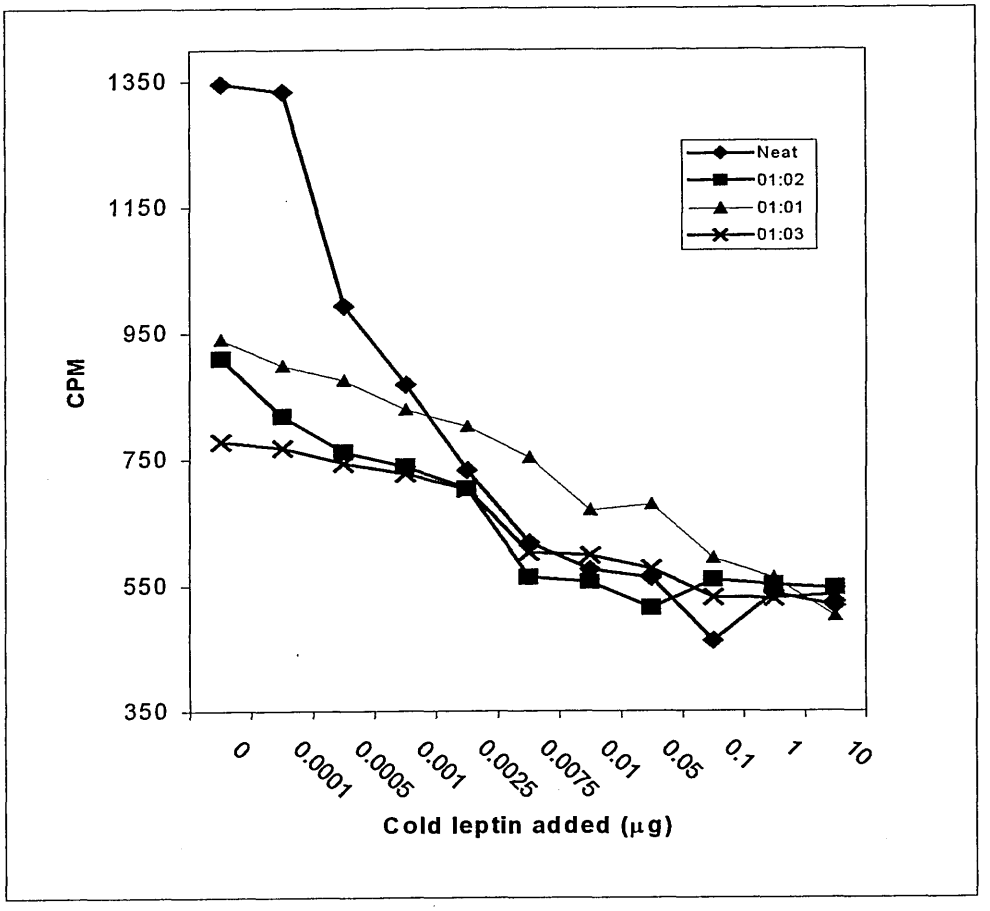


Figure 3.1. Competitive binding curve and parallel displacement of leptin in the LBA assay.

3.5.1.3 Conclusion

The competitive binding experiment provides important information about the LBA assay. Firstly, the amount of dilution is inversely proportional to the counts seen, indicating the binding protein can be diluted away. This suggests that we are measuring a real leptin-binding activity and not a random event. Secondly, increasing the amount of unlabelled leptin added to the assay results in more radioactive ligand being displaced. This indicates that there is increased competition for the binding site.

Scatchard analysis revealed a high affinity ($1.0 - 1.4 \times 10^{-9}$ M^{-1}) binding protein which would be compatible with the expected affinity of the soluble receptor (Liu *et al.*, 1997). The Scatchard analysis appeared to detect only a single species of binding protein, although lower affinity binding proteins may not be detected by this method. It is usual to only see a single binding species, unless the affinities of the two sites are extremely different, or if one of the binding sites is in great excess. In summary, this experiment proves that the binding of radioactive ligand to the binding protein in serum is true. It can be removed with dilution and it is displaced when large amounts of unlabelled leptin are added. Scatchard analysis revealed a single species of binding protein, with high affinity for the ligand. Using charcoal-dextran slurry as the separation method is likely to leave only high affinity binding sites in solution.

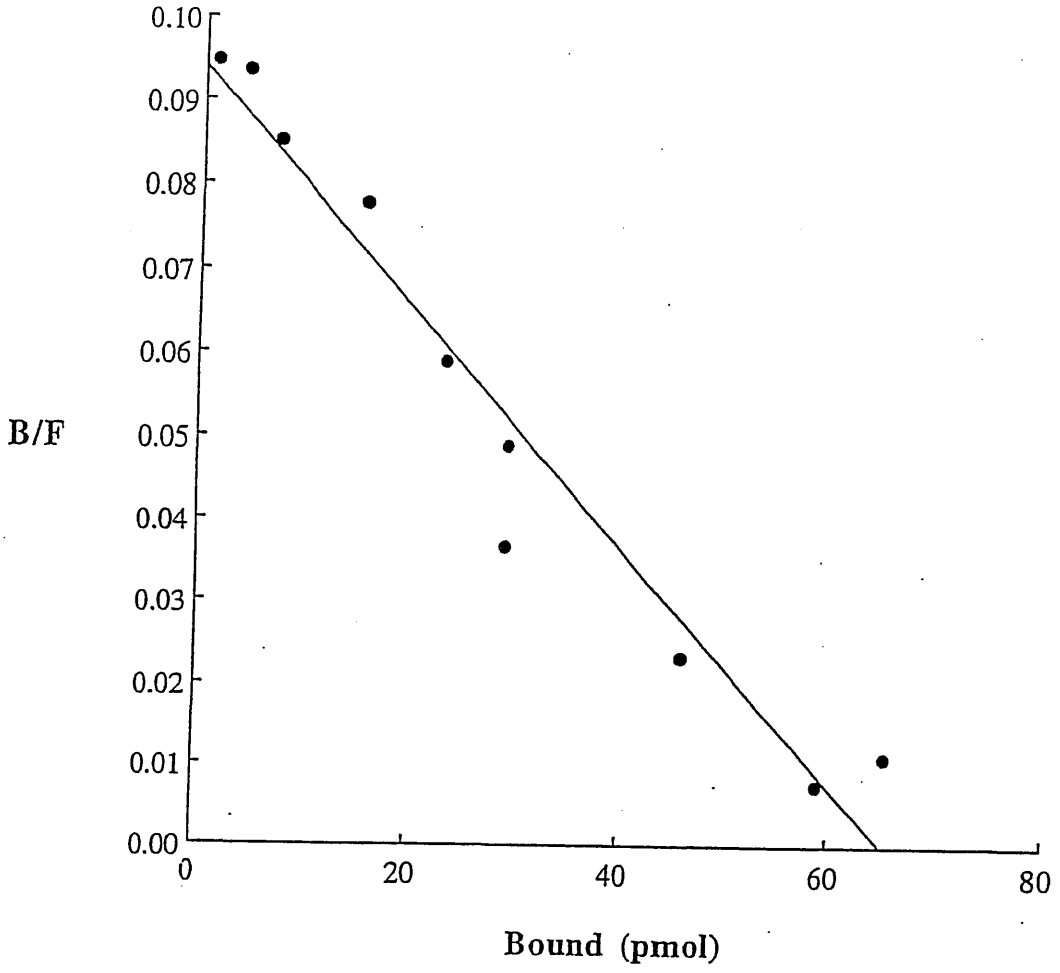


Figure 3.2 Scatchard analysis of LBA in normal human serum. This analysis revealed a high affinity single species of binding protein (1.0-1.4 x 10^9 M^{-1}).

3.5.2 Confirmation of the specificity of the assay for leptin

3.5.2.1 Study Design

Reactions were set up as for the Scatchard analysis, but using increasing amounts of growth hormone and IL-6 in place of leptin. Growth hormone was chosen as it is structurally similar to leptin and is of a comparable size. IL-6 was chosen, as the leptin receptor is structurally similar to the IL-6 receptor family. Samples were assayed in duplicate. Means of these two measures are shown.

3.5.2.2 Results

There was no displacement of the radioactive leptin by either growth hormone or IL-6. (Figure 3.3).

3.5.2.3 Conclusion

The binding species being assessed by the LBA assay appears to be specific for leptin. IL-6 and GH, similar hormones and cytokines, are not able to displace the bound ligand.

3.5.3 Reproducibility and sensitivity of the LBA assay

3.5.3.1 Study Design

In order to assess the reproducibility of the assay, two samples were chosen. The first sample, A, was taken from a normal weight female (BMI = 24.3 Kg/m^2) with a leptin level of 12 ng/ml. The second sample, B, was taken from an obese female subject (BMI = 45.0 Kg/m^2) with a leptin level of 106 ng/ml.

To assess intra-assay variability, 10 aliquots of the same samples were assayed together. The inter-assay variability was continually assessed with each batch of samples. With each batch of samples (n=15), both the A and B samples were assayed alongside. From these collective measurements, the inter-assay variability could be calculated. The assay sensitivity was assessed using 10 repeated measures of a sample not containing any serum leptin-binding proteins. Two zero samples were assayed. Firstly assay buffer was used as the zero samples, as it contained protein (albumin), but not binding proteins to leptin. Secondly, an aliquot of serum was heated to 80°C for 30 minutes and used as a zero sample, this would allow degradation of protein in the serum. This sample would show the limit at which, we could expect to measure LBA. Coefficients of variation were calculated (standard deviation / mean x 100, expressed as a percentage). The CV expresses the standard deviation as a percentage of the mean value and provides a simple appreciation of the precision (reproducibility) of the assay.

3.5.3.2 Results

The sensitivity of the assay was 0.6% ± 0.44 (mean ± 2SD). Binding activity greater than this level can be attributed to leptin binding proteins being present in the sample. Sample A had a specific binding of 12%, with an intra-assay coefficient of variation (CV) of 3.2 % and an inter-assay variation CV of 6.4%. Sample B had a specific binding of 6% with an intra-assay coefficient of variation (CV) of 4.1 % and an inter-assay variation CV of 4.8%.

3.5.3.4 Conclusions

The LBA assay is a reproducible assay. For assays that use dextran-coated charcoal as the method of separation, a CV of less than 10% is considered appropriate. This assay has intra and inter-assay CVs for this assay are less than 10% and therefore are an indication of a highly reproducible assay.

A

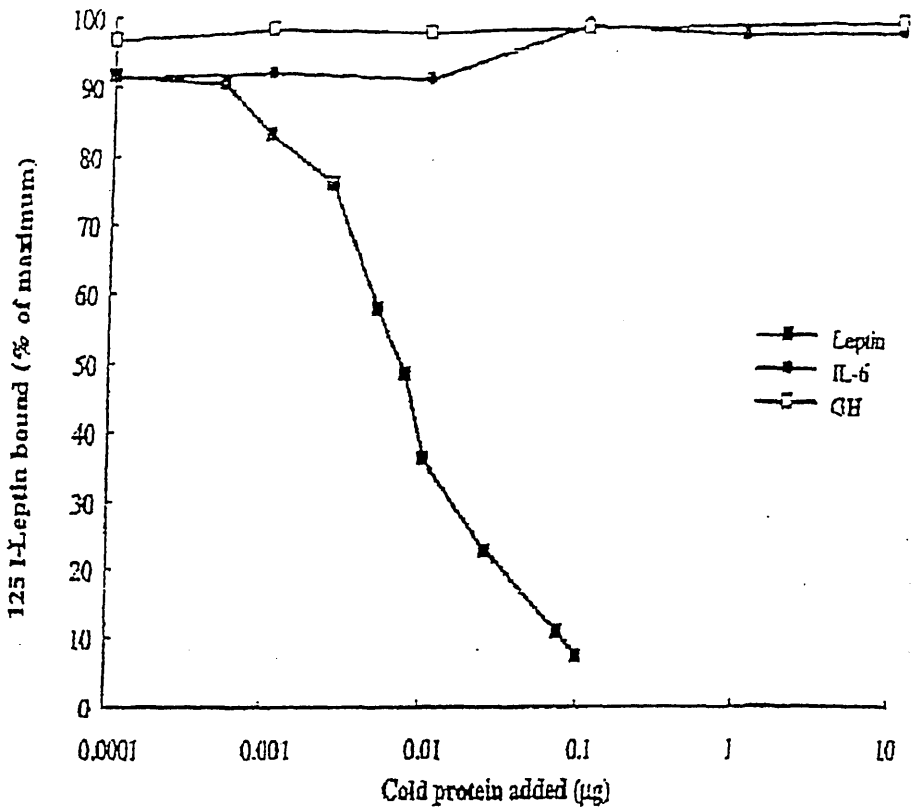


Figure 3.3 Specificity of binding of ^{125}I-Leptin to normal human serum. The specific binding of ^{125}I-Leptin is expressed as a percentage of the maximal binding. Growth hormone and IL-6 were used in place of leptin in the competitive binding assay. Values are the mean of duplicate determinations from a representative experiment.

3.5.4 HPLC Analysis and comparison with the LBA assay

3.5.4.1 Study Design

Samples that had been previously used to investigate the effect of stripping the serum of endogenous leptin were utilised here. Samples were stripped of their endogenous leptin and used unstripped. Eight samples were analysed by the HPLC method described in Chapter 2. This analysis was carried out by Miss Sylvie Simon and Dr Marie-Catherine Postel-Vinay at the Faculte de Medicine Necker in Paris, France. HPLC analysis by Dr Postel-Vinay was utilised as a comparison to the LBA method. The measurement of GHBP is well established in their laboratory.

3.5.4.2 Results

Results from the LBA assays were compared with those obtained by HPLC. A representative profile is shown in Figure 4. HPLC revealed a single peak at the appropriate size for the soluble form of the leptin receptor (~85 kDa). Results of specific binding were comparable considering the different methodology used for the eight samples analysed.

After 10 minutes, a peak eluted off the column. This peak of radioactivity corresponds to the high affinity leptin-binding species seen in the LBA assay. The addition of cold leptin to the reaction mixture caused this peak to be attenuated. The second peak that eluted from the column, after 20 minutes, is the free radioactive leptin. As the large molecules are eluted from the column first, we can be sure that no further peaks will be seen after the large peak of free radioactivity (see discussion re α-2-macroglobulin). There was no detectable measure of radioactivity before 10 minutes.

Sample	Specific Binding (%)	
	LBA	**HPLC**
1	8.1	6.3
2	9.7	8.1
3	6.5	11.7
4	10.5	8.8
5	9.3	7.2
6	12.5	10.4
7	16.5	12.6
8	10.5	10.3
Mean ± SEM	**10.5 ± 1.07**	**9.4 ± 0.78**

Table 3.8. Comparison of results from LBA assay and HPLC analysis.

3.5.4.3 Conclusion

The results from the LBA assay were comparable with the results from the HPLC analysis, confirming the validity of the data. A single peak is seen on HPLC analysis and this compares well with the Scatchard analysis which defined a single site high affinity binding protein. This analysis confirms that the LBA assay is measuring a defined quantity of binding protein that is easily comparable using other methods.

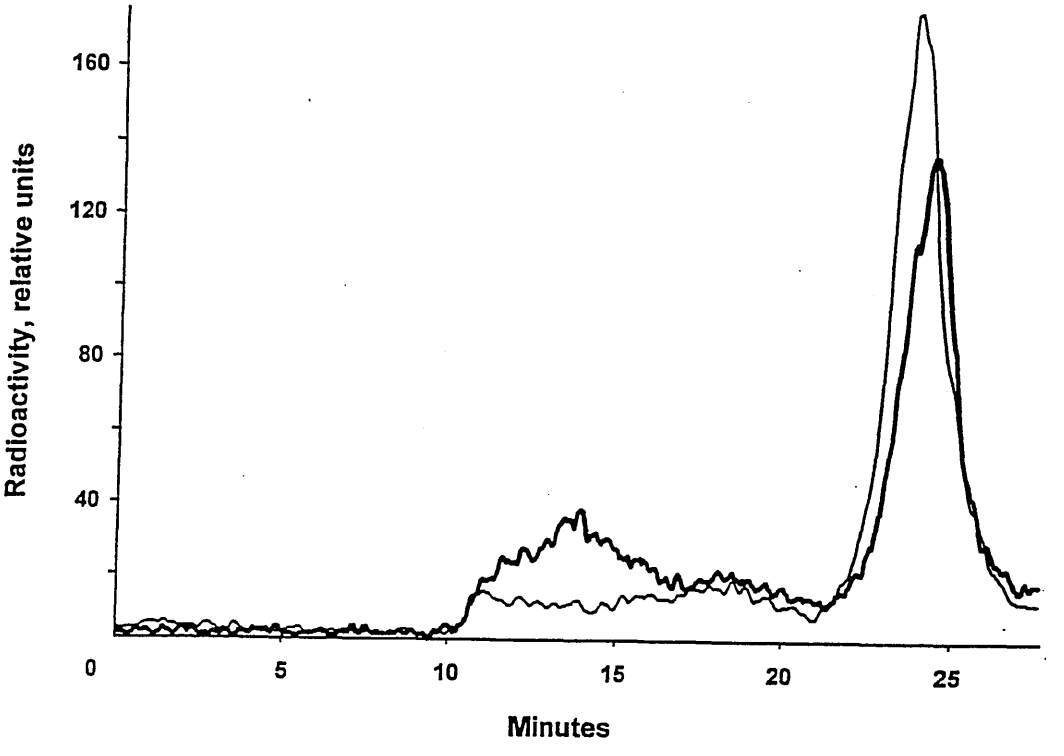


Figure 3.4 Elution profile of ^{125}I-Leptin incubated with human serum. ^{125}I-Leptin (4 x 10^4 cpm) was incubated with 150µl of serum without (thick line) and with (thin line) excess of native leptin. Binding was expressed as a radioactivity in the first peak over the radioactivity in peak 1 and peak 2 (free ^{125}I-Leptin). For the profile shown, total binding was 29.0% and non-specific 13.1% radioactivity.

3.6 Conclusion

The leptin-binding activity assay is a specific, sensitive, and reproducible method for assessing leptin-binding protein in human serum. It was necessary to optimise the assay specifically for leptin binding protein and to validate the results achieved using the assay. Serum samples were stripped of their endogenous leptin to lessen the interference from samples with high levels of leptin. Investigation of sample type revealed that serum or plasma samples (using EDTA or lithium heparin as the anti-coagulant) were appropriate samples for the measurement of LBA in this manner. Validation experiments were able to confirm that:

- The assay is specific for measuring LBA and not binding of other hormones (growth hormone) or cytokines (IL-6).
- A single species of high affinity ($1.0 - 1.4 \times 10^{-9}$ M^{-1}) binding activity was observed.
- The assay is reproducible.
- HPLC analysis also showed a single binding species and this is comparable with data gained from the LBA assay.

At present, exactly what the high affinity binding-protein(s) are has not been elucidated. Previous reports have indicated that at least a portion of the binding activity is attributable to a soluble form of the leptin receptor (Sinha *et al*, 1996, Houseknecht *et al*, 1996). Reports have identified binding proteins from 85 to 240 kDa in size (Houseknecht *et al*, 1996), with a putative soluble receptor reported to be around 85-90 kDa (Liu *et al*, 1997). Some workers have reported in-house ELISA assays for the soluble isoform of the leptin receptor (Lewandowski *et al*, 1998). Diamond *et al*, (1997) described a method for measuring leptin-binding proteins using gel filtration. They observed a number of bound components, including a large protein with a high molecular mass (~450 kDa). Birkenmeier *et al* (1998) have recently reported that leptin is bound to α-2-macroglobulin in serum. α-2-macroglobulin is a major proteinase inhibitor in human blood and tissue (Birkenmeier and Kunath, 1996). It is a large glycoprotein, with a molecular weight of approximately 450 kDa and has the capacity to bind cytokines with low affinity (Mather, 1996). The action of α-2-macroglobulin can be as a carrier, as in the case of transforming growth factor-β (TGF-β) (Feige *et al.*, 1996) or α-2-macroglobulin can assist in sequestering TNF-α, IL-6 and IL-2 after oxidative stress (Wu *et al.*, 1998). Wu *et al.* (1998) postulate that this process allows

differential regulation of cytokines during inflammatory responses such as those that occur in rheumatoid arthritis. Kratzsch *et al*, (1995) identified that growth hormone is also bound to α-2-macroglobulin in serum. It is possible that a portion of the LBA is attributable not only to a soluble form of the leptin receptor, but also to α-2-macroglobulin.

The development of the LBA assay to this stage permits its use in clinical situations. As the role of leptin is pleiotropic, there are many situations, both in health and in disease, which would warrant measurement of LBA. After the establishment of a "normal" range throughout life from early infancy to old age, pregnancy would be a next step. It is known that leptin has an important role in reproduction, particularly in females. Soluble leptin receptor is increased during pregnancy in mice; the placenta is a source of leptin and leptin receptor during pregnancy and it would be interesting to assess how important, if at all, LBA was during this process. For disease situations, the most obvious choice for the measurement of LBA would be obesity, cachexia and anorexic conditions. As leptin is known to reflect fat mass and therefore, be at high levels in most obese humans and at low levels in most anorexic individuals, the measurement of LBA would be pertinent. Any observed difference in LBA in these individuals may assist in the understanding of leptin's complex role in body weight regulation. Measurement of LBA in other disease situations such as, diabetes, renal disease and cancer may aid in relating and expanding the role of leptin to other cytokine and hormonal systems.

Chapter 4

Leptin and Leptin Binding Activity Levels During Pubertal Development

4.1 Introduction

Puberty, the transition from child to adult, is the result of reactivation of the pituitary gonadal axis. Sex steroids reach adult levels in late gestation, driving sexual differentiation in the foetus, and then, shortly after birth, the pituitary gonadal axis switches off and lies dormant until the onset of puberty. The metabolic switches that program this biological clock are now being defined. The importance of body composition has been recognised since the 1970s when Frisch suggested that it was necessary for women to maintain a specific percentage of body fat to achieve menarche and fertility (Frisch *et al.*, 1970).

Two recent reports of humans with mutations in the leptin receptor and leptin gene, who failed to progress through puberty, provide important evidence for the role of leptin in facilitating pubertal development (Clement *et al.*, 1998, Strobel *et al.*, 1998). However, the nature of the link between puberty and leptin is not fully established. Cross-sectional and longitudinal studies of leptin levels before and during puberty indicate that leptin levels follow changes in fat mass (Blum *et al.*, 1997, Garcia-Mayor *et al.*, 1997, Clayton *et al.*, 1997). In males, there is an increase in leptin prior to pubertal development (Mantzoros *et al.*, 1997), this lends weight to the hypothesis that leptin is a signal to the reproductive axis to initiate the start of pubertal maturation. In children, age is also an independent determinant of leptin levels and leptin may act as a permissive signal to puberty over time (Clayton *et al.*, 1997).

Marshall and Tanner (1969 and 1970) described in detail the development of secondary sexual characteristics in both girls and boys. From the studies, the Tanner stages of puberty were defined, with breast and pubic hair development the characteristics for girls and genital development the characteristic for boys. These developmental changes act as a marker to allow the assessment of how far through puberty a child is. The length of time from the onset of puberty to maturity varies between boys and girls. Girls usually complete puberty by the age of 14.5 years and boys by the age of 17.5 years. Growth spurts occur at different times in puberty in boys and girls. The female growth spurt begins in early puberty and is virtually complete by the onset of menarche. The male growth spurt occurs in the later stages of puberty, usually two years after the average female increase in growth. Pre-pubertally both boys and girls have an equal

lean, fat and skeletal mass. At maturity, men have one-and-a-half times more lean, skeletal and muscle mass than women; women have twice the fat mass as men.

4.1.2 Subjects included in the study

Neonatal cord bloods (n = 10) were collected within one hour of delivery (by natural delivery or caesarean section) from the labour ward at the Northern General Hospital. Serum was prepared and leptin levels were measured using the human leptin RIA kit (Linco Research Co.). Serum samples obtained from the children during puberty (n = 132) were collected by Dr Peter Clayton at the Endocrine Science Group, University of Manchester. Leptin levels were measured by Dr Matthew Gill using the human leptin RIA kit (Linco Research Co.). Pubertal staging was performed by an experienced paediatrician using the Tanner stages of puberty. Serum samples from young and elderly adults (n = 35 and n = 19 respectively) were collected by Professor S. Shalet at the Department of Endocrinology at the Christie Hospital. Serum leptin levels were measured by Dr A Rahim using the human leptin RIA kit (Linco Research Co.). Clinical information, including weight, BMI, age and leptin levels are shown in table 4.1. The birthweight of the neonate was recorded, as BMI was not measured. All subjects gave their informed consent, for those under 18 years of age a parent or guardian gave their assent and the studies had the approval of local ethical committees.

		Pre-pubertal		Pubertal					
	Umbilical Cord	**Boys**	**Girls**	**Boys**	**Girls**	**Young Men**	**Young Women**	**Old Men**	**Old Women**
	Median (range)	**Median (range)**	**Median (range)**	**Median (range)**	**Median (range)**	**Median (range)**	**Median (range)**	**Median (range)**	**Median (range)**
Number	10	26	26	39	41	18	17	10	9
Age (years)	NM	7.8 (5-11.8)	8.0 (5.3-11.6)	13.9 (11.1-18.2)	12.6 (8.91-17.2)	22 (20.0-23.5)	21.5 (21.0-25.0)	68 (67.0-76.0)	67.5 (60.0-81.0)
BMI (kg/m^2)	NM	16.1 (13.9-22.9)	15.8 (11.8-23.1)	19.3 (15.0-27.0)	18.9 (15.52-30.24)	24.6 (19.0-26.4)	22.8 (19.0-26.4)	26 (23.1-30.5)	25.8 (20.0-30.0)
Weight (kg)	3.6 (1.7-4.2)	26.0 (17.6-46.8)	26.6 (17.1-49.8)	54.7 (31.4-89.4)	45.5 (27.6-72.4)	NM	NM	77 (62.1-90.2)	60.55 (50.3-77.5)
Leptin (ng/ml)	34.3 (8.4-100)	3.5 (1.7-14.2)	3.3 (1.8-13.9)	2.84 (1.3-24.3)	9.6 (2.0-30.8)	1.3 (0.1-25.2)	9.1 (1.5-26.5)	8.2 (2.5-17.0)	16.0 (3.9-105.0)

Table 4.1 Clinical details of subjects used to establish a normal range of LBA through life

NM Not Measured

NA Not Applicable

4.3 Results

The median LBA at the different ages of life is shown in Figure 4.1. LBA was lowest at birth, highest in prepubertal children, fell during puberty to a mean level of 7-9 % in young men and women and remained at this level through to old age. There were no differences in LBA between males and females at any age. The sex difference in leptin levels observed in pubertal, younger and older males and females was not seen in pre-pubertal children.

In adults, there was no evidence of a relationship between LBA and age, BMI or leptin levels (Table 4.2). In contrast, in children there was an inverse relationship between LBA and age, pubertal status, weight, and BMI (Table 4.2 and figure 4.2). The mean levels of LBA and BMI according to age for girls and boys are shown in Table 4.3 and for Tanner stage in Table 4.4.

In order to determine which is the most important factor in determining LBA, multivariate stepwise regression analysis with LBA as the dependent variable and age, puberty, BMI and leptin as the independent variables was performed. In boys, the model explained 41% of LBA, with only age significantly associated with LBA. In girls, the whole model explained 51% of LBA with age (46%) and BMI (5%) significantly associated with LBA. ANOVA showed a significant effect of pubertal status on LBA ($F = 21.2$, $p < 0.0001$) with LBA at all stages of puberty being significantly lower than levels at stage 1 of puberty, and stage 2 being different from all stages except 3. Pearson correlations between LBA and age, BMI or leptin at each pubertal stage were performed. In girls, LBA was negatively correlated with age during Tanner stage 1. In boys, LBA was inversely correlated with serum leptin during Tanner stage 4

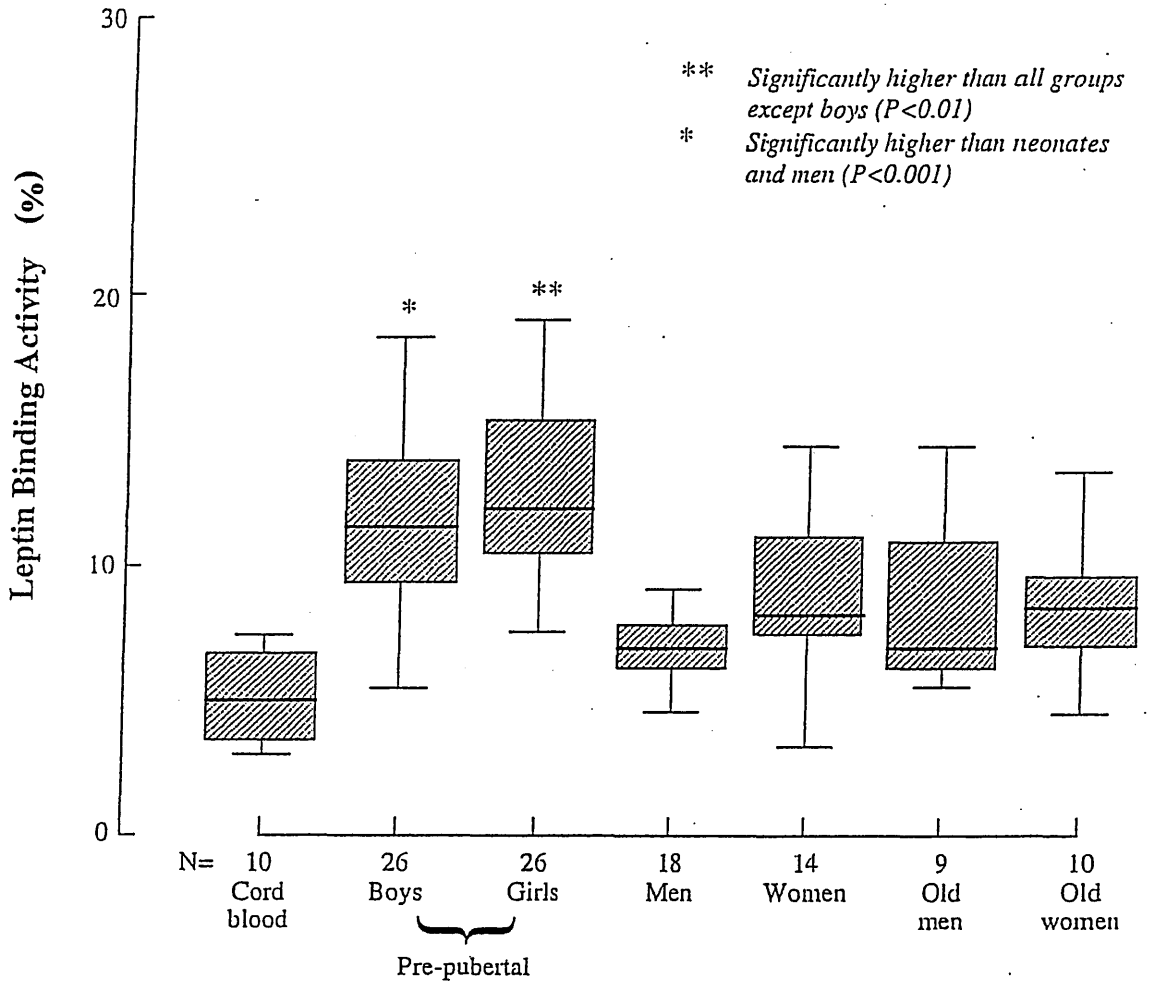
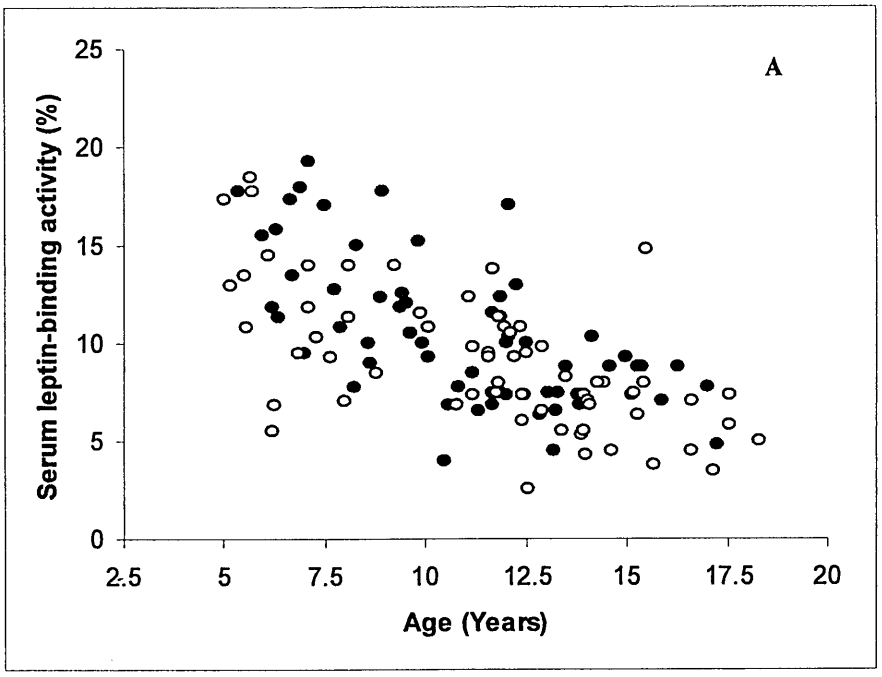


Figure 4.1 Box and Whisker plot of levels of leptin-binding activity through the ages. The median is indicated by the thick black line in the body of the box, the limits of the box are the upper and lower quartiles, the extended bars identify the range of the samples.



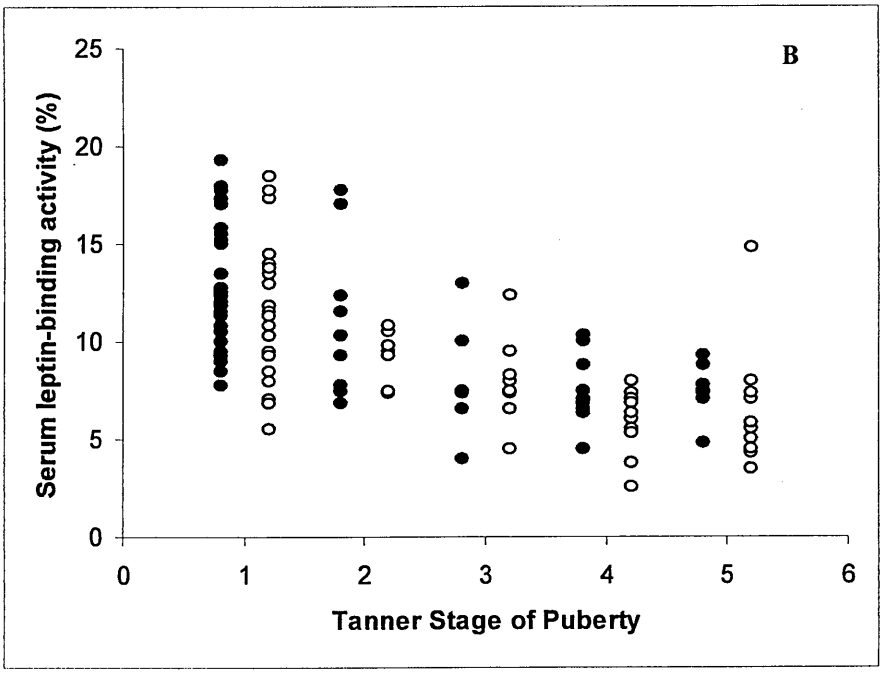


Figure 4.2 Serum leptin-binding activity levels against age (A) and pubertal stage (B) in boys (open circle) and girls (black circles).

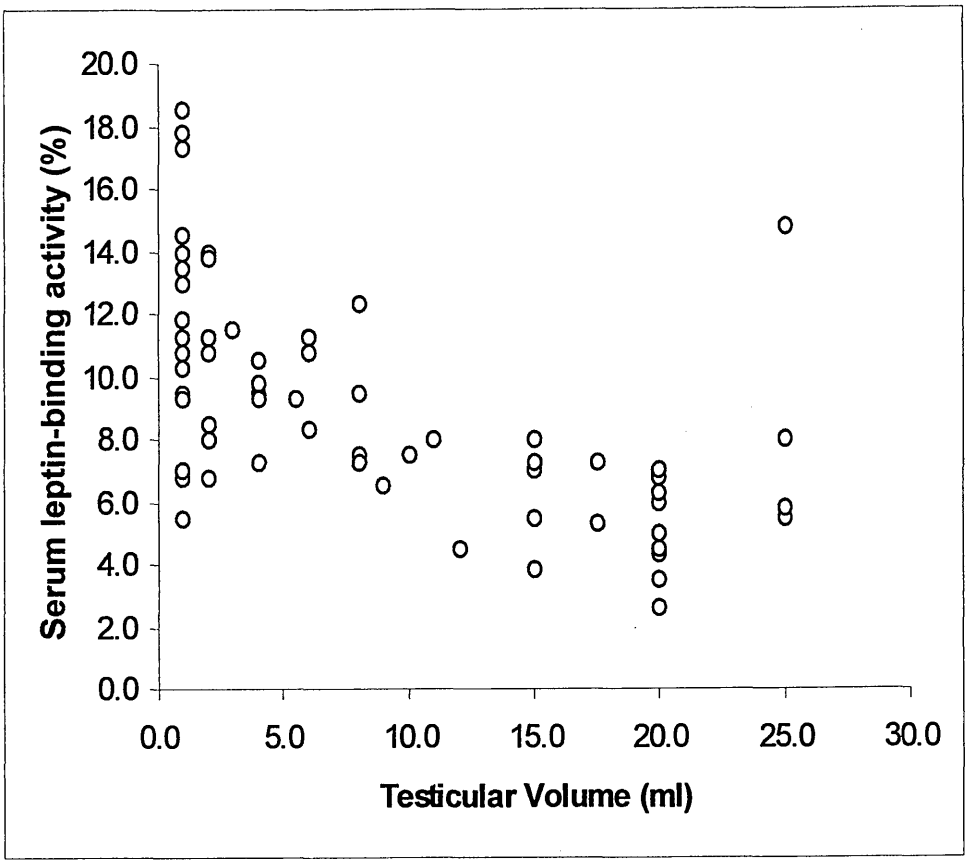


Figure 4.3 Serum leptin-binding activity levels testicular volume in boys.

Variable	Umbilical Cord	Boys	Girls	Men	Women
Number of subjects	10	65	67	18	23
Age	NM	r = -0.64 p = 0.0001	r = -0.66 p = 0.0001	r = -0.09 p = 0.713	r = -0.35 p = 0.226
Pubertal Status	NM	r = -0.62 p = 0.0001	r = -0.61 p = 0.0001	NM	NM
Testicular volume	NM	r = -0.60 p = 0.0001	NA	NM	NA
BMI	NM	r = -0.38 p = 0.002	r = -0.51 p = 0.0001	r = -0.19 p = 0.47	r = -0.35 p = 0.28
Leptin	r = -0.31 p = 0.45	r = -0.03 p = 0.80	r = -0.42 p = 0.001	r = -0.21 p = 0.41	r = -0.06 p = 0.83

Table 4.2: Correlation analysis between different variables and LBA
NM not measured
NA Not applicable

Age	Girls		Boys	
	LBA (%)	**BMI (kg/m^2)**	**LBA (%)**	**BMI (kg/m^2)**
5-6.9	14.5	15.6	12.7	15.9
7-8.9	13.8	17.8	10.8	16.7
9-10.9	10.0	17.7	10.8	17.5
11-12.9	9.5	20.2	9.2	19.6
13-14.9	7.7	19.9	6.4	18.6
15-18.9	7.6	20.7	6.7	22.3

Table 4.3 Mean LBA and BMI for all children according to age.

Tanner Stage	Girls		Boys	
	LBA	**BMI**	**LBA**	**BMI**
1	12.9	16.2	11.1	16.6
2	10.7	18.4	9.5	18.7
3	8.1	18.6	8.0	19.3
4	7.7	19.8	5.9	19.5
5	7.7	21.7	6.6	21.8

Table 4.4 Mean LBA and BMI for all children according to Tanner stage.

4.4 Conclusion

LBA was low at birth, high in early childhood, fell during puberty and remained at the post pubertal level throughout adult life. These changes in LBA parallel the known changes in activity of the pituitary gonadal axis with high levels of LBA being present during the childhood years when the pituitary gonadal axis is quiescent In pubertal children, LBA showed a significant relationship with age, pubertal status, BMI and testicular volume in boys. Multivariate analysis suggested that the most important factor was age. There is an absolute requirement for leptin for the initiation and progression of puberty as demonstrated by the hypogonadal state of leptin resistant or deficient human subjects (Clement *et al*, 1998, Strobel *et al.*, 1998). However, leptin may only provide a tonic "background signal" on which other initiators act, as leptin levels in puberty primarily relate to fat mass (O'Rahilly, 1998). Our finding, of the direct inverse relationship between LBA and pubertal status or age, with a fall in LBA seen at the earliest stages of puberty, suggests that this is a link from leptin to the progression of puberty.

In both the children and in adult subjects, there were no differences in LBA between the sexes. This is in sharp contrast to the well-recognised sexual dimorphism in serum leptin levels (Rosenbaum *et al.*, 1998). If the change in LBA is related to a change in leptin receptor subtype then this may be an alteration that occurs at puberty and remains unaltered through adult life. A relationship between BMI and LBA was not observed in the adult groups. These studies were carried out on a relatively small normal population. Previous studies have suggested that LBA is reduced in obesity (Sinha *et al.*, 1996, Houseknecht *et al.*, 1996, Diamond *et al.*, 1997).

LBA has been reported to relate inversely to leptin levels, however this may have been an artefact due to interference in the assay by endogenous leptin (Diamond *et al.*, 1996). In the present study, endogenous leptin was removed prior to assaying LBA and no statistical evidence of a relationship was found between leptin levels and binding activity except in girls. In girls, leptin increases with age and puberty as LBA is decreasing. The negative correlation would therefore be expected.

These data confirm that the measurement of serum LBA is biologically meaningful, and as such, suggest that this assay may be a useful indicator of leptin activity in other situations.

In conclusion, we have demonstrated an inverse relationship between age and LBA and a strong correlation between puberty and a decrease in LBA. The fall in the earliest stages of puberty suggests that this is a primary event, not driven by changes in sex steroids. The fall in LBA could be explained by a change in leptin receptor expression, which may be the link between the leptin system and the initiation and maintenance of puberty.

5.1 Introduction

Recent studies have suggested that leptin has a role in female reproduction although the exact nature of the role is not fully understood (Cioffi *et al.*, 1996, Butte *et al.*, 1997, Conway *et al.*, 1997). Female *ob/ob* mice are infertile and their fertility is restored with administration of leptin (Barash *et al.*, 1996). Circulating leptin concentrations are higher in females than males, even when corrected for differences in body mass index (BMI) (Hickey *et al.*, 1996, Kohrt *et al.*, 1996). Further evidence for a role for leptin in reproduction is that pregnancy causes changes in the serum concentration of leptin with levels at 36 weeks gestation higher than at 3 and 6 months postpartum (Butte *et al*, 1997). Other studies have suggested that this increase in leptin may be brought about by the progressive increases in plasma progesterone, oestrogen and human placental lactogen during pregnancy (Cioffi *et al*, 1996, Conway *et al.*, 1997).

It has been postulated that leptin is a peripheral signal that integrates fat stores, nutrition and reproductive viability. Female athletes can develop amenorrhoea; this loss of menstrual function often correlates directly with the proportion of weight that is lost (Goldfien and Monroe, 1997). Decreased leptin levels were found in undernourished women of reproductive age, mainly presenting with oligo- or amenorrhoea (Macut *et al.*, 1998). This supports the hypothesis of Frisch (1990), that adequate fat stores are required for normal reproductive function to occur and that leptin acts as a peripheral signal, from the fat stores to the brain, indicating that the body has the ability to support a pregnancy.

Leptin interacts with the hypothalamo-pituitary-ovarian axis (Yu *et al.*, 1997). In women undergoing pituitary down-regulation and ovarian stimulation for *in vitro* fertilisation (IVF), serum leptin levels have been shown to correlate with oestradiol and follicle stimulating hormone (FSH) (Butzow *et al.*, 1997), and other studies have suggested that leptin may be involved in bringing about the ovarian response to gonadotrophin stimulation (Strowitzki *et al.*, 1997). This hypothesis is supported by the presence of leptin receptor in ovarian tissue (Cioffi *et al.*, 1996) and the presence of leptin within the pre-ovulatory follicle and mature oocyte (Cioffi *et al.*, 1997).

5.1.1 Cytokines and soluble cytokine receptors through the menstrual cycle

The majority of previous investigations on the role of leptin in female reproduction have been carried out in pregnant women or those undergoing IVF treatment using stimulated cycles. Less is known about the variation in serum leptin levels and leptin binding activity throughout the menstrual cycle in normal fertile women. Some cytokines, such as IL-1 (Lynch *et al.*, 1994) and soluble cytokine receptors, such as the IL-6 soluble receptor (Gorai *et al.*, 1998), are known to fluctuate during the menstrual cycle while others, such as IL-10, do not vary in this way (Maskill *et al.*, 1998).

A report by Gorai *et al* (1998) suggests that there are significant fluctuations of the soluble IL-6 receptor during the menstrual cycle in their cohort of Japanese women. They demonstrated a rise in soluble IL-6 receptor in the early and late follicular phase of the menstrual cycle. As a proportion of the LBA is reported to be the soluble form of the leptin receptor, and soluble cytokine receptors have been reported to fluctuate during the menstrual cycle, it is possible that LBA would also fluctuate during the cycle in response to varying leptin levels. However, other workers do not report any fluctuation in soluble IL-6 receptor during the menstrual cycle (Angstwurm *et al.*, 1997). Other binding proteins, such as growth hormone-binding protein (GH-BP) and IGF-BP have also been studied during the menstrual cycle. Klein *et al.*, (1996) measured GH-BP in two groups of regularly cycling women aged 20-25 years and 40-45 years. They found that GH-BP does not fluctuate in either of the two groups studied. Trainer *et al.* (1998) studied corticotrophin-releasing hormone binding protein, they found no variation of this binding protein in the menstrual cycle. Controversy surrounds the variation of IGF-BPs during the menstrual cycle. Many workers report no significant difference in levels of either IGF-BP 1 or IGF-BP 3 at any point of the menstrual cycle (Juul *et al.*, 1997, Ovesen *et al.*, 1998, Van Dessel *et al.*, 1996). Helle *et al.* (1998) report that IGF-BP 3 is highest in the follicular phase of the cycle, but that there is no change in levels of IGF-BP I. Westwood *et al.* (1999) disagree, these workers found that IGF-BP I varied with the menstrual cycle, with a peak occurring in the late secretory phase.

5.1.2 Leptin and pregnancy

During pregnancy, there is a significant rise in leptin above those levels seen in non-pregnant women (Butte *et al.*, 1997, Hassink *et al.*, 1997). Tamas *et al.* (1998) performed longitudinal analysis of leptin levels during the pregnancies of nine women.

In each case, there was a steady rise in leptin levels throughout pregnancy until week 28 where they observed a small decline. They report a positive correlation between BMI and leptin levels in the first and second trimester of pregnancy but, not in the third trimester; this may be due to the difficulties in obtaining an accurate BMI during pregnancy. Leptin is produced primarily by adipose tissue. It is now known, however, to be expressed and secreted by a number of tissues including the placenta. At this time, it is not known what contribution placental leptin production makes to the total leptin level during pregnancy. Leptin receptor isoforms have been identified in the placenta. Henson *et al.* (1998) localised leptin receptor transcripts to the syncytiotrophoblast cells. These cells produce hormones necessary for maintaining pregnancy and the authors postulate that placental leptin may be a factor in maintaining pregnancy. Bodner *et al.* (1999) agree with the previous studies on the placental distribution of the leptin receptor. They identified the long form of the leptin receptor in the cytoplasm of the syncytiotrophoblast cells and other receptor isoforms in the apical membrane of the syncytiotrophoblast cells, intervillous space and in foetal vessels.

There is evidence to suggest that leptin is an important factor in foetal growth and development. Leptin mRNA has been identified in human foetal adipose tissue (Bernard *et al.*, 1999) and in murine foetal hair follicles, bone, and cartilage (Hoggard *et al.*, 1999). Cord blood leptin is associated with intrauterine growth, even after allowing for placental weight, adiposity, maternal leptin concentration and cord blood insulin level (Varvarigou *et al.*, 1999). Harigaya *et al.* (1999) suggest that leptin has an important function in the regulation of weight gain in early infancy. Hytinantti *et al.* (1999) investigated changes in leptin levels in the early postnatal period. Leptin is markedly reduced in the three days after birth and the gender difference observed in adults is already present in their study. It is possible that leptin, produced by the placenta, may be an important factor in intrauterine growth and development and thus be a mechanism by which poor placentation can lead to a poor pregnancy outcome. Alternatively, lower serum leptin levels may reflect lower production by a poorly-functioning placenta.

5.1.3 Leptin and cigarette smoking

Cigarette smoking is associated with lower BMI, smokers weigh less than age-matched non-smokers (Nicklas *et al.*, 1999). A major deterrent to stopping smoking, especially amongst women, is weight gain (Spring *et al.*, 1992). Leptin, expressed by adipose tissue, is proposed to regulate body weight via hypothalamic control of energy intake

and expenditure. Increased energy intake is believed to be the most likely cause of weight gain after smoking cessation. There is some evidence that smoking increases the metabolic rate and that this rate may decrease on cessation of smoking (Moffatt *et al.*, 1991). Several groups of workers have reported that leptin levels vary between smokers and non-smokers; these reports are in conflict. Nicklas *et al.* (1999) measured leptin levels in middle-aged and elderly male subjects, they found that smokers have significantly higher leptin levels than non-smokers whereas Hodge *et al.*, (1997), report that leptin levels are lower in their Nauruan, West Samoan and Mauritian male population of smokers than non-smokers. Wei *et al.* (1997) also reports lower leptin levels in their population of Mexican-American smokers.

It is well documented that smoking during pregnancy can have a detrimental effect on the foetus and may lead to premature birth or the infant being light for gestational age (LGA) (Abel, 1980). Recent reports have suggested the effect of *in utero* exposure to tobacco smoke may have long term consequences on the child's health. Smoking during pregnancy is a risk factor for the development of congenital heart defects (Kallen, 1999) and for behavioural and neurodevelopmental difficulties such as, reduced intellectual ability and decreased attention span (Eskenazi and Castorina, 1999). Mathews *et al.*, (1999) demonstrated that the level of cotinine, a nicotine metabolite, at week 16 of pregnancy was predictive of a poor outcome. At cotinine levels of greater than 225 ng/ml, the subjects were more likely to have a pregnancy that resulted in a poor outcome.

5.1.4 Objectives

In order to expand current knowledge of leptin in female reproductive processes, studies of leptin and leptin binding activity were carried out in three cohorts of women; two studies within the menstrual cycle and a study of leptin during pregnancy.

There were three main aims of these studies:

- To test the hypothesis that levels of leptin and leptin binding activity alter throughout the menstrual cycle.
- To aid in the elucidation of the role of LBA in adulthood and assist in understanding the role of leptin in reproduction.
- To provide some understanding of the role of leptin during pregnancy, the possible relationship with pregnancy outcome and the effect of smoking during pregnancy.

5.2 Serum leptin and LBA levels during the menstrual cycle

5.2.1 Study Design

5.2.1.2 Cohort A

Serum samples were obtained from 17 normal fertile women, these were collected by Mr T.C. Li at the Jessop Hospital for Women in Sheffield. All the women were aged 30-39 years and had a BMI of between 19.8 and 27.9 kg/m^2. The subjects were not required to fast before sampling took place. The number of serum samples collected from each subject ranged from 1 to 3 in the follicular phase and 2 to 3 in the luteal phase. All subjects had normal menstrual cycles as confirmed by detection of luteinising hormone (LH) surge and luteal phase progesterone levels. Each woman had had at least one successful pregnancy. None of the subjects were currently prescribed oral contraceptives or corticosteroids.

The samples obtained were timed precisely according to the LH surge. From day 9 of the cycle, early morning urine or daily serum samples were collected from each subject. LH was measured in each sample by an established immunoenzymatic assay (Serono Diagnostics Ltd, Surrey, UK.) The day in which the LH surge occurred was designated as day LH+0 (Li *et al.*, 1993).

Leptin levels were measured using a specific human leptin RIA from Linco Research Co. as described in Chapter 2. Serum progesterone had previously been measured in samples taken ten days after the LH surge (LH+10), at the point where progesterone is maximal, in 14 of the women. The progesterone was measured using a Progesterone EIA kit (Serozyme, Welwyn Garden City, Herts, UK.). Analysis of serum progesterone levels and urinary or serum LH was carried out at the Jessop Hospital for Women. All subjects gave informed consent and the studies had the approval of the local ethical committee (South Sheffield Ethics Committee).

2.5.1.3 Cohort B

Serum samples were obtained from eight women throughout the menstrual cycle; these were collected by Dr Melissa Westwood at the University of Manchester. All the women had a BMI between 19.8 and 28.7 kg/m^2 and were aged between 21 and 40 years. The women had normal length menstrual cycles of between 25 and 35 days. None of the women were currently prescribed oral contraceptives or corticosteroids.

The subjects were required to fast prior to the blood samples being taken. Menstrual cycle date was determined by measurement of the LH surge. The number of serum samples collected from each subject ranged from 1 to 3 in the follicular phase and 2 to 3 in the luteal phase. The measurement of serum and urinary LH was carried out by Dr Melissa Westwood. Leptin levels were assessed using a specific human leptin ELISA (R&D Systems) and LBA was measured as described in Chapter 2. The women who provided samples for these studies gave their informed consent and the studies had the approval of the local ethical committee.

Statistical Analysis

The difference between the mean levels of leptin in the follicular and luteal phases for each woman was assessed by a paired t test. The relationship between leptin and BMI was assessed using Pearson product moment testing.

5.2.2 Results

5.2.2.1 Cohort A

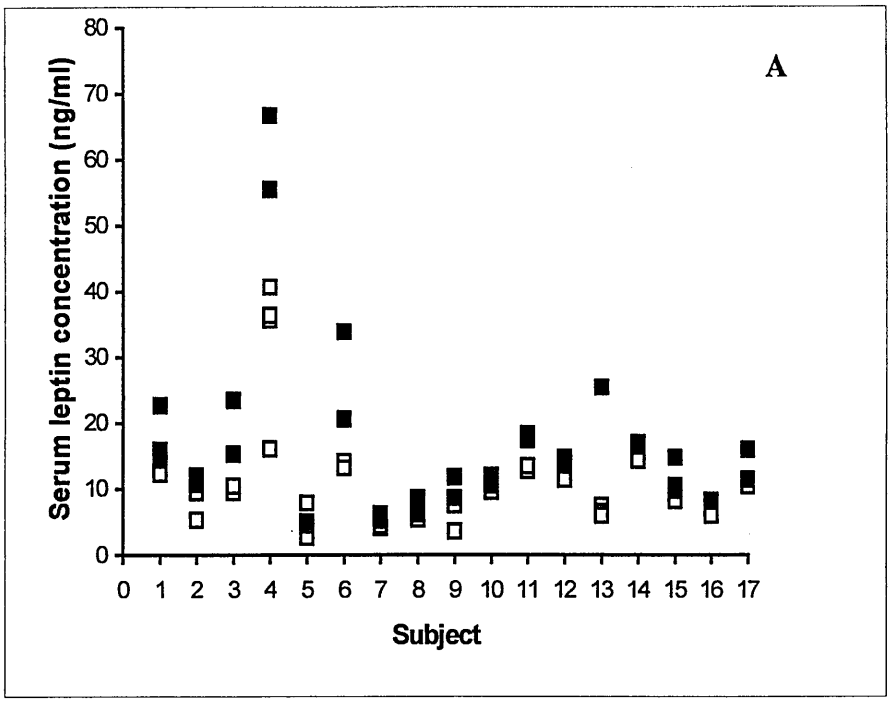
Levels of leptin gradually increased throughout the cycle and were maximal in the luteal phase. In 14 out of the 17 subjects, mean luteal levels of leptin were arithmetically higher than the mean follicular leptin levels. The mean follicular and luteal levels of leptin in the remaining 3 subjects were unchanged.

Serum follicular and luteal phase leptin levels for each individual woman is shown in Figure 5.1. Leptin levels were significantly higher ($p<0.01$) in serum obtained during the luteal phase than in serum collected during the follicular phase. The overall median serum leptin during the follicular phase was 10.0 ng/ml with a range of 4.3-32.2 ng/ml. The median level of leptin during the luteal phase was 11.4 ng/ml with a range of 4.9-66.7 ng/ml.

BMI measurements were not available for subjects 2 and 10. For the 15 subjects whose values were available, a significant correlation was seen between leptin levels (mean of all menstrual cycle measurements) and BMI. ($r = 0.54$, $p < 0.05$) (Figure 5.2). Luteal phase progesterone levels were obtained in 14 of the 17 subjects. No correlation was seen, however between leptin and progesterone at the peak progesterone level ten days after the LH surge ($r = -0.34$, $p < 0.370$) (Figure 5.3).

5.2.2.2 Cohort B

Leptin levels were measured in samples from eight women throughout the menstrual cycle. As expected, the mean leptin level (from each subjects samples throughout the cycle) correlated with BMI ($r = 0.892$, $p=0.003$) (Figure 5.2). The mean leptin level for the 8 subjects was 13.9 ± 4.9 (mean $\pm$ SEM). The mean level of leptin in the follicular phase was 12.6 ± 4.5 ng/ml (mean $\pm$SEM) and in the luteal phase was 15.3 ± 5.4 ng/ml (mean $\pm$ SEM). Leptin levels were significantly higher in the luteal phase of the menstrual cycle ($p = 0.04$) and this agrees with the previous study. The follicular and luteal phase leptin levels are shown in Figure 5.1. Serum LBA was measured in 8 subjects; LBA was higher in the luteal phase than the follicular phase in three individuals, lower in the luteal phase than the follicular phase in three individuals and remained unchanged throughout the cycle in 2 subjects (Figure 5.4). The mean level of LBA in the follicular phase was 8.5 ± 3.0 % (mean $\pm$ SEM) and in the luteal phase was 8.3 ± 2.9 (mean $\pm$ SEM). There was no significant difference between the mean follicular level of LBA and the mean luteal level of LBA using a t test for paired data. A significant correlation between LBA and BMI was not seen ($r = -0.693$, $p = 0.06$) (Figure 5.5), although a negative trend was observed: with a higher BMI, a lower LBA is present.



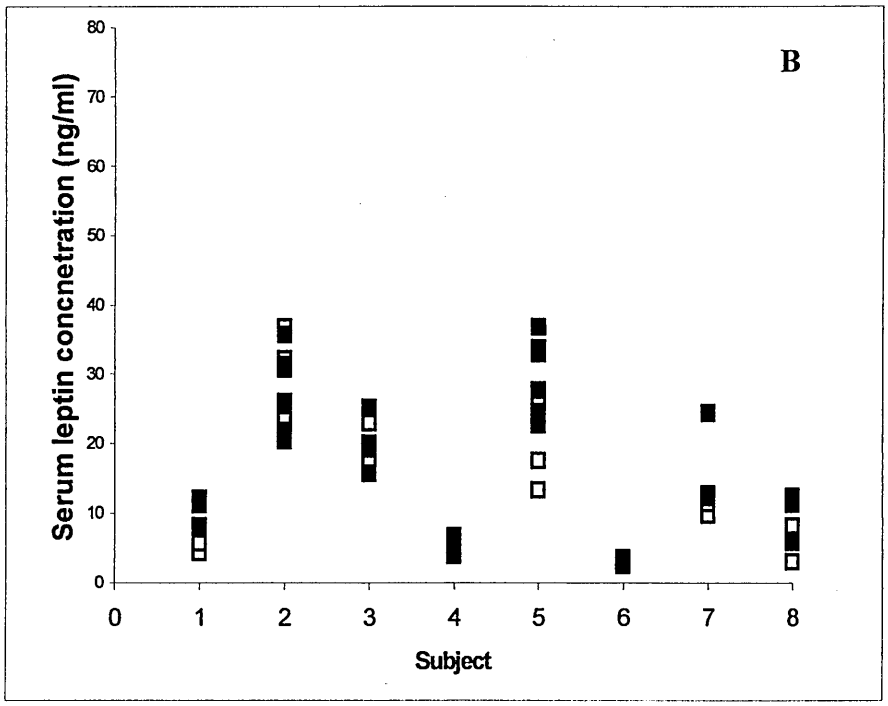
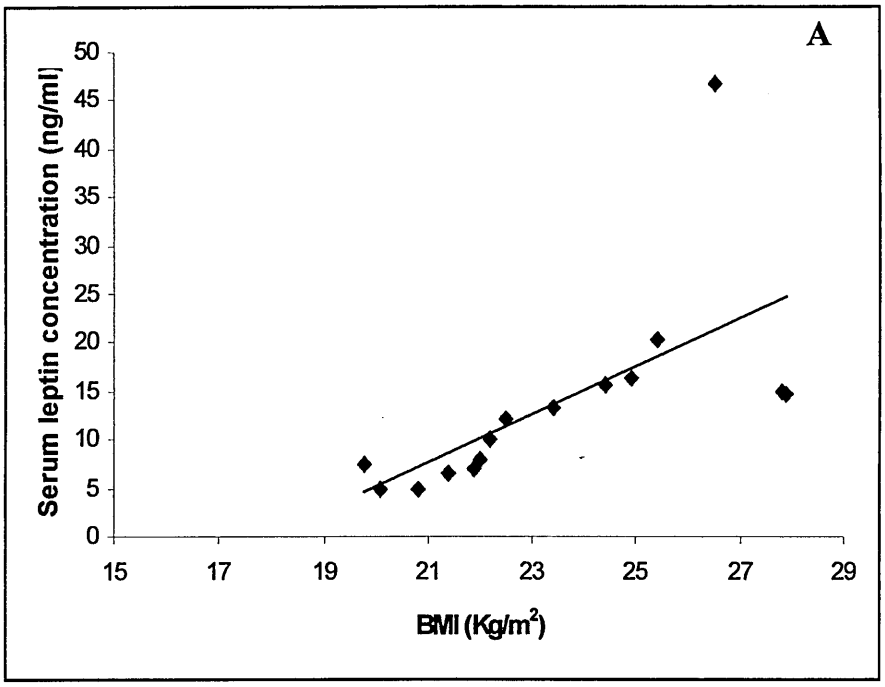


Figure 5.1 Levels of leptin for each individual subject in the follicular (open squares) and luteal (black squares) phases in cohort A and B.



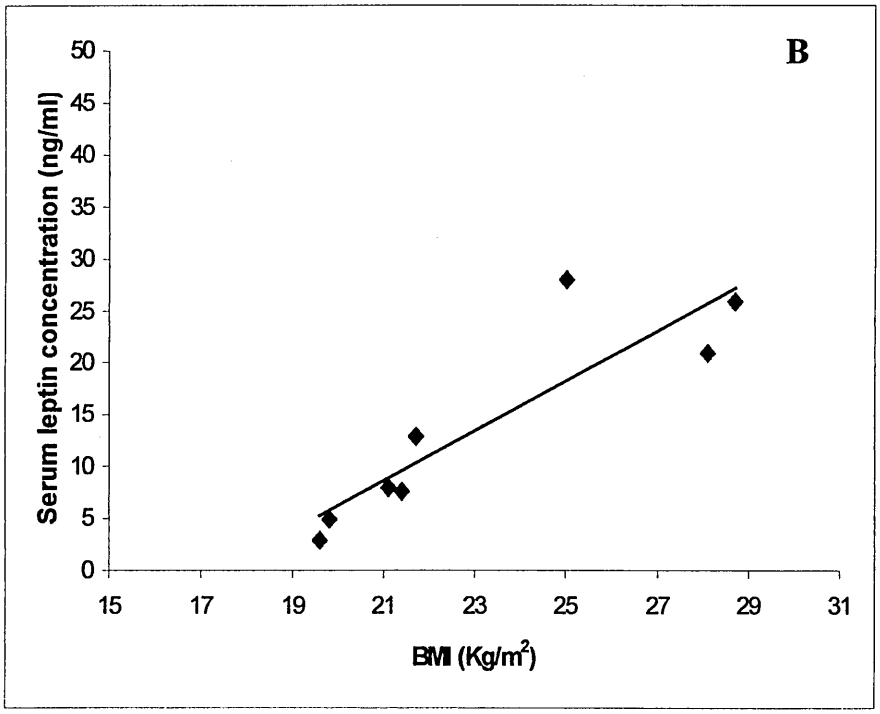


Figure 5.2 Mean serum leptin levels correlated with BMI in both cohort A and B. A significant correlation was seen in cohort A ($r = 0.54$, $p < 0.05$) and cohort B ($r = 0.892$, $p = 0.003$).

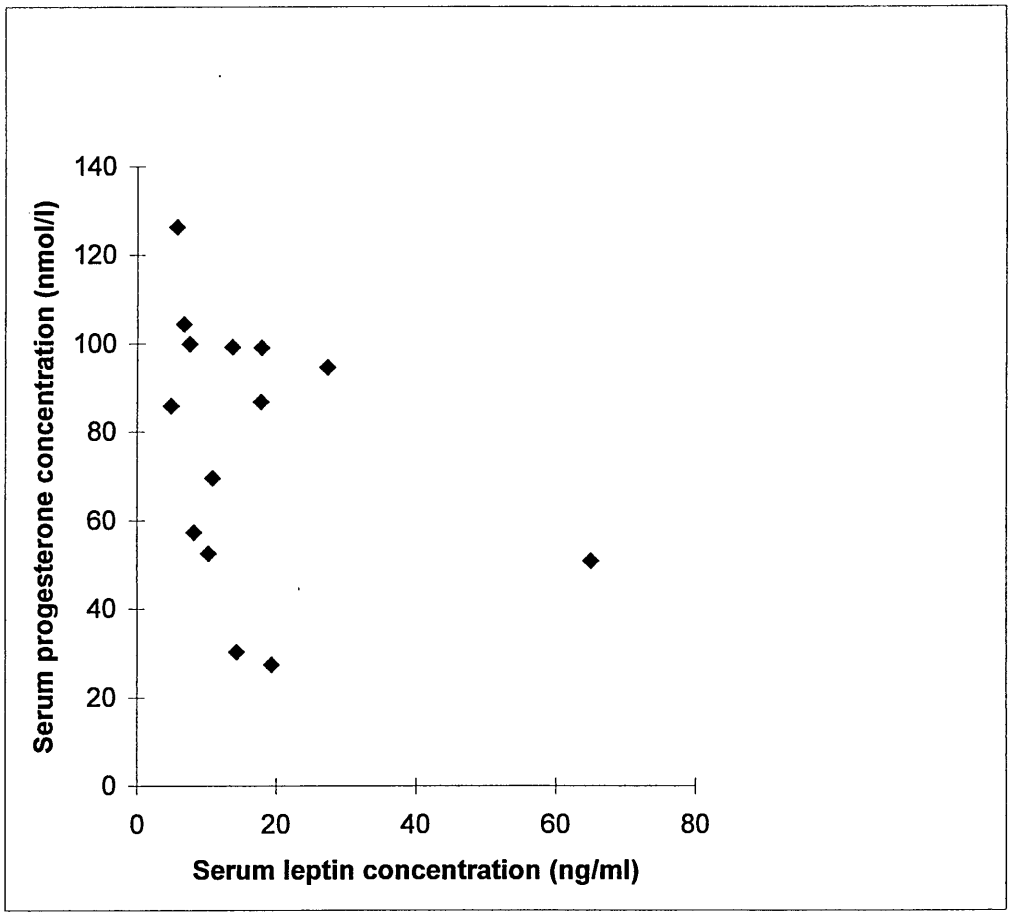


Figure 5.3 In cohort A, there is no relationship between leptin and progesterone on day LH + 10.

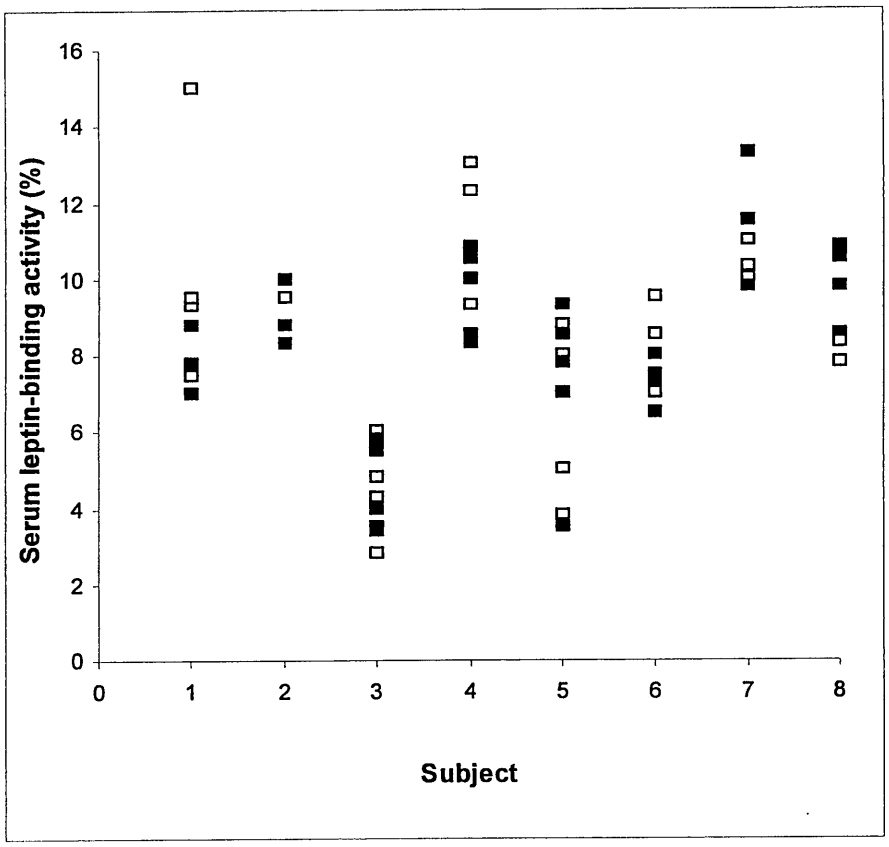


Figure 5.4 Levels of LBA for each individual subject (in cohort B) in the follicular (open squares) and luteal (black squares) phases.

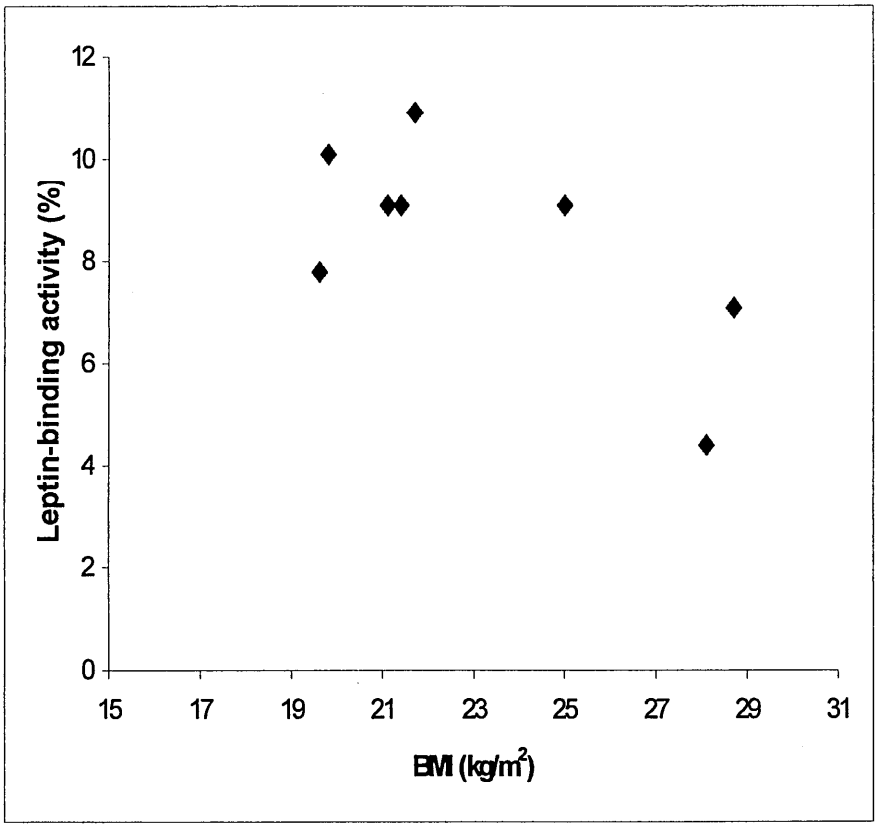


Figure 5.5 Serum leptin-binding activity (%) did not show significant correlation with BMI in cohort B ($r = -0.693$, $p = 0.06$)

5.3 Effect of smoking status and pregnancy outcome on serum leptin levels during pregnancy

5.3.1 Study design

As leptin is known to fluctuate:

a) during the menstrual cycle;

b) in pregnancy;

c) with smoking status,

The aims of the present study were to assess if maternal serum leptin concentrations varied in subjects who had a poor pregnancy outcome compared to those with a normal pregnancy outcome, and if there was any variation in serum leptin levels in pregnant smokers compared to pregnant non-smokers.

Subjects used in this study were recruited by Dr Fiona Mathews at the University of Oxford as part of a larger study of more than 1000 nulliparous, pregnant women investigating antioxidant status and birthweight. At the first antenatal booking-in appointment the women's height, weight and smoking status were recorded. During routine antenatal blood testing (at weeks 16 and 28), samples were obtained for cotinine and leptin analysis. In total, 154 samples were obtained from 77 women (paired samples at both 16 and 28 weeks) for cotinine and leptin analysis. Poor pregnancy outcomes of light for gestational age and pre-term birth were chosen *a priori*. Data on each subject's pregnancy outcome was obtained retrospectively.

Cotinine levels were measured with an in-house RIA (as described in chapter 2) to assign smoking status. This analysis was carried out in collaboration with Dr Robert Smith. Any subject with a cotinine level of >15ng/ml was considered a smoker. Serum leptin levels were measured using the leptin ELISA (R&D Systems), as described in chapter 2.

This study had the approval of the local ethical committee and all participants gave their informed consent.

5.3.2 Results

In this cohort of pregnant women, mean serum leptin levels at week 16 were 24.5 ± 2.8 ng/ml (Mean ± SEM). This increased to 25.0 ± 2.9 ng/ml at week 28. In the total cohort, there was no significant increase in serum leptin concentration between weeks 16 and 28. At 16 weeks of gestation, BMI was calculated. Serum leptin levels correlated with BMI ($r = 0.536$, $p = 0.001$) at week 16 (Figure 5.6). Clinical data for all subjects is shown in Table 5.1a. Mean serum leptin levels at 16 and 28 weeks and cotinine levels are shown in Table 5.1b.

Mean serum leptin levels were lower in subjects who had a poor pregnancy outcome compared to those who had a normal pregnancy outcome at week 16 and week 28 although this did not reach significance (Figure 5.7). Clinical details for subjects separated by pregnancy outcome are shown in Table 5.2a. Mean BMI was significantly lower in those women with poor pregnancy outcome compared to women who had a normal pregnancy outcome. Mean serum leptin concentrations at 16 and 28 weeks and serum cotinine levels for women separated according to pregnancy outcome are shown in Table 5.2b.

Leptin levels correlated with BMI in both the women with a normal pregnancy outcome ($r = 0.661$, $p = 0.0001$) and poor pregnancy outcome ($r = 0.429$, $p = 0.004$) at week 16 of the pregnancy. By week 28, only the women with poor pregnancy outcome had leptin levels that correlated with BMI (taken at week 16) ($r = 0.619$, $p = 0.0001$). There was a significant correlation between BMI and birthweight in those subjects with a normal pregnancy outcome at 16 weeks ($r = 0.376$, $p = 0.03$), but this was not apparent at 28 weeks or in subjects with a poor pregnancy outcome. A relationship between maternal serum leptin level and infant's birthweight was not observed.

Mean serum leptin levels were significantly lower in pregnant smokers than in pregnant non-smokers at week 16 and week 28 of pregnancy (Figure 5.8), although this may be an effect of smokers having a lower BMI than non-smokers. BMI was significantly lower in those women who smoked compared to the non-smokers. Serum leptin level correlated with BMI at week 16 of pregnancy in both the smokers and the non-smokers. However, at week 28, leptin level correlated with BMI (taken at week 16) in the non-smoking group. There was no correlation between serum leptin level and serum cotinine level in these subjects. There was no significant correlation between BMI and

birthweight at either week 16 or 28 in the smokers. In the non-smokers, BMI correlated with birthweight at 16 weeks but, this relationship had disappeared by week 28. There was no correlation between leptin levels and birthweight at week 16 or 28.

ANOVA (with Bonferroni correction) was used to determine differences between the groups. At week 16 there was no significant difference between the four groups of pregnant subjects. By week 28, however, non-smokers with normal pregnancy outcome had significantly higher leptin levels than normal outcome smokers ($p = 0.03$) and poor outcome smokers ($p = 0.009$). The change in serum leptin levels between 16 and 28 weeks of pregnancy is shown in those with poor and normal pregnancy outcome, in smokers and non-smokers and subjects separated according to smoking status and pregnancy outcome (Figure 5.9). The individual changes in serum leptin concentration between 16 and 28 weeks of pregnancy in subjects separated according to smoking status are shown in Figure 5.10. The change in serum leptin levels between 16 and 28 weeks separated according to pregnancy outcome and smoking status is represented in Figure 5.11 and Table 5.4.

	Mean ± SEM	Number
Age (years)	24.7 ± 2.9	77
Weight (kg)	65.7 ± 7.7	76
BMI (kg/m^2)	24.7 ± 2.9	76
Systolic blood pressure (mmHg)	125.4 ± 14.8	77
Diastolic blood pressure (mmHg)	78 ± 9.2	77
Gestation period (weeks)	38.4 ± 4.5	77
Birthweight of infant (kg)	2.9 ± 0.3	77

Table 5.1a Clinical details for all subjects in this study of pregnancy.

	Mean ± SEM	Number
Leptin at 16 weeks (ng/ml)	24.5 ± 2.8	77
Leptin at 28 weeks (ng/ml)	25.6 ± 3.0	77
Cotinine (ng/ml)	59.4 ± 7.0	77

Table 5.1b Mean serum leptin concentrations and cotinine levels (mean of week 16 and 28) in all subjects in this study of pregnancy.

	Normal Outcome	n	Poor Outcome	n	p value
Age (years)	25.5 ± 4.4	34	24.8 ± 3.8	43	NS
Weight (kg)	68.9 ± 12	33	62.8 ± 9.5	43	0.03
BMI (kg/m^2)	25.5 ± 4.4	33	23.7 ± 3.6	43	0.04
Systolic blood pressure (mmHg)	114.1 ± 19.6	34	132.7 ± 20.1	43	NS
Diastolic blood pressure (mmHg)	65.9 ± 11.5	34	86.3 ±13.1	43	NS
Gestation period (weeks)	40.3 ± 6.9	34	36.9 ±5.6	43	0.0001
Birthweight of infant (kg)	3.4 ± 0.6	34	2.5 ±0.4	43	0.0001

Table 5.2a Mean clinical data for subjects grouped according to pregnancy outcome

	Normal Outcome	n	Poor Outcome	n	p value
Leptin at 16 weeks (ng/ml)	24.6 ± 4.2	34	22.0 ± 3.3	43	NS
Leptin at 28 weeks (ng/ml)	28.0 ± 4.8	34	22.6 ± 3.4	43	0.08
Cotinine (ng/ml)	39.4 ± 6.8	34	82.2 ± 12.5	43	0.03

Table 5.2b Mean serum leptin concentrations and cotinine levels (mean of week 16 and 28) grouped according to pregnancy outcome.

	Non-Smokers	n	Smokers	n	p value
Age (years)	25.9 ± 4.2	38	24.3 ± 3.9	39	0.06
Weight (kg)	68.4 ± 11.1	37	62.7 ± 10.0	39	0.03
BMI (kg/m^2)	25.5 ± 4.2	37	23.4 ± 3.8	39	0.02
Systolic blood pressure (mmHg)	136.8 ± 22.2	38	112.4 ± 18	39	NS
Diastolic blood pressure (mmHg)	90.0 ± 14.6	38	64.9 ± 10.4	39	NS
Gestation period (weeks)	37.8 ± 6.2	38	39.1 ± 6.3	39	0.03
Birthweight of infant (kg)	2.9 ± 0.5	38	2.95 ± 0.5	39	NS

Table 5.3a Mean clinical data for subjects grouped according to smoking status.

	Non-Smokers	n	Smokers	n	p value
Leptin at 16 weeks (ng/ml)	27.2 ± 4.4	38	19.2 ± 3.1	39	0.01
Leptin at 28 weeks (ng/ml)	29.3 ± 4.8	38	20.8 ± 3.3	39	0.009
Cotinine (ng/ml)	6.3 ± 1.0	38	118.8 ±19.0	39	0.0001

Table 5.3b Mean serum leptin concentrations and cotinine levels (mean of week 16 and 28) grouped according to smoking status.

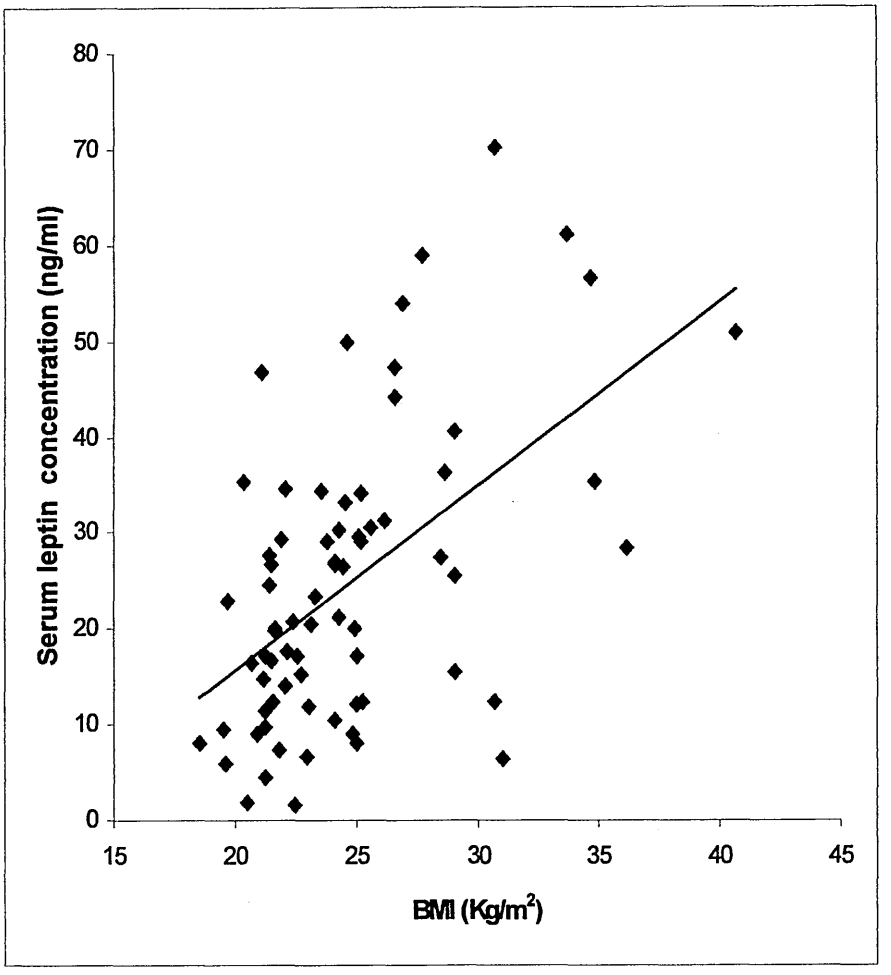


Figure 5.6 Serum leptin levels correlate with BMI at week 16 of pregnancy in a cohort of 77 women.

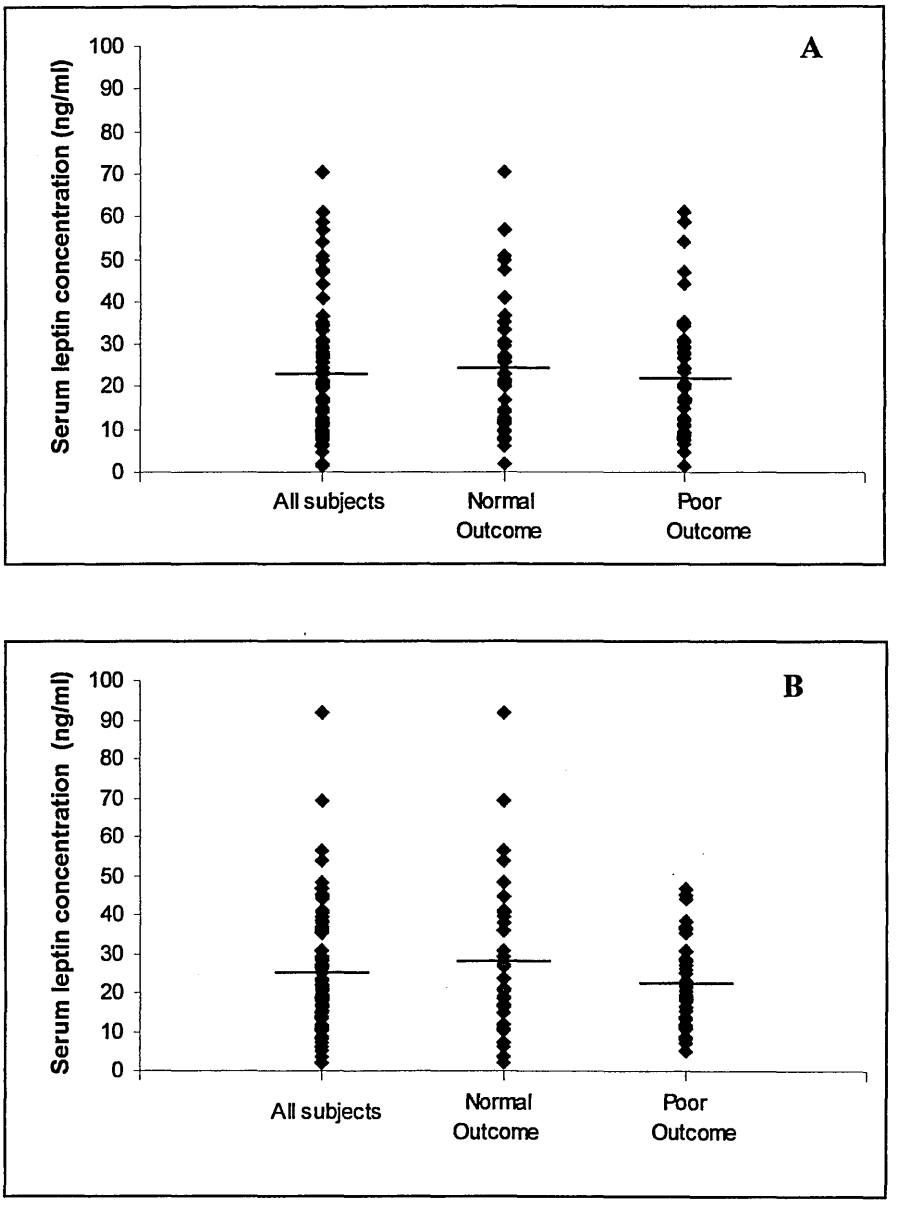
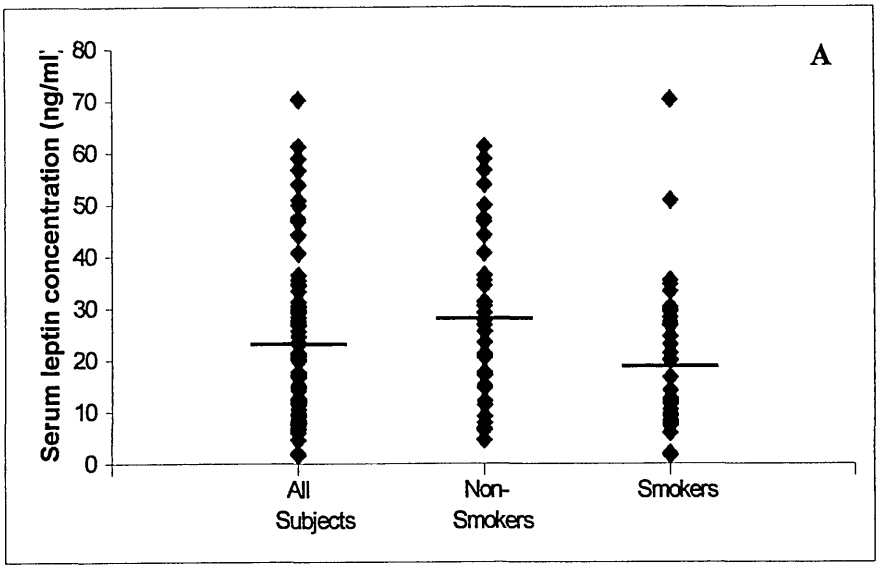


Figure 5.7 Serum leptin levels in all subjects, and separated into groups according to pregnancy outcome, at 16 (A) and 28 (B) weeks



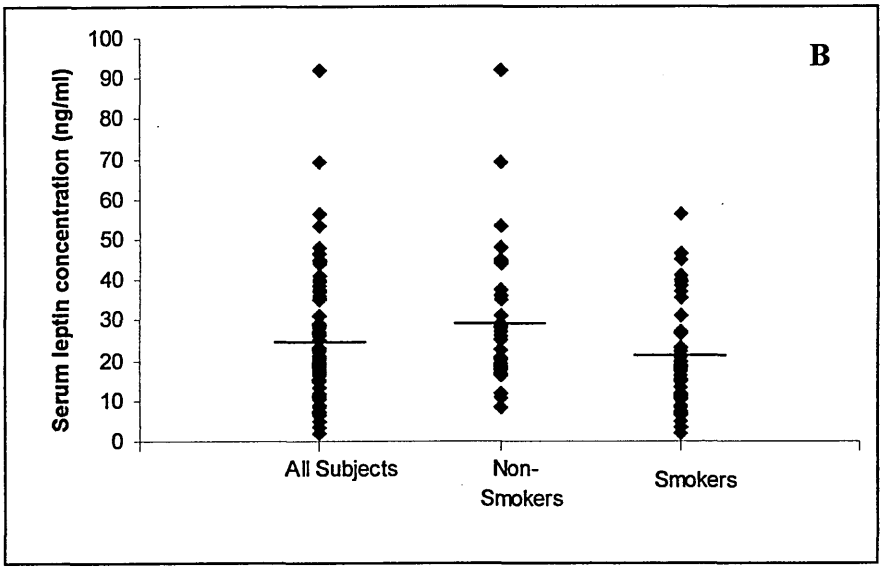
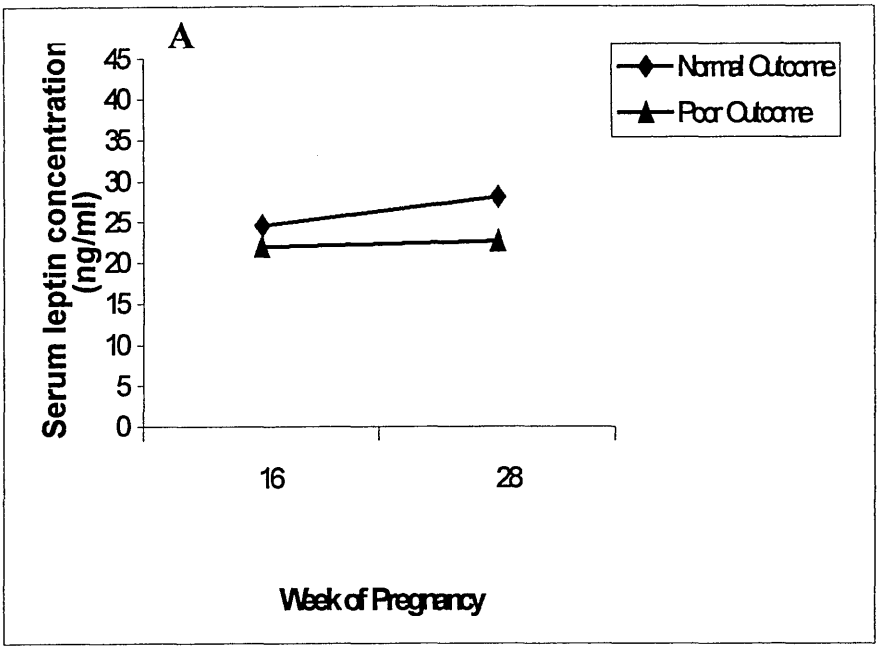


Figure 5.8 Serum leptin levels in all subjects, and separated into groups according to smoking status, at 16 (A) and 28 (B) weeks



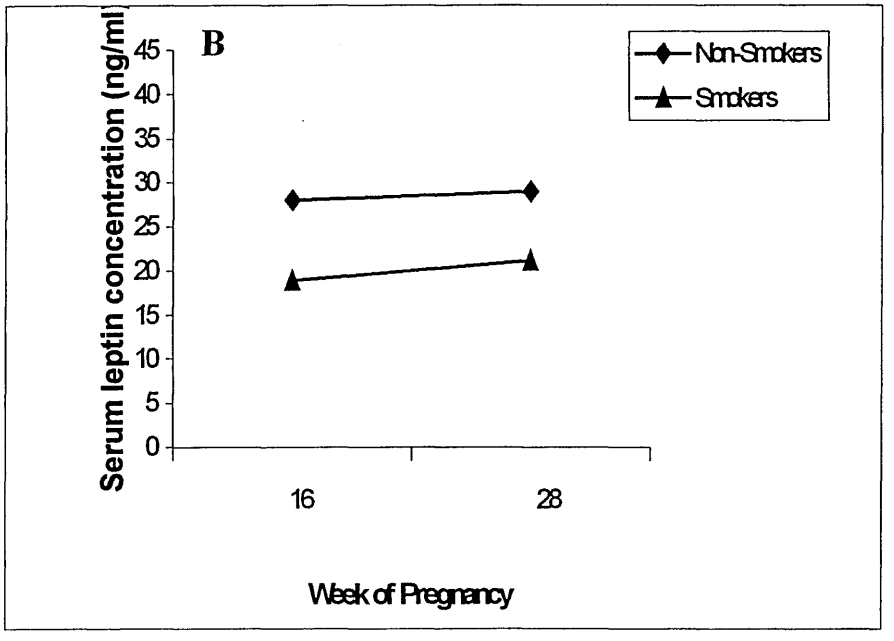


Figure 5.9 The overall change in serum leptin concentration between 16 and 28 weeks, observed in subjects with normal and poor pregnancy outcome (A), smokers and non-smokers (B)

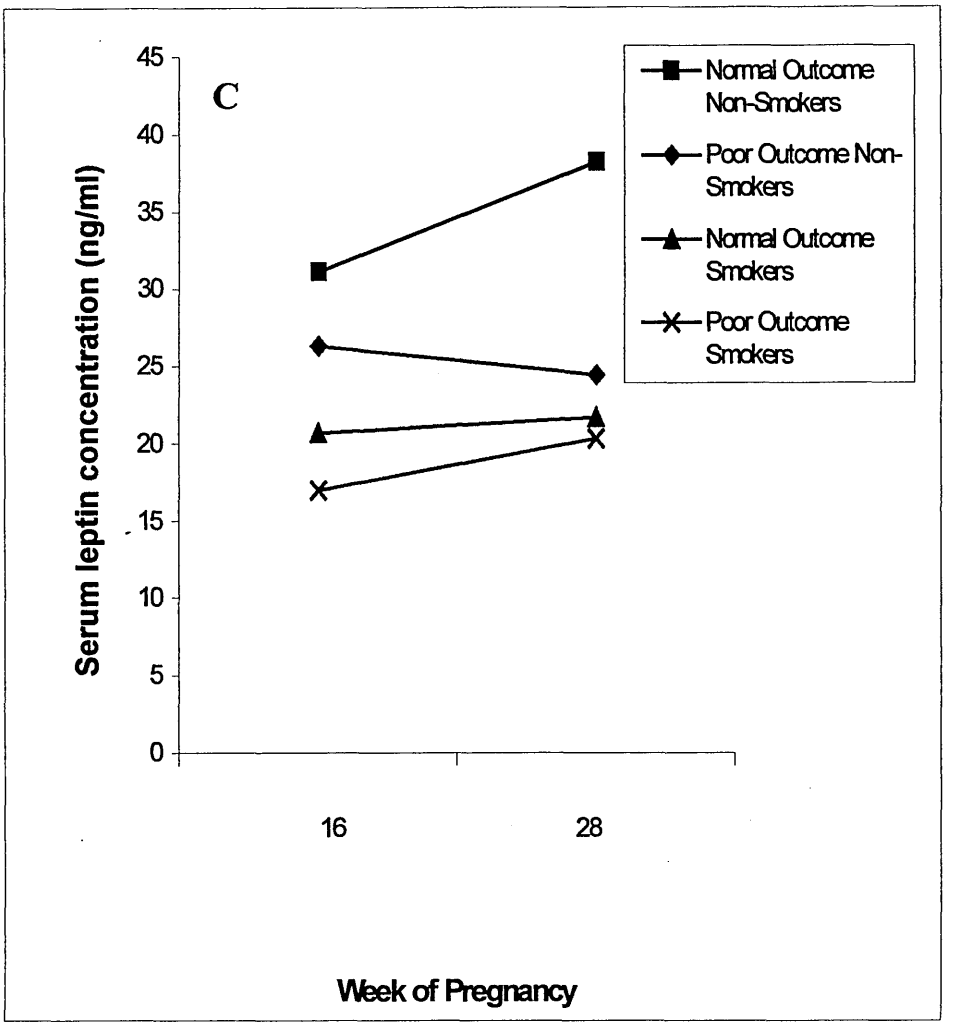


Figure 5.10 The overall change in serum leptin concentration between 16 and 28 weeks, observed in subjects separated according to pregnancy outcome and smoking status

	Mean serum leptin concentration (ng/ml) (Mean ± SEM)		Numbers Increased		Numbers Decreased	
	16 weeks	**28 weeks**	**>2 ng/ml**	**<2 ng/ml**	**>2 ng/ml**	**<2 ng/ml**
Normal Outcome Non-Smoker	31.1 ± 8.6	38.2 ± 10.6	7	0	5	1
Normal Outcome Smoker	20.7 ± 4.5	21.7 ± 4.7	7	3	11	2
Poor Outcome Non-Smoker	26.3 ± 5.5	24.4 ± 5.1	6	1	14	0
Poor Outcome Smoker	17.0 ± 3.8	20.5 ± 4.6	11	2	6	1

Table 5.4 Mean serum leptin concentrations (Mean ± SEM) according to smoking status and pregnancy outcome, with numbers of subjects that increased and decreased between 16 and 28 weeks of pregnancy.

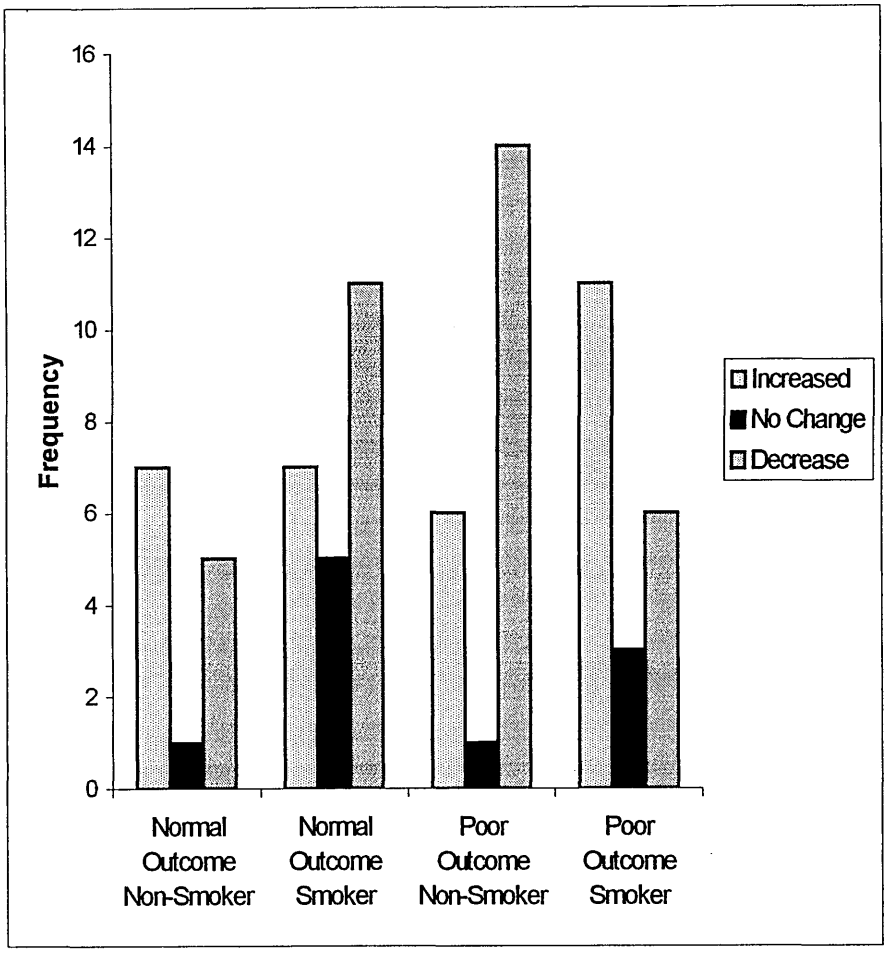


Figure 5.11 Histogram representation of the change in leptin between 16 and 28 weeks in subjects separated according to pregnancy outcome and smoking status.

5.3.3 Conclusion

5.3.3.1 Serum leptin and LBA levels during the menstrual cycle

The results of this study indicate that circulating leptin concentrations increase after ovulation in the luteal phase of the menstrual cycle. This increase was seen in 14 out of the 17 women included in cohort A. Levels of leptin showed no change in 3 women. The reason for this is unknown, but it may be due to differences in levels of reproductive hormones. This same increase in the luteal phase of the menstrual cycle was also seen in cohort B. In this study, leptin levels increased in 7 of the 8 women, the remaining woman's leptin level remained constant throughout the cycle. In a similar study, Hardie *et al.* (1997) also found that levels of leptin increased during the luteal phase of the menstrual cycle. Their study was, however, limited by the fact that it included only 6 regularly cycling women. In addition to similar changes seen throughout the menstrual cycle in both studies, the absolute levels of leptin in the serum were similar in both these studies and that of Hardie *et al.* (1997).

The observation that levels of leptin are higher during the luteal phase of the cycle than the follicular phase would agree with suggestions for a role for leptin in reproduction (Montague *et al.*, 1997) and, possibly, implantation (Cioffi *et al.*, 1997). The presence of leptin receptors in the ovary (Cioffi *et al.*, 1997) also suggests that it may have a role to play in ovarian function and the rise in leptin levels seen during the luteal phase supports the hypothesis that it interacts with the hypothalamo-pituitary-ovarian axis to help maintain the corpus luteum. The corpus luteum is the glandular body that develops in the ovary in place of the ovum that has been released at ovulation. It produces female sex steroids: progesterone and oestrogen. The maintenance of the corpus luteum is important for implantation processes.

Several studies have shown that serum leptin levels correlate well with BMI (Butte *et al.*, 1997). In this study, a significant correlation of BMI with serum leptin levels was also seen. The relationship between BMI and reproductive ability is well established. Gymnasts, marathon runners and others with low percentage body fat often have amenorrhoea, and puberty is delayed in females with low percentage body fat mass (Vogel, 1996). In addition, it has been shown that a critical fat mass (>10th centile) is required for ovulation in women (Frisch, 1990). In relation to this, it has been suggested

that leptin may serve as a signal to the reproductive system to inform it that stores are sufficient to sustain reproduction (Barash *et al.*, 1996).

It was noted that there was some interpersonal variation in the leptin levels measured, the reason for this is unknown although a study has shown that leptin levels in women are highly variable even when they have been normalised for BMI. However, subject 4 of cohort A, had a far higher serum leptin level than other subjects, with only a moderately raised BMI of 26.5 kg/m^2. A possible explanation for this anomaly is that she may have eaten prior to having the sample taken and hence, had a short-term change in metabolism. An alternative explanation may be the presence of a leptin receptor defect or a much greater adiposity than the other subjects. BMI is not a measure of adiposity but of total body weight compared with height and leptin levels have been shown to correlate better with measures of adiposity than with BMI (Conway *et al.*, 1997). In addition, there are other factors, which may effect serum leptin concentrations for example exercise (Friederich *et al.*, 1995), which were not controlled for in this study. A number of other factors including diurnal rhythms, food intake, cytokine and hormones are known to influence leptin levels. We can offer no clear explanation of the raised levels of leptin in this subject, which were consistent in all her samples.

In contrast with other reports (Hardie *et al.*, 1997) no correlation was seen between luteal phase progesterone levels and mean luteal phase leptin levels. In our study, progesterone measurements were only obtained at one point in the cycle, whereas they were obtained throughout the cycle in the study by Hardie *et al* (1997). Other studies, in women undergoing IVF have suggested levels of leptin correlate with oestradiol and FSH. We have not measured these in this study and it may be that leptin correlates better with these reproductive hormones than it does with progesterone.

In cohort B, LBA was measured. There was no significant difference between mean serum LBA in the follicular phase and mean serum LBA in the luteal phase of the menstrual cycle in this cohort of eight regularly cycling women. It is possible that a variation in serum LBA during the menstrual cycle may become apparent if more subjects were studied, although other studies investigating the fluctuation of cytokines and soluble receptors during the menstrual cycle used similar numbers of subjects. A trend towards lower LBA in those subjects with a higher BMI was observed, although this did not reach a level of significance. This observation, of a negative correlation

between serum LBA and BMI, has been reported by other workers measuring LBA by varying methods (Sinha *et al.*, 1996, Houseknecht *et al.*, 1996, Diamond *et al.*, 1997) and was significant in a larger study of postmenopausal Caucasian women (reported in chapter 6). As there is still some disagreement concerning the fluctuation of IGF-BPs during the menstrual cycle, it can be postulated that serum LBA may also be as contentious. The possibility that the soluble leptin receptor portion of the LBA varies with menstrual cycle phase cannot be excluded.

In summary, we have shown, in two cohorts of normal women, that serum levels of leptin increase during the luteal phase of the menstrual cycle, supporting the contention that leptin may have a role in reproduction. In addition, this fluctuation should be taken into account when studies, involving the measurement of leptin, are performed in female subjects. There is no evidence, from this study, that LBA has a role in the human menstrual cycle. Perhaps in this small cohort of normal fertile women, a small fluctuation or variation across the menstrual cycle would not have been recognised or, as with IGF-BP, the full role of LBA is still to be elucidated.

5.3.3.2 Effect of smoking status and pregnancy outcome on serum leptin levels during pregnancy

In this study, serum leptin levels were arithmetically lower in subjects with a poor pregnancy outcome compared to a normal pregnancy outcome at both 16 and 28 weeks: this reached a level of significance at 28 weeks. Lage *et al.*, (1999) reported that serum leptin levels increased throughout the first six months of pregnancy, but were low in women who had a spontaneous abortion in the first trimester of pregnancy.

In this study of pregnant women, two subjects had extremely high leptin concentrations at week 16 of pregnancy, these levels had decreased dramatically by week 28 of pregnancy. Both of these women were obese with BMIs of 30.7 and 40.5 kg/m^2. It is likely that this is the reason for their extremely high leptin level at week 16. Both of these women had a normal pregnancy outcome. We can conclude, therefore, that this decrease in leptin level by 28 weeks of pregnancy did not affect their pregnancy outcome.

Leptin levels are reported to be lower in smokers than non-smokers (Wei *et al.*, 1997). This study confirms that leptin is reduced in smokers compared to non-smokers even in pregnancy. In this study, as in others, leptin levels were higher than the reported levels for non-pregnant subjects. It is possible that the difference in leptin levels seen in smokers and non-smokers may be due to differences in BMI. Smokers have a lower BMI than non-smokers (Nicklas *et al.*, 1999). This may be a direct effect of increased lipolysis. Smoking increases secretion of catecholamines, which in turn increases lipolysis and thus the breakdown of adipose tissue occurs. If there is less fat mass present, the amount of leptin secreted will also be less. This is complicated by the fact that during pregnancy, other sites of leptin production are also present. Festa *et al.* (1999) report that the increase in leptin levels in their study of pregnant women were independent of BMI and fasting insulin. During pregnancy, BMI is not an adequate measure of adiposity, however in this and other studies of pregnant subjects, BMI does correlate with serum leptin levels (Hassink *et al.*, 1997, Helland *et al.*, 1998).

Smoking during pregnancy is associated with a reduction in foetal growth and an increase in foetal mortality. Smoking during pregnancy, in fact, carries a low risk for poor pregnancy outcome, as many women who do not smoke have a poor pregnancy outcome. Mathews *et al.* (1999) have shown an increase in the likelihood of a poor pregnancy outcome with a dose-dependent increase in cotinine levels. However, there is now evidence to suggest that smoking is responsible for adverse affects on child development (Trasti *et al*, 1999). The placentae of women who are heavy smokers are extremely large at delivery. The placental weight, in these cases, is primarily made-up of the peripheral villous tree. This section of the placenta in known to adapt to stress, particularly hypoxia (Kadyrov *et al*, 1998). As leptin is an angiogenic factor, this increase in the peripheral villous tree may be reflected by the amount of leptin produced by the placenta. Smoking causes impairment in the transport of oxygen across the placenta. It is speculated that the placenta, in trying to increase its surface area to increase oxygen exchange, in turn causes the disproportionately large placenta at birth. During pregnancy, nicotine metabolism is altered. Various cytochrome P450 elements are responsible for nicotine metabolism in the non-pregnant situation. However, these genes are not expressed by the placenta and therefore, metabolism of nicotine by the placenta is altered (Pasanen, 1999).

In these studies, a decrease in serum leptin concentration was observed between 16 and 28 weeks in subjects who were non-smokers but had a poor pregnancy outcome. It can be postulated that leptin is a necessary factor in placental development and therefore in having a good pregnancy outcome. Perhaps, the leptin levels were decreased in this group of pregnant women, as placental function, or even the foetus itself is not undergoing the correct developmental procedure. Poor pregnancy outcome may occur as a result of poor placental function. This decrease in placental function may cause a decrease in placental leptin production and secretion and as such a decrease in maternal serum leptin levels. Leptin's action as an angiogenic factor, may increase the formation of new blood vessels (Bouloumie *et al.*, 1998, Sierra -Honigmann *et al.*, 1998) and, therefore, it may act in an autocrine manner in the placenta. Feedback to leptin receptors expressed in the placenta may occur, resulting in the formation of blood vessels and increasing responses to the needs of the foetus. Leptin is known to be highly elevated in severe pre-eclampsia, this may be the placenta responding to the hypoxic situation however, leptin levels in mild pre-eclampsia are not elevated (Mise *et al.*, 1997). It is possible, as leptin is believed to have importance in implantation processes, that the change in leptin is most important in the early weeks of pregnancy, prior to this study being performed. The change in leptin observed between the two time-points is likely to be of more clinical significance. This is primarily due to the fact that starting BMI does not have as much influence on this measurement. By 28 weeks of gestation, the outcome of the pregnancy can be assessed. It is possible that measurement of leptin at two earlier time-points in pregnancy would be an indicator of poor pregnancy outcome, whether the subject does or does not smoke.

As only a small proportion (10%) of all births result in a poor pregnancy outcome, and of these deliveries, smoking affects only a few of these cases, it may be that a low leptin level is a more notable risk factor, as it will encompass all poor pregnancy outcomes, not just those seen in smokers. From this study we can state, that there is no single determinant of poor pregnancy outcome, although leptin does appear to be a constituent of this multifactoral disorder.

Chapter 6

Leptin and its Receptor in the Human Endometrium

6.1 Introduction

Numerous studies have shown that leptin has an important function in mammalian reproductive processes. *Ob/ob* mice do not produce a functional leptin protein. Both males and females are extremely obese and infertile. This infertility can be completely reversed when the mice are treated with recombinant leptin (Ahima *et al.*, 1997). Supplementation of leptin allows the mice to progress through puberty and to become fertile. Female hamsters that are treated with recombinant mouse leptin show increased sexual behaviour, providing evidence for a role for leptin in female reproduction. These processes may be brought about by leptin signalling. Leptin binds to receptors in the hypothalamus, whereon a neuroendocrine signal is passed to the pituitary gland and onto the gonadal axis via gonadotrophin releasing hormone. However, leptin receptors are also expressed in many peripheral tissue including those of the reproductive tract, such as the ovary and testes (Cioffi *et al.*, 1997, Hoggard *et al.*, 1997). The presence of leptin receptor isoforms, particularly the long signalling form, in the reproductive tract, suggests that leptin, in addition to the effect on hypothalamo-pituitary-gonadal axis, may also have a direct effect on these tissues and consequently a direct effect on reproductive function.

6.1.1 The endometrium

The human uterus consists of three histologically separate layers,

1. the perimetrium, the outer layer this aids in anchoring the uterus in the abdominal cavity
2. the myometrium, the thick middle layer of the uterus, it consists of three layers of smooth muscle (shown in figure 6.1)
3. the endometrium, this is the inner layer of the uterus, this layer is vitally important for the implantation of the embryo and is the layer studied in this chapter (shown in figure 6.1).

The endometrium can be subdivided into two layers, the *stratum functionalis* and the *stratum basalis* (Figure 6.1). The *stratum functionalis* surrounds the entire lumen of the uterine cavity and is shed during menstruation. The *stratum basalis* is in direct contact with the myometrium and is a permanent layer; it is this layer that gives rise to the

stratum functionalis. The *stratum functionalis* is made up of two types of cells, stromal and epithelial cells. During each cycle, the uterus prepares to receive an embryo and readies itself to provide nourishment for a foetus. These preparations are reflected in the cellular changes taking place at the level of the endometrium. The *stratum functionalis* proliferates during the menstrual cycle, the layers of cells (both stromal and epithelial) thicken and after ovulation, deep endometrial glands develop. The stromal and epithelial cells of the endometrium have very different functions. The stromal cells are destined to become the decidua and then the maternal side of the placenta. The epithelial endometrial cells are in two forms: the luminal epithelium, cells that line the surface of the endometrium and the glandular epithelium, cells that line the surface of the endometrial glands. The epithelial cells are important in the early stages of implantation, as it is these cells that the blastocyst align against and then attach to. If fertilisation and then implantation do not occur, the excess endometrial tissue is shed, this is menstruation.

The endometrium is extremely important in implantation. Implantation involves the attachment of the blastocyst to the uterine surface. There are two important factors necessary for successful implantation, the production of a blastocyst able to implant and the development of an endometrial surface that is receptive to the blastocyst.

It is well established that the preparation of the endometrial lining for implantation and pregnancy is directly influenced by hormones, specifically the female sex steroid hormones, oestrogen and progesterone. It is believed that these hormones act as the initiators of a complex molecular pathway, involving cytokines and growth factors, that eventually leads to successful implantation. Many of the tissues involved in implantation produce and/or respond to cytokines. The human endometrium actively secretes and reacts to cytokine stimulation. Cytokines of particular importance are the interleukin (IL)-1 family, IL-6, tumour necrosis factor-α (TNF-α) and leukaemia inhibitory factor (LIF). Implantation is believed to occur at a point in the menstrual cycle where the endometrium is optimally receptive to receive an embryo. This is known as "the implantation window" (Simon *et al.*, 1996). Cytokines and growth factors are thought to have important roles in controlling the receptivity of the endometrium for an implanting embryo.

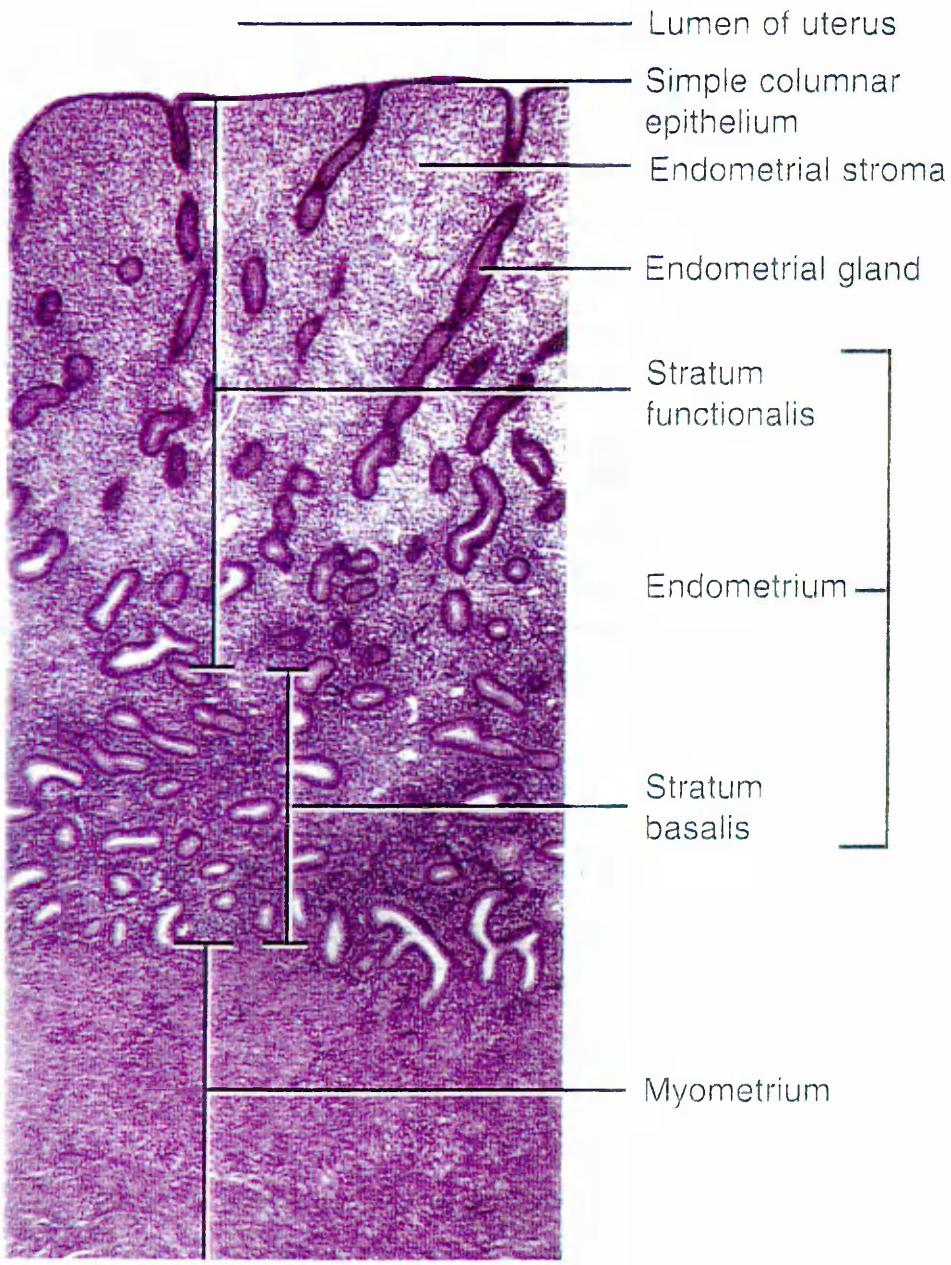


Figure 6.1 The histology of the human endometrium and myometrium. (Taken from Tortora, 1997)

Several studies have identified the importance of IL-1 in the human endometrium. IL-1β mRNA and protein have been detected in the endometrial layer (Simon *et al.*, 1995), and IL-1 type I receptor is present throughout the menstrual cycle (Simon *et al.*, 1993). Plasma IL-1 levels increase in the luteal phase of the menstrual cycle (Cannon *et al.*, 1995), indicating that IL-1 may be under the control of female sex hormones. *In vitro* studies have shown that IL-1 induces IL-6 production in endometrial stromal (Semer *et al.*, 1991) and epithelial cell cultures (Laird *et al.*, 1994). TNF-α is present in the human endometrium, and studies have suggested that production is greatest during the late proliferative phase of the cycle, although a second peak during the mid-secretory phase has been described (Laird *et al.*, 1996, Von Wolff *et al.*, 1999). The production of TNF-α, in epithelial endometrial cell cultures, is stimulated by IL-1 in a concentration-dependent manner (Laird *et al.*, 1996b). Further to this, the production of LIF in human endometrial cell cultures is controlled in part by IL-1 and TNF-α (Arici *et al.*, 1995, Laird *et al.*, 1997).

Leptin is known to interact with cytokines in other tissues. Murine studies have shown that leptin stimulates proliferation and TGF-β expression in the glomerular endothelial cells of the kidney (Wolf *et al.*, 1999). In studies of a murine adipose cell line, 3T3-L1, Granowitz (1997) demonstrated that treatment of the cultures with TGF-β caused an increase in *ob* gene expression. Treatment of the cultures with IL-1β, IL-6, IL-11 and TNF-α caused a decrease in *ob* gene expression. Luheshi *et al.*, (1999) have demonstrated that leptin actions in the brain depend on IL-1. In their studies of rats, they showed that leptin causes the release of IL-1 in the central nervous system (CNS). They state that the actions of leptin on appetite and temperature regulation are controlled by two different mechanisms, with only temperature involving cyclooxygenase products.

Leptin has an important role in many female reproductive processes. As cytokines are important factors for successful implantation and plasma leptin levels vary throughout the menstrual cycle it can be postulated that leptin may also have a role in endometrial function and embryo implantation. This chapter described studies which were carried out in order to determine if any leptin receptor isoforms were present in endometrial tissue, if leptin had a direct effect on the proliferation of the cells of the endometrium and, finally, if leptin brought about any changes in cytokine secretion from these cells.

6.2 RT-PCR Analysis of three leptin receptor isoforms in human endometrial cells in culture.

RT-PCR was used to determine if the various leptin receptor isoforms were expressed in endometrial stromal and epithelial cells in primary culture. Endometrial biopsy samples were collected and cultured as described in chapter 2. The biopsies were each collected from a different woman, all of whom had regular menstrual cycles of 25-35 days in length. The biopsies, from which the endometrial cells were cultured, were collected on days 7, 14, 19 and 33 of the menstrual cycle. RNA from epithelial and stromal endometrial cells in culture was extracted, as described in the materials and methods chapter. Specific primers for three leptin receptor isoforms (listed in appendix II) were used to identify leptin receptor mRNA expression in the endometrial stromal and epithelial cultured cells. A positive control for each isoform of the leptin receptor was analysed alongside the stromal and epithelial cells. RNA extracted from human embryonic kidney 293 (HEK293) cells, or from normal human liver was used as the positive control for the short and variant forms of the leptin receptor. For the long form of the leptin receptor, RNA extracted from normal human liver was used, as the HEK 293 cells do not express this isoform of the leptin receptor. RT-PCR was carried out twice on mRNA extracted from stromal cell cultures and epithelial cell cultures prepared from the four different biopsies. Examples of expression in the cells prepared from biopsies taken in the early, mid and late phases of the menstrual cycle are shown.

6.2.1 Results of RT-PCR studies

Expression of the leptin receptor isoforms is shown in Figure 7.2. Figure 7.2a shows the expression of the long form of the leptin receptor. A band corresponding to the long form of the leptin receptor can be observed in the positive control of human liver and in endometrial epithelial cell cultures taken from biopsies on days 7, 14, and 33. A band was also observed, of the size corresponding to the long form of the leptin receptor, in the endometrial epithelial cell culture taken from a biopsy on day 19. There was no positive RT-PCR result for the long form of the leptin receptor in RNA from any of the endometrial stromal cell cultures taken from biopsies on days 7,14, 19 and 33.

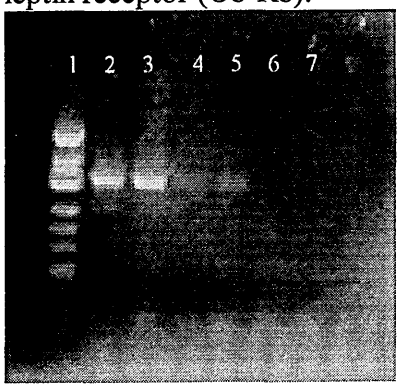
Figure 7.2b shows the expression of the short form of the leptin receptor in RNA extracted from endometrial epithelial and stromal cells in culture taken from biopsies on days 7, 14, and 33. A band corresponding to the short isoform of the leptin receptor can

be seen in the positive control of HEK293 cells but, not in the human liver sample. Expression of the short form of the leptin receptor in this samples has been observed (photograph not shown). Expression of the short form of the leptin receptor was observed in both stromal and epithelial cell cultures prepared from biopsies taken on days 7, 14, and 33. Expression of the short isoform of the leptin receptor was also observed in endometrial stromal and epithelial cell cultures prepared from a biopsy taken on day 19 of the menstrual cycle.

Figure 7.2c shows the expression of the variant form of the leptin receptor. This isoform has an intermediate length intracellular domain. Expression of the variant form of the leptin receptor was been observed in human liver. Both HEK293 cells and human liver have been shown to express this form of the leptin receptor (photograph not shown). Expression of the variant form of the leptin receptor was observed in both endometrial stromal and epithelial cell cultures prepared from biopsies taken on days 7, 14, and 33. Expression of this isoform was also observed in endometrial stromal and epithelial cultured cells prepared from a biopsy taken on day 19 of the menstrual cycle.

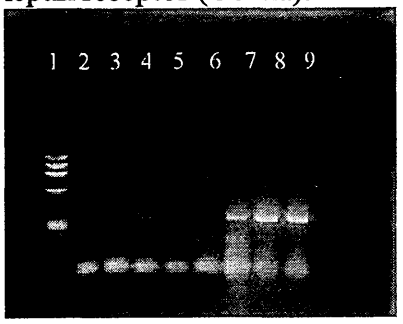
Each RT-PCR reaction contained the same amount of RNA (4μg); therefore, it is possible to comment on the amount of expression in each of the samples. These studies have shown that there is a high level of expression in the liver of the long form of the leptin receptor. Comparing the three endometrial epithelial cell culture samples, there is a higher level of expression of the long form of the leptin receptor in the cells cultured from the biopsy taken on day 7 of the menstrual cycle, than in those samples taken on days 14 and 33. When considering the expression of the short form of the leptin receptor in endometrial stromal and epithelial cell cultures, there is very little expression of this isoform of the leptin receptor in the epithelial cells compared to the stromal cells. The levels of expression appear uniform in the epithelial cells, but the level of expression is varied in the stromal cells. Expression of the short form of the leptin receptor is higher in the samples taken on days 14 and 33 than on day 7. The variant isoform of the leptin receptor is expressed at a consistently high level in all samples of stromal cells in culture. Expression of this isoform in the epithelial cells in culture were lower compared to the stromal cells, however there was little difference in expression in each individual sample, suggesting uniform expression throughout the menstrual cycle.

Figure 6.2a. The long isoform of the leptin receptor (Ob-Rb).



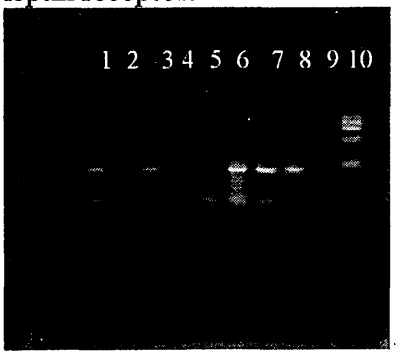
Lane	Sample
1	DNA Hinf I Marker
2	Human Liver
3	Epithelial cells Day 7
4	Epithelial cells Day 14
5	Epithelial cells Day 33
6	Stromal cells Day 14
7	Negative

Figure 6.2b. The short isoform of the leptin receptor (Ob-Ra).



Lane	Sample
1	DNA Hae III Marker
2	Human liver
3	Epithelial cells Day 7
4	Epithelial cells Day 14
5	Epithelial cells Day 33
6	Stromal cells Day 7
7	Stromal cells Day 14
8	Stromal cells Day 33
9	HEK 293 cells

Figure 6.2c. The variant isoform of the leptin receptor.



Lane	Sample
1	Epithelial cells Day 7
2	Epithelial cells Day 14
3	Epithelial cells Day 33
4	HEK293 cells
5	Human Liver
6	Stromal cells Day 7
7	Stromal cells Day 14
8	Stromal cells Day 33
9	Negative
10	DNA Hae III Marker

Day of Cycle	Leptin Receptor Isoform		
	Short (Ob-Ra)	Long (Ob-Rb)	Variant
7	++	+++	++
14	+	+	+
19	+	+	+
33	+/-	++	++

Table 6.1a Leptin receptor isoform expression in endometrial epithelial cells in culture.

Day of Cycle	Leptin Receptor Isoform		
	Short (Ob-Ra)	Long (Ob-Rb)	Variant
7	+++	-	++
14	+++	-	++
19	+++	-	++
33	+++	-	++

Table 6.1b Leptin receptor isoform expression in endometrial stromal cells in culture.

6.3 Proliferation of endometrial cells in culture in response to leptin

Since leptin receptors were expressed by both stromal and epithelial endometrial cells in culture, a tritiated-thymidine uptake assay was utilised to assess if the leptin receptor isoforms that were expressed in the endometrium, had a functional role in endometrial cell proliferation. This technique assesses DNA synthesis, transport of thymidine across the plasma membrane and phosphorylation of the thymidine by thymidine kinase prior to use in DNA synthesis (Burke *et al.*, 1995). The endometrial cells in culture (both stromal and epithelial) were seeded into 96-well plates, grown to confluency and incubated with tritiated thymidine in the absence or presence of various concentrations of leptin for 48 hours as described in chapter 2. There are large differences in the counts per minute (CPM) in two of the proliferation assays. This is due to a change in specific activity of the tritiated thymidine used in those assays. Statistical analyses were performed (f test, followed by the appropriate t test) to look at differences between control cells and cells stimulated with various concentrations of leptin. P values are expressed as follows: $p<0.05$ - *, $p<0.01$ - **, $p<0.001$ - ***

6.3.1 Results

The results obtained were very varied. In two of three experiments, leptin caused a significant increase in tritiated thymidine uptake by endometrial epithelial cells, while in the third experiment no significant effect was seen (Figure 6.3). For the endometrial stromal cells a significant increase in cell proliferation was also seen in three of the four experiments carried out (Figure 6.4). In all other experiments an increase in cell proliferation was also seen however, the effect was no significant. Every care was taken in order to keep culturing times the same; however, cells were used when they had reached confluence. Occasional cultures took longer to reach confluency.

In order to fully explain the effect of leptin on the expression of leptin receptor isoforms and on proliferation of the stromal and epithelial cells in culture, more biopsy samples from all stages (days) of the menstrual cycle should be studied.

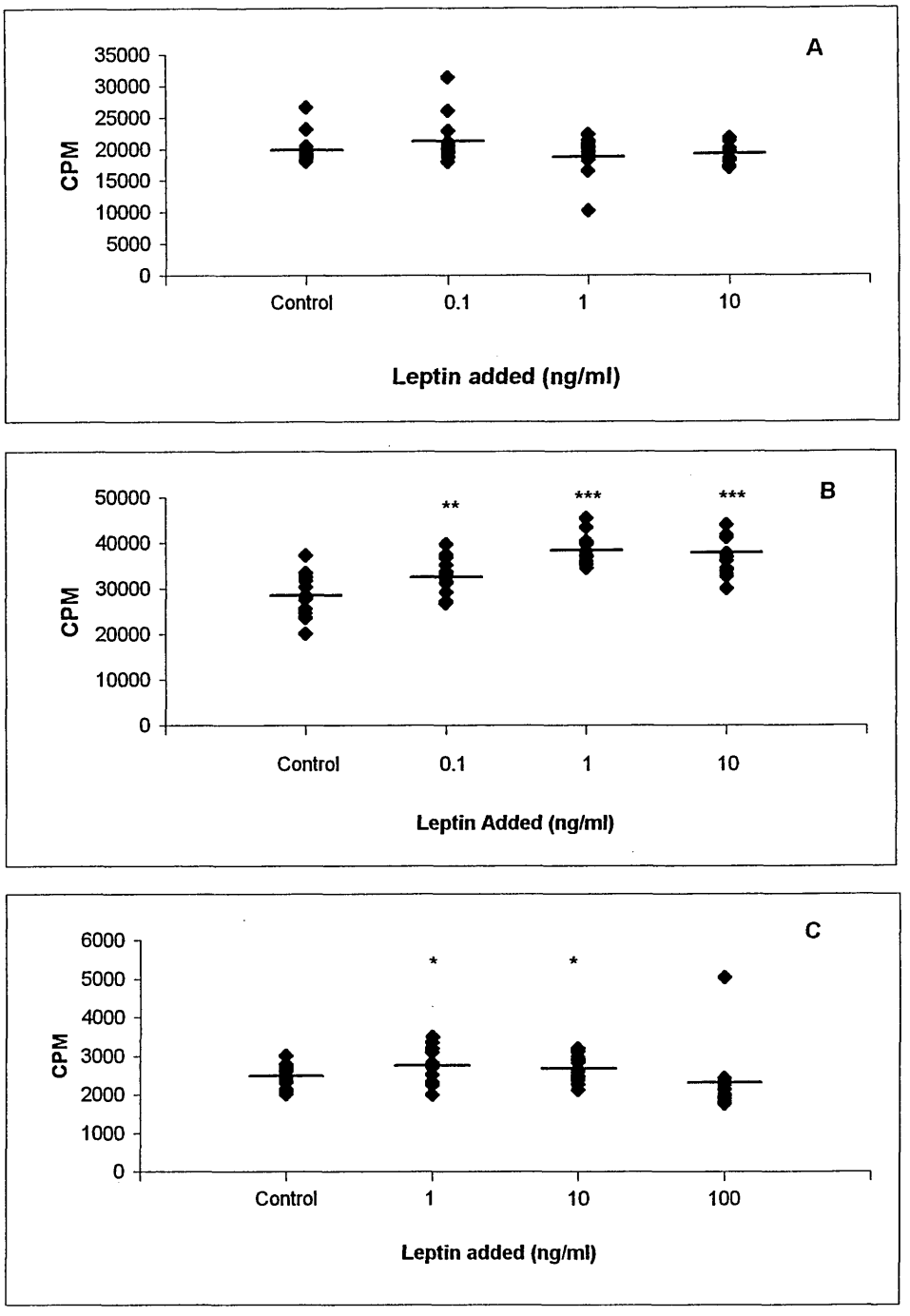
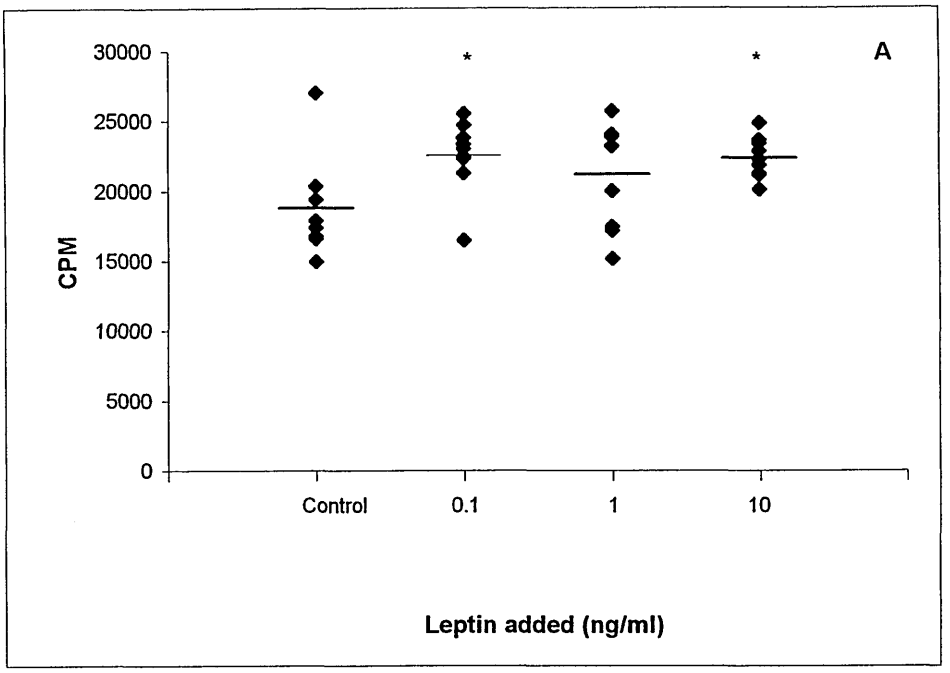


Figure 6.3 Proliferation of endometrial epithelial cells in response to leptin.
The cells were prepared from biopsies taken on days 8 (A), 9 (B), and 25 (C).
(Mean ± SEM). Statistical analyses were performed (f test, followed by the appropriate t test)
to look at differences between control cells and cells stimulated with
various concentrations of leptin.



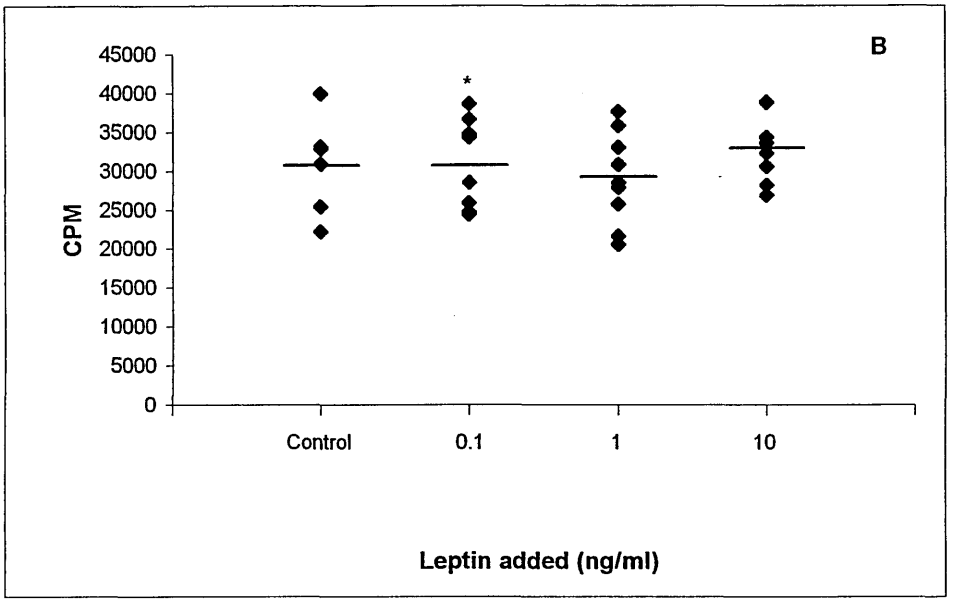
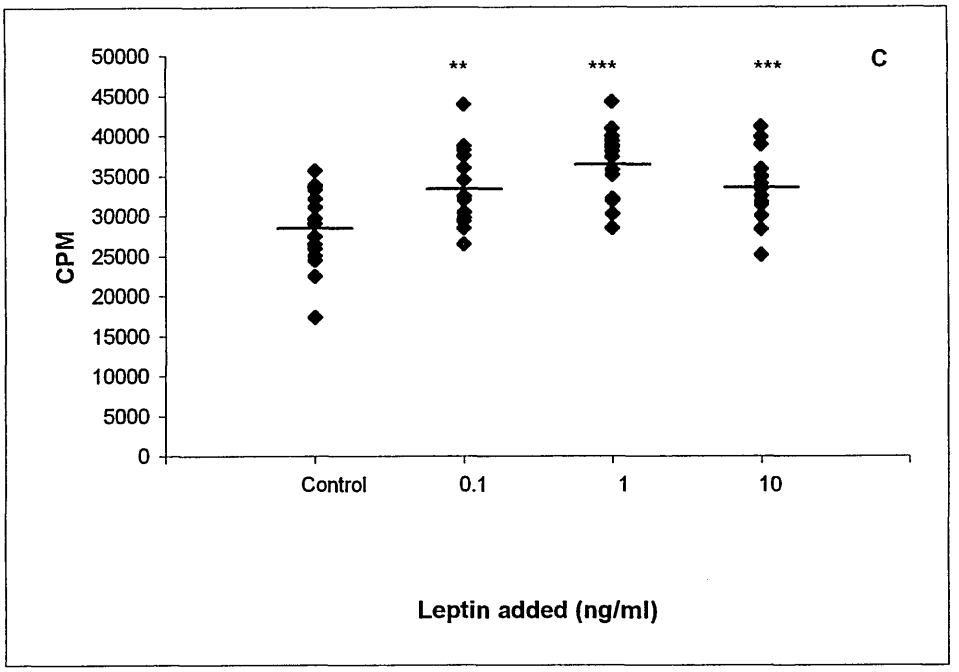


Figure 6.4a Proliferation of endometrial stromal cells in response to leptin.
The cells were prepared from biopsies taken on days 8 (A), 9 (B).
(Mean ± SEM). Statistical analyses were performed (f test, followed by the appropriate t test)
to look at differences between control cells and cells stimulated with
various concentrations of leptin.



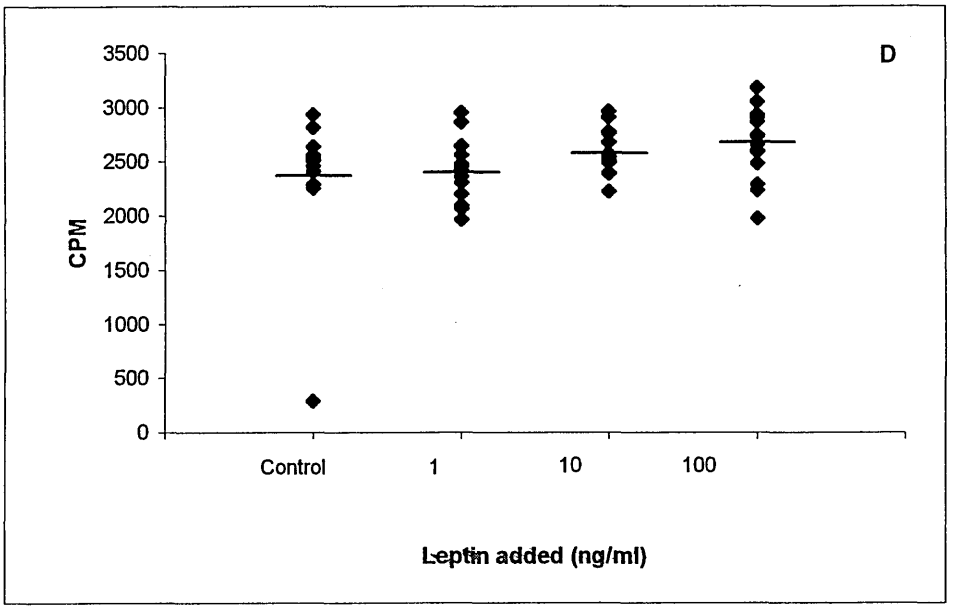


Figure 6.4b Proliferation of endometrial stromal cells in response to leptin.
The cells were prepared from biopsies taken on days 21(C) and 25 (D).
(Mean ± SEM). Statistical analyses were performed (f test, followed by the appropriate t test)
to look at differences between control cells and cells stimulated with
various concentrations of leptin.

6.4 The effect of leptin on the production of LIF and TNF-α by epithelial endometrial cells

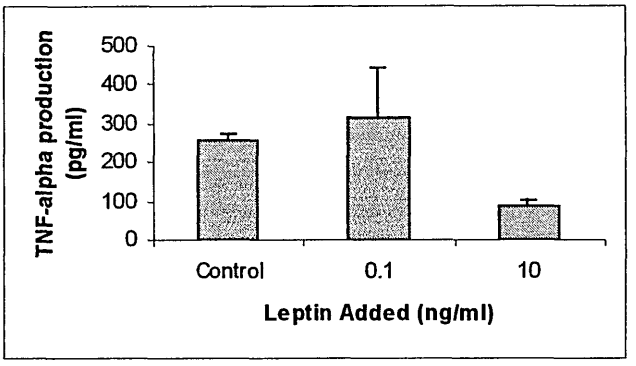
As incubation of endometrial epithelial cells in culture with leptin caused an increase in cell proliferation, studies to investigate the effect of leptin on cytokine production by these cells were carried out.

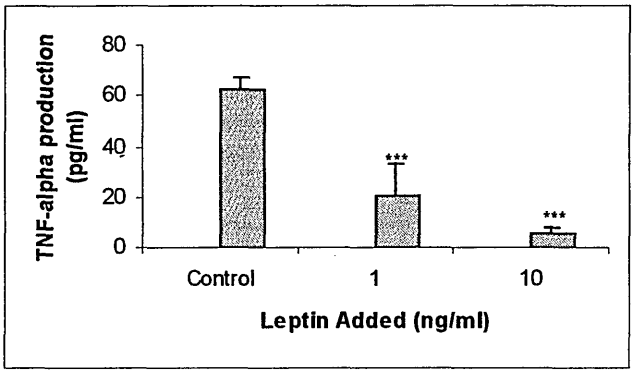
The supernatants from the proliferation assays were collected and stored at -20°C until required; these were then utilised to assess the production of the cytokines, LIF and TNF-α, by the epithelial cells of the endometrium in response to leptin. TNF-α and LIF production were only measured in the supernatants of epithelial endometrial cells as TNF-α and LIF are not normally detectable in the supernatants of stromal cells (Laird *et al.*, 1996, Laird *et al.*, 1997). Differences in TNF-α and LIF production by these cells in culture were assessed using an f test, followed by the appropriate t test. P values are expressed as follows: $p<0.05$ - *, $p<0.01$ - **, $p<0.001$ - ***

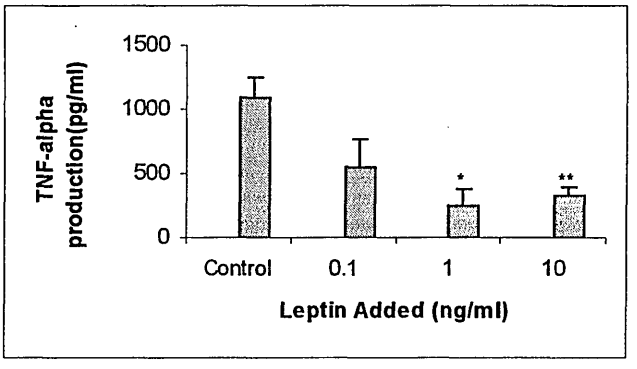
6.4.1 Results

Figure 6.5 shows the effects of leptin on TNF-α production by epithelial endometrial cells prepared from four different biopsies. Leptin caused a concentration-dependent decrease in TNF-α production, with significant decreases observed over a concentration range of 1.0-100 ng/ml of leptin. These cells had a concentration-dependent increase in proliferation in response to leptin. This is in contrast to the decrease in TNF-α production by the epithelial endometrial cells in culture. From these data, it can be concluded that the decrease in TNF-α secretion is a true response and is not affected by cell proliferation. More TNF-α was being produced by epithelial cells taken from biopsies in the early menstrual cycle on days 8 and 9. A similar decrease of TNF-α in response to leptin was observed in the cells taken form each biopsy.

Figure 6.6 shows the effects of leptin on LIF production by epithelial endometrial cells prepared from three different biopsies. In contrast to its effect on TNF-α, leptin had no significant effect on LIF production by endometrial epithelial cells.







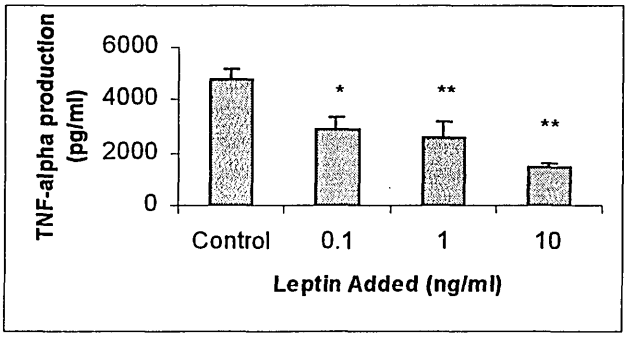
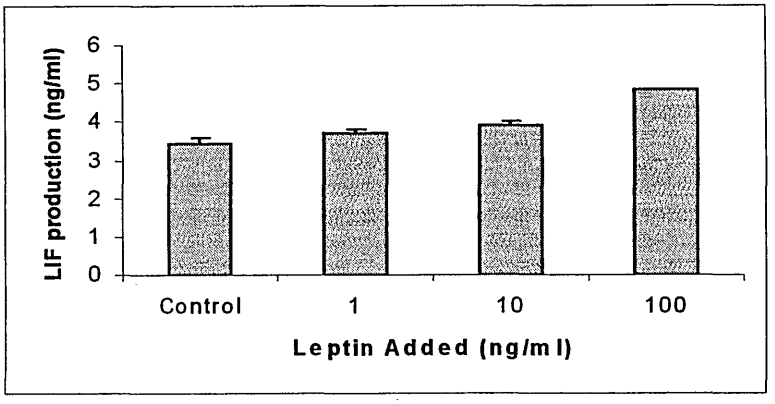
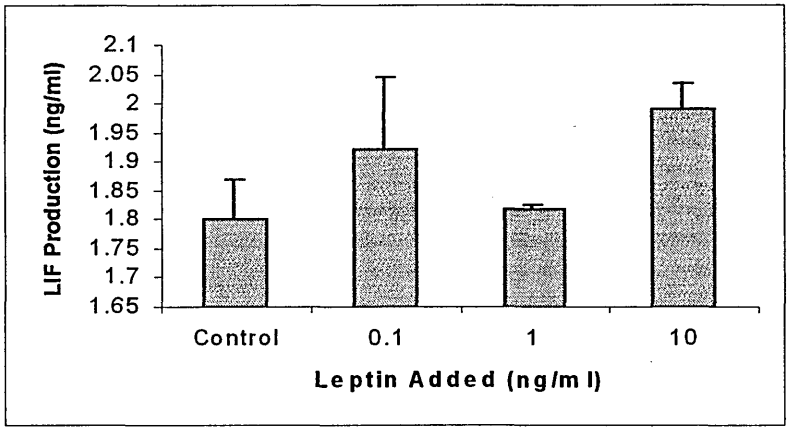


Figure 6.5 Production of TNF-α by the epithelial cells of the endometrial in response to leptin (0-100ng/ml) (Mean ± SEM). Statistical analyses were performed (f test, followed by the appropriate t test) to look at differences between control cells and cells stimulated with various concentrations of leptin.





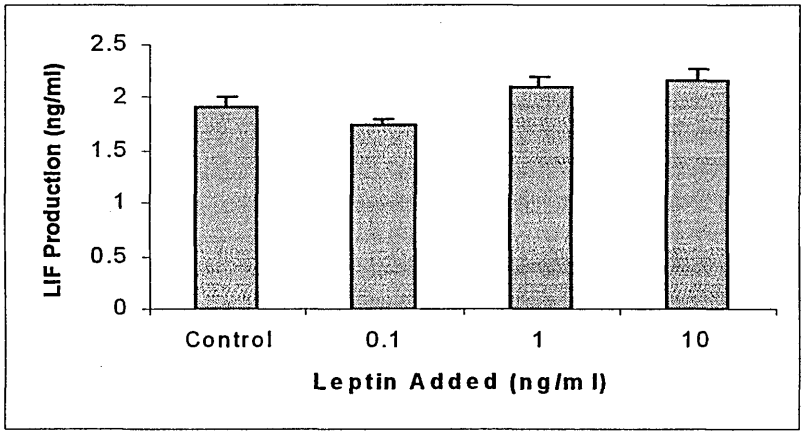


Figure 6.6 Production of LIF by the epithelial cells of the endometrium in response to leptin (0-100ng/ml) (Mean ± SEM). Statistical analyses were performed (f test, followed by the appropriate t test) to look at differences between control cells and cells stimulated with various concentrations of leptin.).

6.5 Conclusion

6.5.1 RT-PCR studies

The results from this study suggest that all three of the leptin receptor isoforms are expressed in the endometrium. The pattern of expression varies with the cell type, although all forms were expressed by epithelial cells while only the short and variant forms were expressed by the stromal cells. The results *in vitro* suggest that leptin receptor expression does vary during the menstrual cycle, although care should be taken between extrapolating between the *in vitro* and *in vivo* systems. These RT-PCR studies were not quantitative; however, as the same amount of RNA was used in each reaction, commenting on the response in the menstrual cycle is possible. The presence of the various isoforms of the leptin receptor in both cultured stromal and epithelial cells suggests that the leptin receptor is expressed *in vivo* and therefore, that leptin may have a direct functional effect on endometrial function and embryo implantation. Therefore, further work was carried out, in order to determine if these receptors were able to function in human endometrial cells.

6.5.2 Proliferation of endometrial cells in culture

The results from this initial study suggest that leptin may increase the growth of epithelial and stromal endometrial cells in culture. However, there was a lot of variability in the response seen and only a limited number of experiments were carried out. One reason for the variability seen may be that the cells were not cultured to complete confluency, although every effort was made to use the cells in the same state. It is known that cell density is an important factor affecting cell growth and therefore, different states of confluency may effect the cells proliferative response to leptin. This would lead to variation in the cell number and therefore to differences in proliferative response. If further studies confirm these results it would suggest that leptin may effect cellular growth *in vivo*. The proliferation in response to leptin at various concentrations in both stromal and epithelial cells in culture indicates that the leptin receptors present in these cell types are functional. As all the endometrial cells in culture are taken from different women, there may be some variation in leptin receptor function. Polymorphisms in the leptin receptor gene have been shown to affect ligand binding (Chapter 6), it is possible these genetic changes would effect cell proliferation in these studies. These results can be tentatively associated with expression of leptin receptor isoforms in the stromal and epithelial cells in culture. At the time when the epithelial

cells in culture express the long form of the leptin receptor (day 7), there was a significant increase in cell proliferation (day 9). When expression of the long form of the leptin receptor was reduced (day 14 and 19) there was a small increase in cell proliferation (day 25) in epithelial endometrial cells. This pattern is also seen with the short and variant forms of the leptin receptor in epithelial endometrial cells in culture. As the stromal endometrial cells do not express the long form of the leptin receptor, changes in cell proliferation were due to the short and variant isoforms. An increase in cell proliferation was observed early in the menstrual cycle (day 8 and 9), this is when the expression of the short isoform is low and variant isoform is high. Later in the cycle (day 21 and 25) when expression of the short form of the receptor increases, there is no increase in cell proliferation. In this situation, it can be postulated that this isoform is acting as a dominant negative element, with leptin binding to both the short and the variant forms, thus preventing cell proliferation.

6.5.3 The effect of leptin on the production of TNF-α and LIF by epithelial endometrial cells

Leptin caused a significant decrease in the production of TNF-α by epithelial endometrial cells. In these studies, epithelial cell TNF-α secretion decreases, in a concentration-dependent manner, in response to leptin. As leptin causes epithelial endometrial cells to proliferate and TNF-α secretion to decrease, it can be concluded that there must be a direct effect on TNF-α secretion, as a decrease in secretion would not reflect an increase in cell number. Laird *et al.* (1996) and Von Wolff *et al.* (1999) describes the production of TNF-α in epithelial endometrial cells throughout the menstrual cycle. TNF-α levels are maximal in the late proliferative phase, just prior to the LH surge and then decrease again in the secretory phase. This is in agreement with levels of TNF-α in serum (Brannstrom *et al.*, 1999). This decrease in TNF-α in the endometrium during the secretory phase, when serum leptin levels are high, together with the results of this study suggest that leptin reduces the amount of TNF-α locally in the endometrium. It is possible that leptin may be an important factor in reducing TNF-α by the endometrium in the late secretory phase, thus aiding implantation. At present, it is not known if leptin is expressed by human uterine tissues. Bovine studies have shown, by RT-PCR, the expression of leptin by uterine tissues during pregnancy (Takahashi *et al.*, 1998). RT-PCR for leptin transcripts in the endometrium or the

measurement of leptin levels in uterine flushings may allow consolidation of the hypothesis that leptin has a role, perhaps only indirectly, in implantation.

LIF production by epithelial endometrial cells was not affected by the addition of leptin to the growing cells in culture. This suggests that the effect of leptin on cytokine modulation in the epithelial cells of the endometrium may be specific for TNF-α.

The effect of leptin on the proliferation of endometrial stromal and epithelial cells and the decrease in TNF-α production by endometrial epithelial cells in response to leptin, suggests that functional leptin receptor isoforms are present in the endometrium.

As the long signalling form of the leptin receptor was present in the epithelial endometrial cells, proliferation of these cells in response to leptin may be controlled by this isoform of the receptor. The stromal endometrial cells did not express the long form of the leptin receptor but still proliferated in response to leptin. It is possible that in these tissues another form of the leptin receptor, either the short or variant form, can signal via the Jak/STAT pathway or by some other signalling pathway. Other workers have suggested that the short form of the leptin receptor can cause signalling to be induced. Murakami *et al.*, (1997) provide evidence of the short form of the leptin receptor initiating production of the early genes c-fos, c-jun and jun-B. Yamashita *et al.*, (1998) detected phosphorylation of the MAP kinase pathway in cells transfected with the short form of the leptin receptor. Therefore, it is possible that in the endometrium, leptin may initiate signalling through the short or variant forms of the leptin receptor, via a pathway other than Jak/STAT.

Chapter 7

Investigation of three single nucleotide polymorphisms in the leptin receptor

7.1 Introduction

Single nucleotide polymorphisms (SNP) are the most common genetic variants, with one occurring every 100-300 kb. The National Human Genome Research Institute (NHGRI) in the United States has recently reported its intensive effort to identify 50 000 new SNPs within three years. It is hoped that this will accelerate the identification of disease associated genetic changes. SNPs can be used to associate sequence variation with observed phenotypes and to investigate the functional aspects of the variation. Populations analysis or case-control groups can be used to identify associations between sequence variation and phenotype. This approach is far simpler than pedigree analysis as family data from successive generations is not required.

7.1.1 Linkage Disequilibrium

Linkage disequilibrium occurs when a combination of alleles of a set of genetic markers (a haplotype), occurs significantly more or less frequently in a population than would be expected. This information suggests that as the alleles have been inherited together, there has been less opportunity for recombination events to occur at meiosis and therefore, the physical distance between the alleles is small. In this case, the common allele of one polymorphism is inherited in combination with the common allele of the another polymorphism. Theoretically, a specific genotype within a population may be consistently associated with a phenotypic difference. As linkage disequilibrium exists, a haplotype of markers may also be associated with the phenotypic change. It is possible that polymorphisms within the leptin receptor gene are associated with phenotypic differences in body weight. One or more of these genetic changes may have a direct functional effect on phenotype or reflect linkage disequilibrium with another polymorphism nearby, possible in another gene.

7.1.2 Genetic studies of leptin receptor polymorphisms in a cohort of postmenopausal women

Inter-individual differences in BMI are highly heritable (Austin *et al.*, 1997, Rice *et al.*, 1999) and a proportion of this heritability may reflect genetic variations in the leptin and leptin receptor genes.

Several single nucleotide polymorphisms (SNPs) have been described in the human leptin receptor gene (Considine *et al.*, 1996, Matsouka *et al.*,1997). Three polymorphisms, in the extracellular domain of the leptin receptor gene, GLN223ARG, LYS656ASN and LYS204ARG, have been studied in this chapter. These three SNPs each result in an amino acid change in the coding region and, therefore, may have an effect on ligand binding or receptor dimerisation. The SNP, GLN223ARG, is associated with circulating leptin levels but not with body mass index (BMI) in non-diabetic Pima Indians (Thompson *et al.*, 1997). Homozygosity for the G allele, of this SNP, is associated with lower plasma leptin levels after correction for obesity and sex. LYS656ASN has been reported to be associated with lower BMI in a population of lean males (Gotoda *et al.*, 1997) and with waist circumference in young obese females (Wauters *et al.*, 1999). Similar associations may be observed in other populations. To investigate this, an allelic association study on a population of postmenopausal women from the Sheffield area was undertaken.

There are profound differences in the leptin system between males and females. Leptin has an important, though not fully understood, role in reproduction. Circulating leptin levels are higher in females than in males, even when corrected for differences in BMI (Hickey *et al.*, 1996) and vary significantly with the menstrual cycle (as discussed in Chapter 5 and reported by Hardie *et al.*, 1997). This may represent an important confounding factor in leptin studies and, as such, postmenopausal women were used in these investigations in order to minimise hormonal effects. GLN223ARG and LYS656ASN genotypes were determined with BMI, fat mass and serum leptin levels measured in order to investigate whether the functional correlations reported are also observed in female Northern European Caucasians.

Significant genetic associations with a particular SNP may indicate a direct effect on protein function, or simply reflect linkage disequilibrium with other polymorphisms within the same gene or nearby. In order to determine whether there is a measurable functional difference in ligand-binding capacity of the leptin receptor associated with the SNPs studied here, serum leptin-binding activity (LBA) was also measured. An amino acid change in the extracellular region of the leptin receptor may change the functional characteristics of the receptor. A soluble form of the leptin receptor exists in serum: serum leptin binding activity has been described in both humans and rodents, at least a proportion of this binding is attributable to the soluble extracellular domain of

the leptin receptor. Modulation of leptin activity by a soluble form of the receptor is consistent with observations in other cytokine systems, including IL-1, TNF- and growth hormone. Since all the receptor isoforms share the same extracellular domain, a difference in serum leptin-binding activity (LBA), associated with a particular allele, may indicate that other forms of the leptin receptor also have altered ligand-binding capacity or that it may affect receptor dimerisation or the affect the production of soluble receptor by the cell.

7.1.3 Aims of the study

Three leptin receptor polymorphisms were studied, LYS204ARG, GLN223ARG and LYS656ASN. All of these variations result in a change in an amino acid and therefore, may potentially cause variation in leptin binding to the extracellular domain of the receptor or in the signal transmitted on ligand binding. The aims of these studies were to:

- Genotype female postmenopausal subjects for the three SNPs present in the extracellular domain of the leptin receptor.
- To seek associations between these polymorphisms and BMI, fat mass and leptin levels.
- To investigate the effect of the amino acid changes on ligand-binding using the LBA assay.
- To determine the extent of linkage disequilibrium between these polymorphisms in the leptin receptor gene.

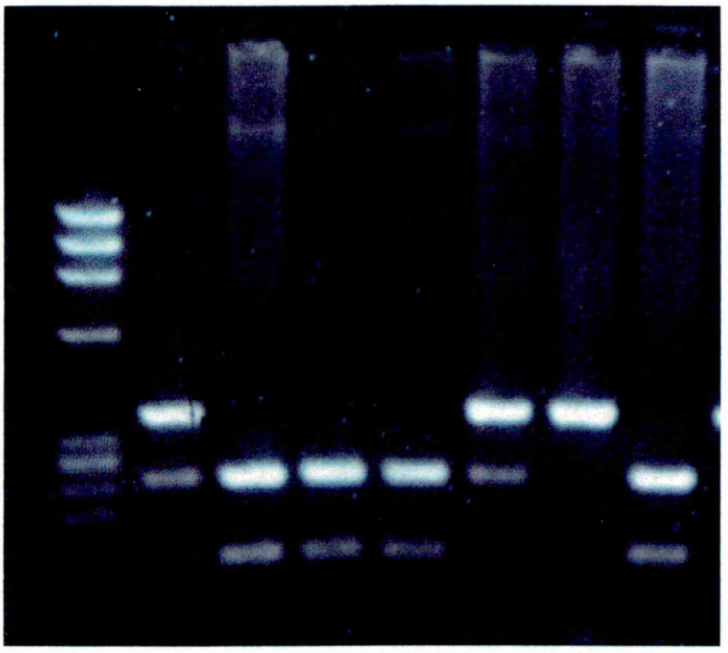
7.2 Study Design

Blood samples were 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 epidemiology study). Blood for genotyping studies (n = 225) was spotted onto standard neonatal blood cards and dried overnight before storage at room temperature. The mean age was 67.7 ± 4.6 years (mean ± SEM) (n = 220) and the mean BMI of the women was 26.9 ± 1.8 kg/m^2 (mean ± SEM) (n = 220). Clinical details were recorded at the time of sample collection. All subjects gave informed consent and the studies had the approval of the North Sheffield Local Research Ethics Committee local ethical committee. The women included in the study of leptin levels and LBA (n = 84) were selected as they were not currently

prescribed hormone replacement therapy (HRT) or corticosteroids, neither were any of them diabetic. Serum leptin levels were measured by Allison Lee (at the University of Sheffield) using the Linco Research Co. RIA kit. Serum LBA was measured in subjects where sufficient serum was available for the purpose.

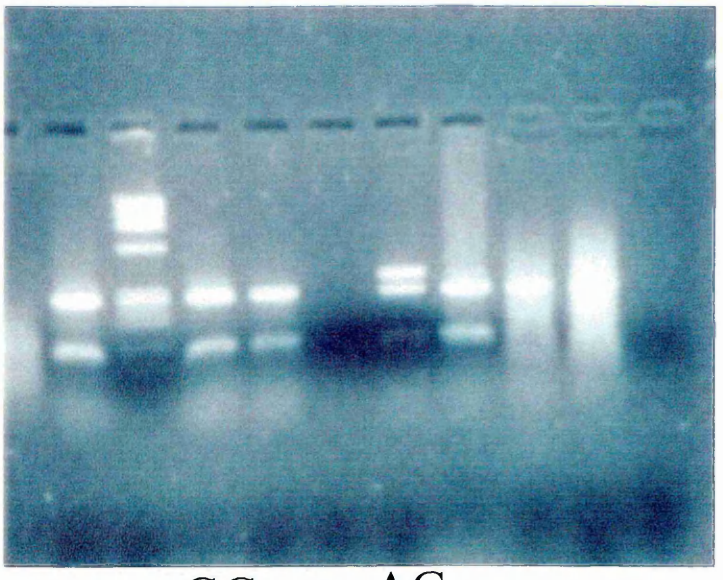
Genotyping was carried out as described in Chapter 2. The visualisation of the alleles for GLN223ARG is shown in Figure 7.1a, for LYS204ARG in Figure 7.1b and for LYS656ASN in Figure 7.1c.

A



GG AG AA

B



GG AG

Figure 7.1 Visualisation of the different genotypes of GLN223ARG (A) and LYS204ARG (B) single nucleotide polymorphisms of the leptin receptor.

C

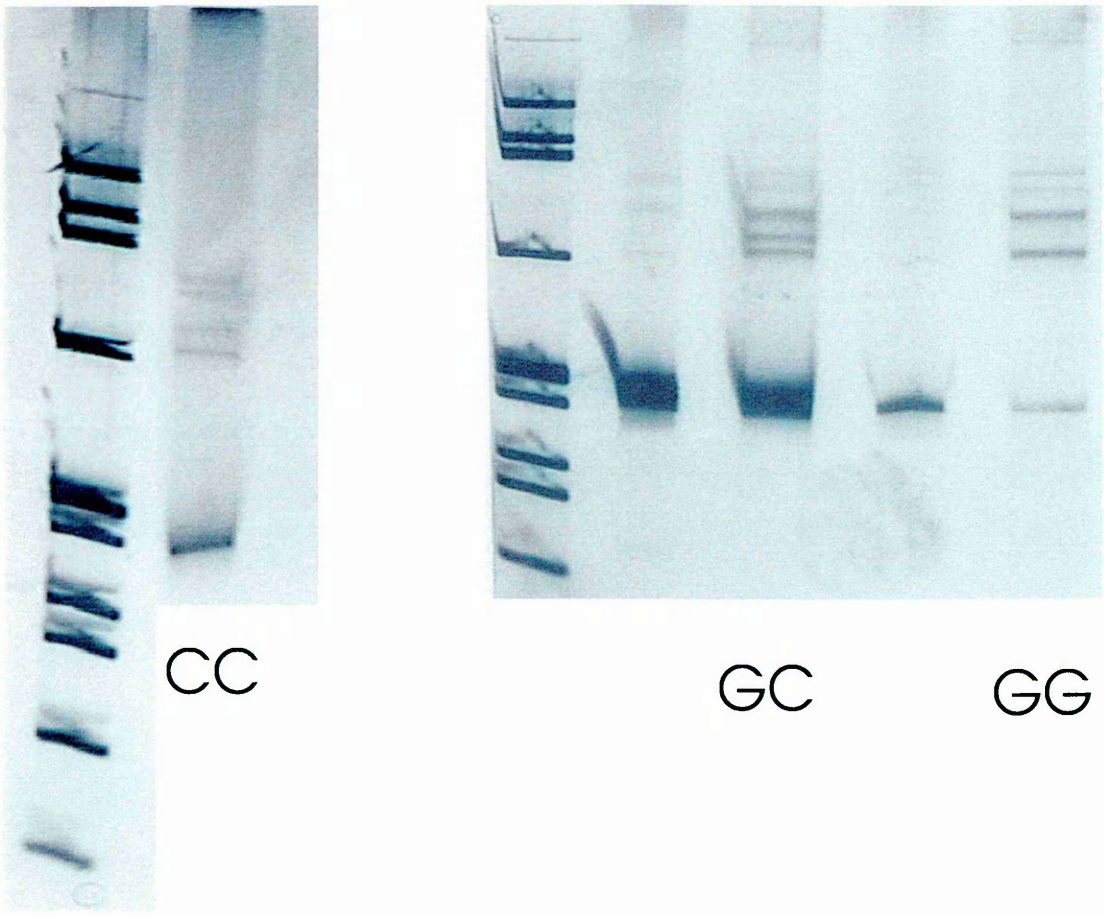


Figure 7.1 Visualisation of the genotypes of LYS656ASN (C) by SSCP.

7.3 Results

7.3.1 GLN223ARG

In order to determine allele and genotype frequencies, 223 subjects have been genotyped for the GLN223ARG SNP. The calculated allele frequencies correspond well with published work (Table 7.1). The balance of homozygotes and heterozygotes observed was as predicted by the Hardy-Weinberg equation from these allele frequencies (Table 7.2).

Data on BMI was available for 220 subjects. Homozygotes for the G allele had a lower mean BMI (25.4 $kg/m^2 \pm 4.5$) (Mean ± SEM) than A homozygotes (27.2 $kg/m^2 \pm 3.1$) and heterozygotes (27.2 $kg/m^2 \pm 2.6$) (Figure 7.2). Levels of fat and lean mass were assessed in a number of subjects (n = 89) where data was available. Homozygotes for the G allele had a significantly lower fat mass (22021g ± 5505) compared to the other genotypes (A homozygotes (26393g ± 4816) and heterozygotes (28895g ± 4405) (p = 0.006)) (Figure 7.3a). Levels of lean mass in those homozygous for the G allele (36186g ± 9047) did not differ when compared to the other genotypes (A homozygotes 35990g ± 6568) and heterozygotes (36613 ± 5581) (p = NS) (Figure 7.3b).

Serum leptin levels were measured in a subset of this cohort (n = 84). As expected, these correlated with BMI (n = 84, r = 0.687, p <0.001) (Figure 7.4) and with fat mass (n = 81, r = 0.719, p = 0.0001) (Figure 7.5). Homozygotes for the G allele had lower mean circulating leptin levels (12.6 ng/ml ± 3.2) than A homozygotes (18.3 ng/ml ± 3.6) and heterozygotes (23.8 ng/ml ± 3.6) (Figure 7.6)

In order to clarify whether the observed associations between the polymorphism GLN223ARG and BMI and leptin levels could be the result of a direct functional effect on the leptin receptor binding parameters, serum LBA was measured in a number of subjects in our cohort (n = 60), where sufficient serum was available. Serum LBA was significantly higher in individuals homozygous for the G allele (11.6% ± 3.1) compared to those homozygous for the A allele (10.5% ± 2.3) and the heterozygotes (9.7% ± 1.9) (p = 0.04) (Figure 7.7). A negative correlation was observed between BMI and LBA (n = 58, r = -0.253, p <0.05) (Figure 7.8), although no significant correlation was observed between LBA and serum leptin levels (Figure 7.9). This data is summarised in table 7.3.

Allele	Post-menopausal women (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*	Black males (n = 79) *Chagnon et al., 2000*	Japanese non-obese (n = 68) *Matsouka et al., 1998*	Japanese obese (n = 47) *Matsouka et al., 1998*
A	0.6	0.57	0.56				?
G	0.4	0.43	0.44				?

Table 7.1 Comparison of allele frequencies for GLN223ARG between this study and other published work

	Observed Genotype Frequency	Expected Genotype frequency	
	n	%	n
Heterozygotes	115	48	108
Homozygous A	78	36	81
Homozygous G	32	16	36
Total	225	100	225

Table 7.2 Data for Hardy-Weinberg equation and χ^2 analysis for GLN223ARG in this population of postmenopausal women using allele frequencies previously described.

	Genotype			P value
	AA	AG	GG	
BMI (kg/m^2) n = 220	27.2 ± 3.1	27.2 ± 2.6	25.4 ± 4.5	0.001
Fat mass (g) n = 89	26393 ± 4816	28895 ± 4405	22021 ± 5505	0.006
Lean mass (g) n = 89	35990 ± 6568	36613 ± 5581	36186 ± 9047	NS
Serum leptin concentration (ng/ml) n = 84	18.3 ± 3.6	23.8 ± 3.6	12.6 ± 3.2	0.0001
Serum LBA (%) n = 60	10.5 ± 2.3	9.7 ± 1.9	11.6 ± 3.1	0.04

Table 7.3 Mean levels of BMI, fat and lean mass, leptin and serum LBA for each genotype of GLN223ARG. P value is calculated by comparing those homozygous for the G allele with the other genotypes using the appropriate t test.

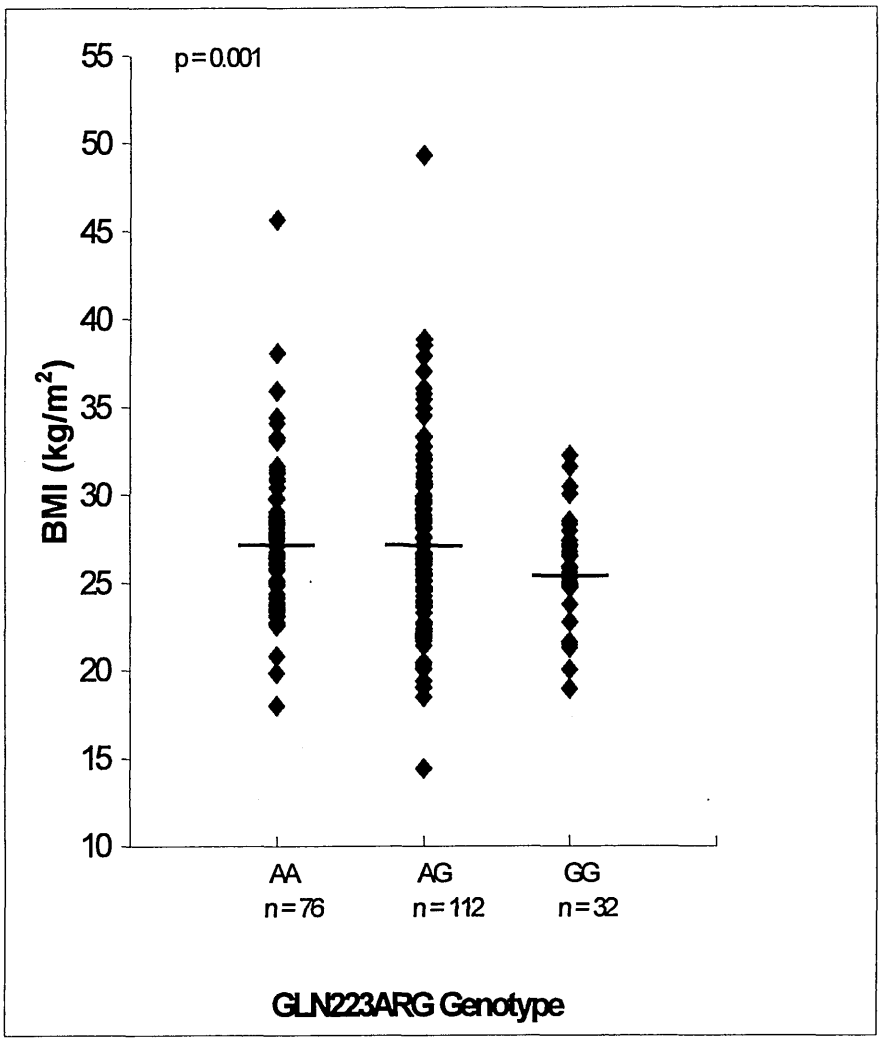
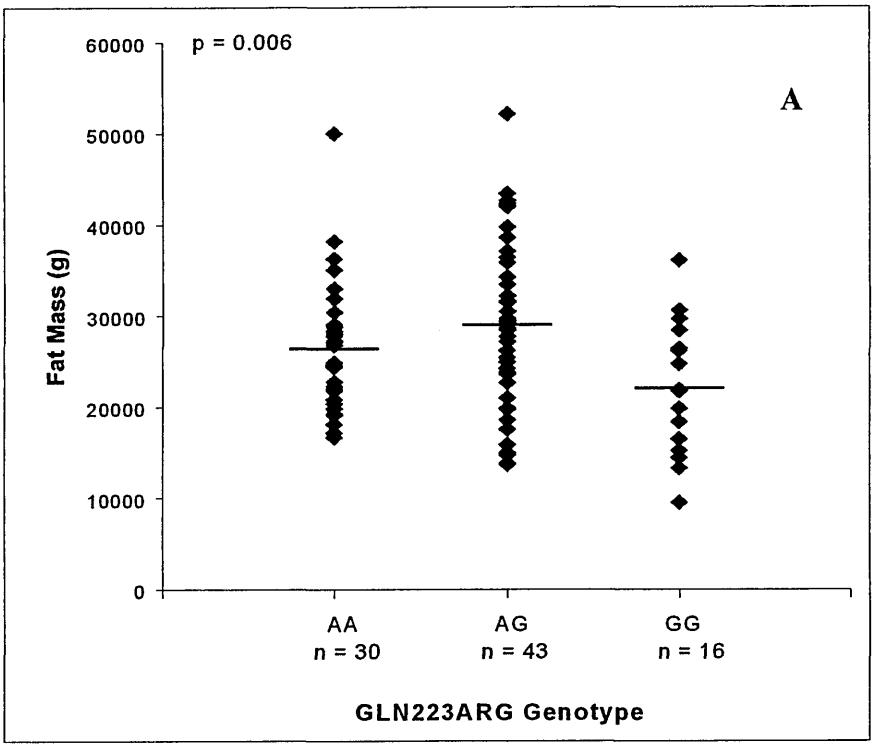


Figure 7.2 BMI and GLN223ARG genotype in the whole population group: mean BMI is significantly lower in those homozygous for the G allele compared to heterozygotes and those homozygous for the A allele.



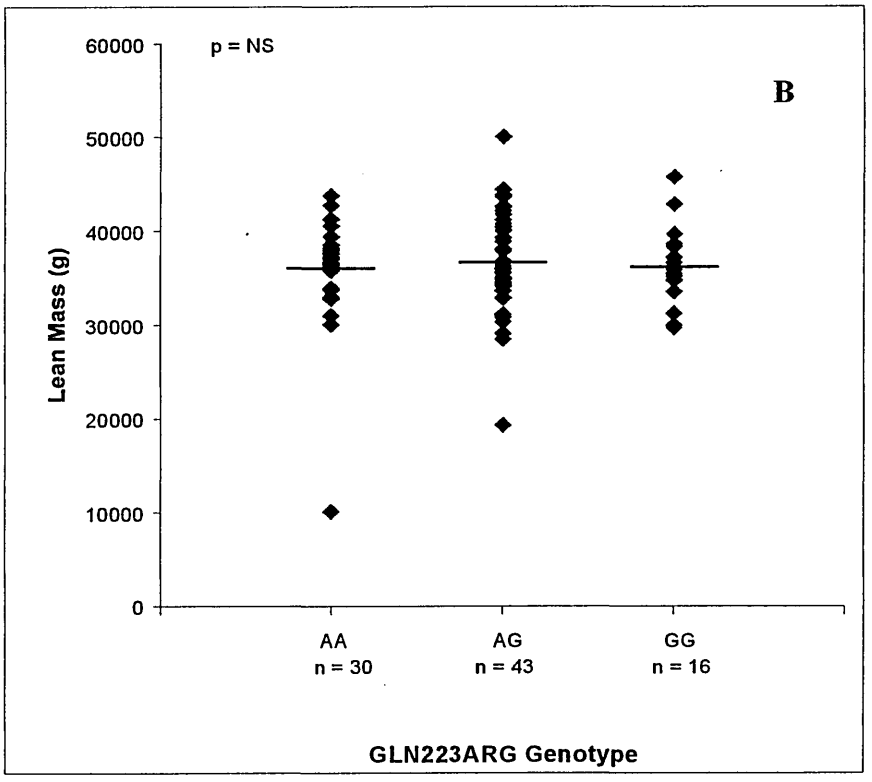


Figure 7.3 Fat mass (A) and GLN223ARG genotype: mean BMI is significantly lower in those homozygous for the G allele compared to heterozygotes and those homozygous for the A allele. Lean mass (B) and GLN223ARG genotype: no significant difference was observed.

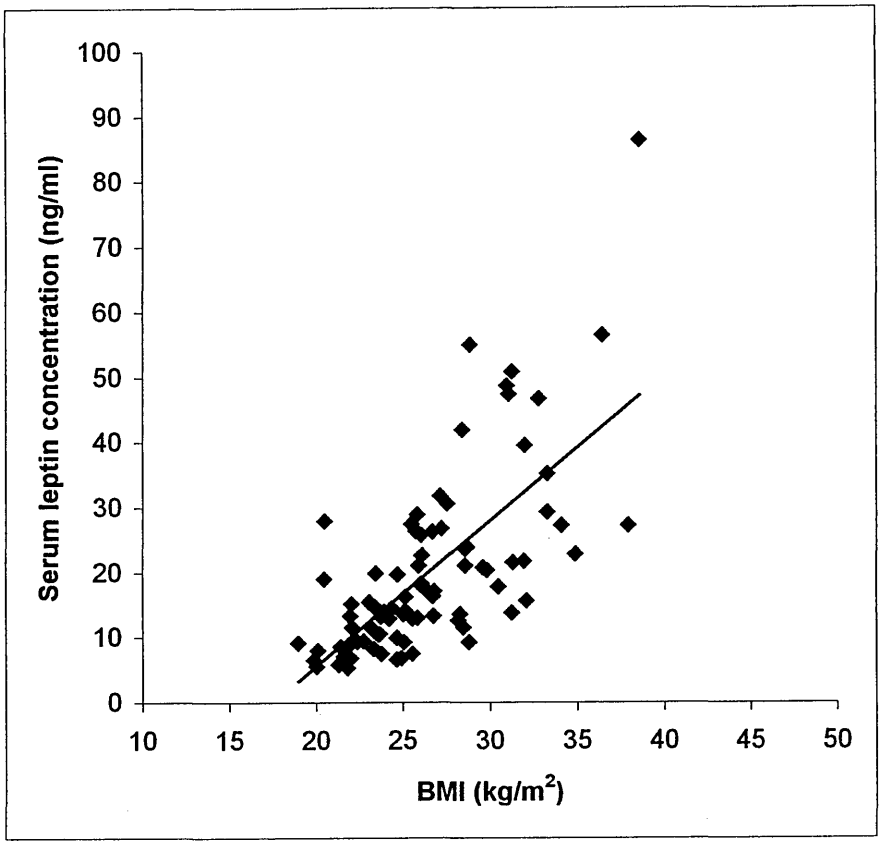


Figure 7.4 Correlation of serum leptin levels with BMI in this cohort ($r = 0.687$, $p = 0.001$)

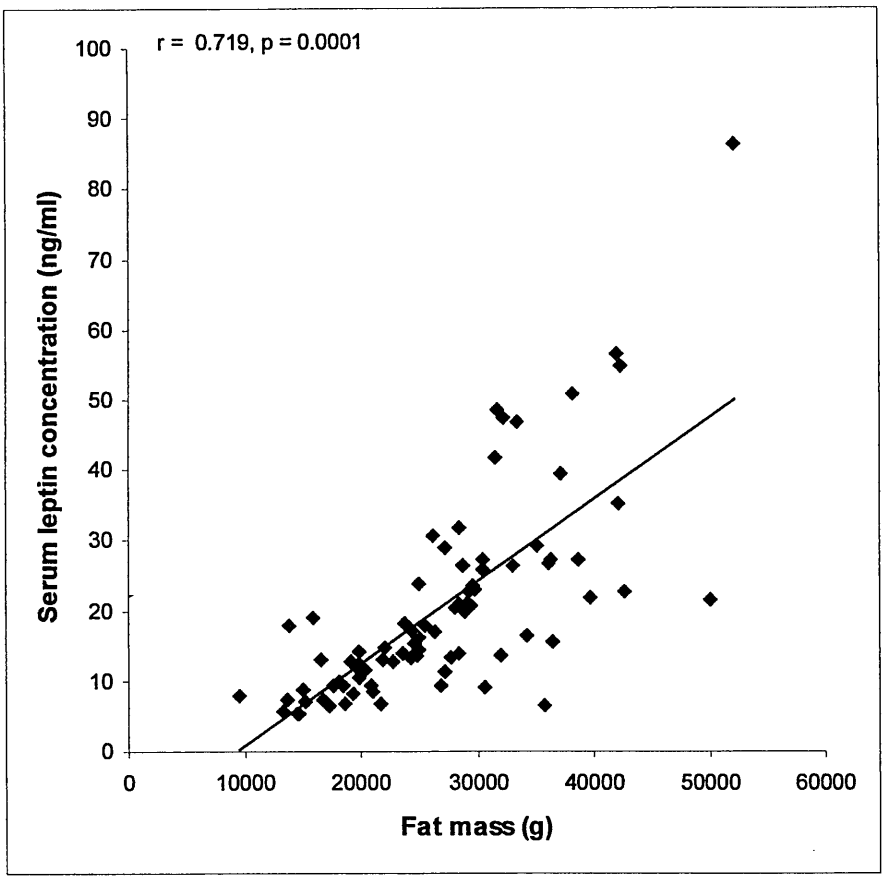


Figure 7.5 Serum leptin concentration correlates with fat mass in this cohort (r = 0.719, p = 0.0001)

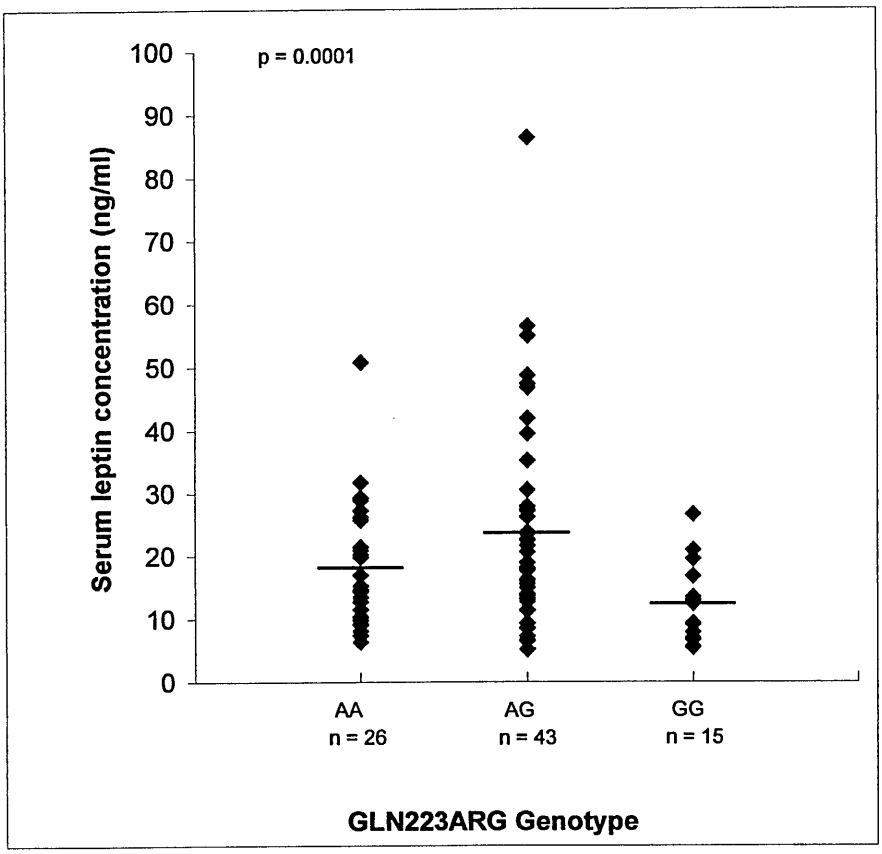


Figure 7.6 Serum leptin levels and GLN223ARG genotype: mean serum leptin levels are lower in those homozygous for the G allele compared to heterozygotes and those homozygous for the A allele ($p = 0.0001$).

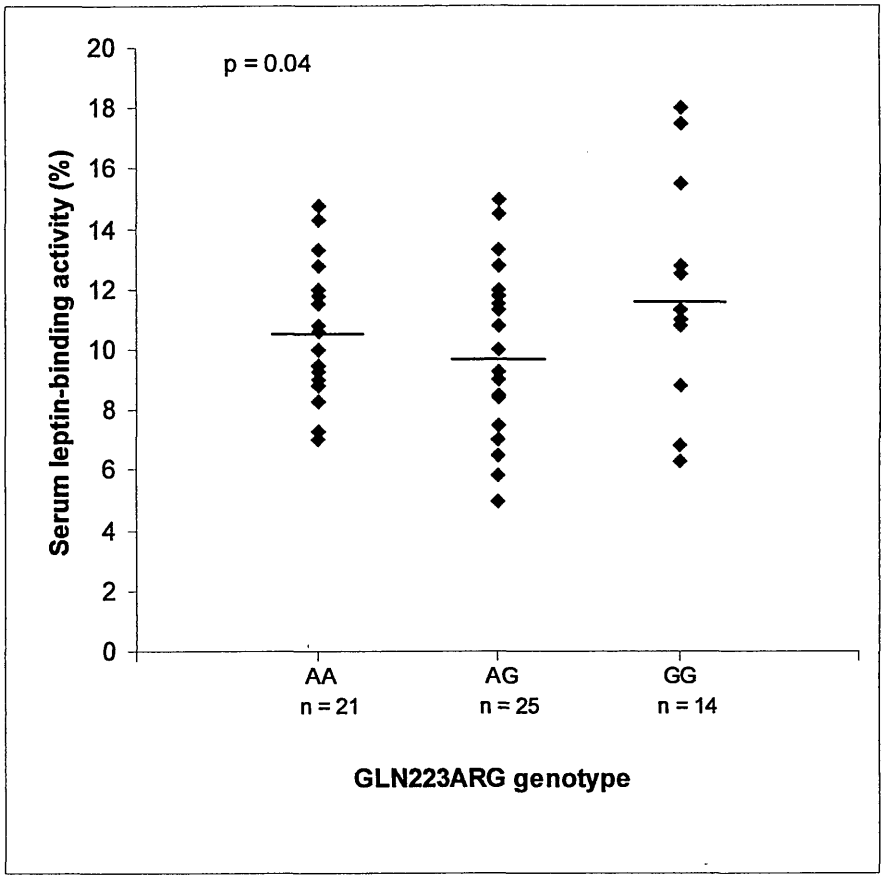


Figure 7.7 Serum leptin-binding activity and GLN223ARG genotype: mean LBA levels are higher in those homozygous for the G allele compared to heterozygotes and those homozygous for the A allele.

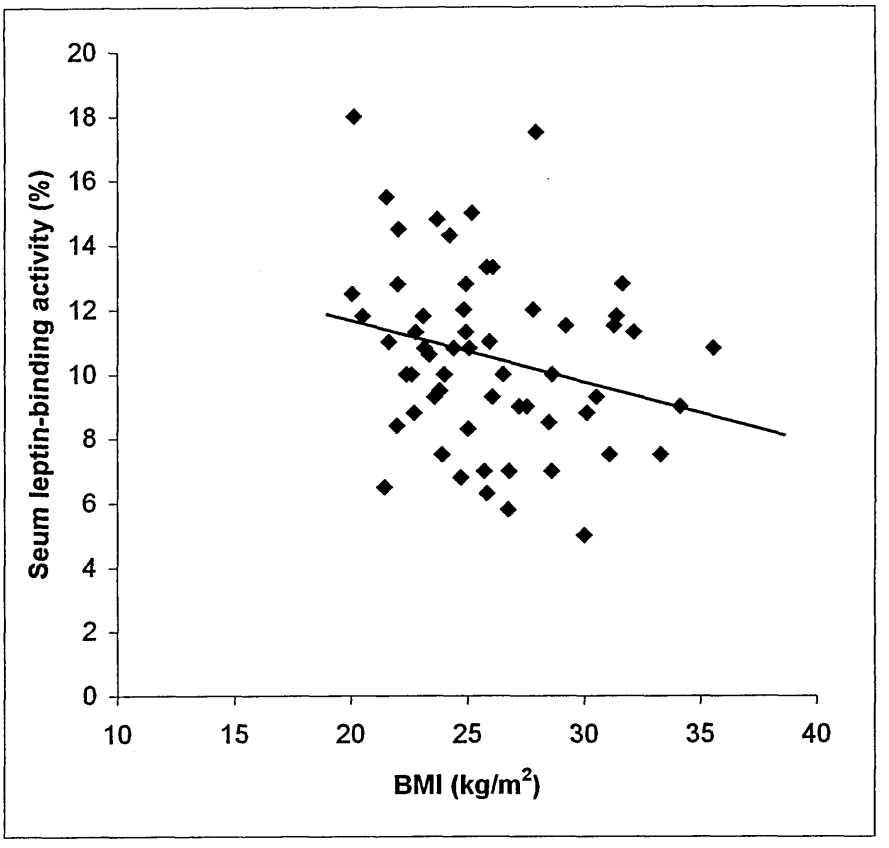


Figure 7.8 Correlation of serum leptin-binding activity with BMI ($r = -0.253$, $p = 0.05$, $n = 58$).

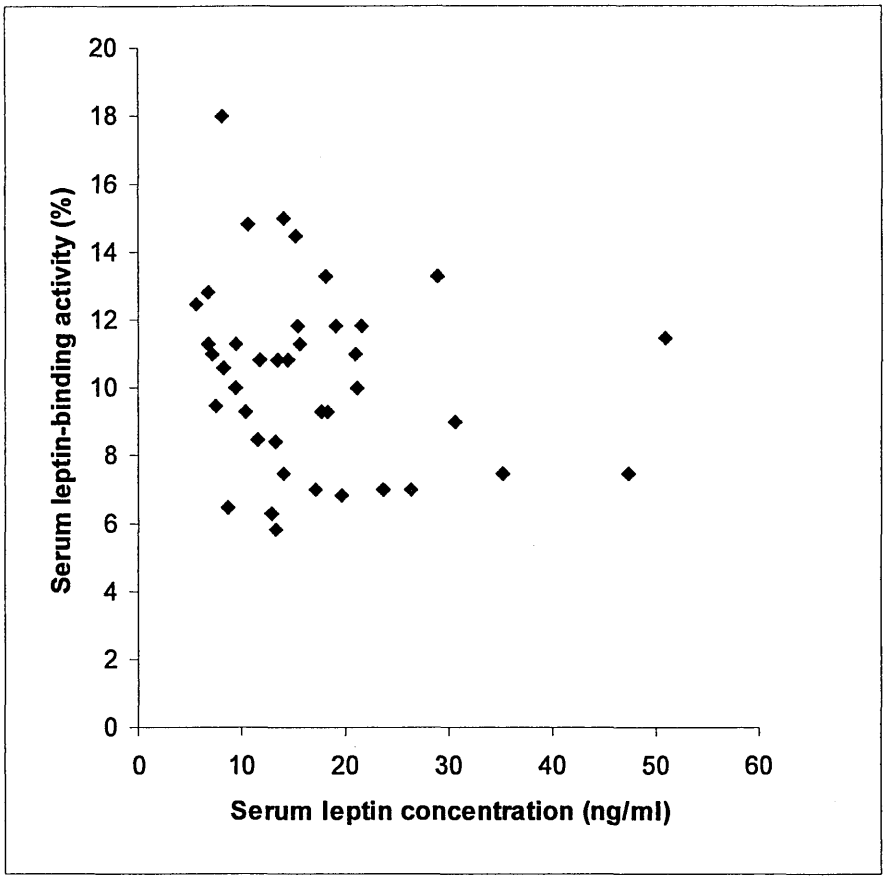


Figure 7.9 No significant correlation between serum leptin levels and LBA was observed.

7.3.2 LYS656ASN

In order to determine allele and genotype frequencies for this SNP, 152 subjects were genotyped. The calculated allele frequencies (G allele 0.69 and C allele 0.31) are shown in Table 7.4 with other published allele frequencies. The balance of homozygotes and heterozygotes was as predicted by the Hardy-Weinberg equation from these allele frequencies (Table 7.5).

Data on BMI was available for 137 subjects. Mean BMI for those homozygous for the C allele was 26.6 kg/m^2 ± 6.8 (Mean ± SEM), this did not differ significantly from the heterozygotes (27.1 kg/m^2 ± 3.7) or those homozygous for the G allele (27.7 kg/m^2 ± 3.4) (Figure 7.10). Fat and lean masses were also assessed in 76 subjects. Mean levels of fat mass for those homozygous for the C allele (24761g ± 9523) were not significantly different from the other genotypes (heterozygotes 27626g ± 5116, those homozygous for G allele 25302g ± 4081) (Figure 7.11a). Similarly, the mean lean mass in those homozygous for the C allele (36589g ± 14073) did not differ significantly from the other genotypes (heterozygotes, 35775g ± 6225 and G allele homozygotes, 36246g ± 5846) (Figure 7.11b). Serum leptin levels were measured in 72 subjects. Mean levels of leptin for those homozygous for the C allele (15.3 ng/ml ± 5.9) (Mean ± SEM) did not differ significantly from the heterozygotes (22.3 ng/ml ± 4.3) and those homozygous for the G allele (18.0 ng/ml ± 2.9) (Figure 7.12).

Serum levels of LBA were measured in a subset of the population (n = 57). As there were no observed associations between this SNP and BMI and leptin levels, a significant difference in the level of serum LBA was not expected. Mean levels of LBA for those homozygous for the C allele (9.9% ± 5.0) (Mean ± SEM) did not differ from the heterozygotes (10.4% ± 2.2) or those homozygous for the G allele (10.6% ± 1.9) (Figure 7.13). This data is summarised in table 7.6.

Allele	Post-menopausal women (n = 225) (This study)	Lean British males (n = 132) *Gotoda et al., 1997*	Obese British males (n = 190) *Gotoda et al., 1997*	Japanese obese (n = 47) Matsouka *et al.*, 1998	Japanese non-obese (n = 68) Matsouka *et al.*, 1998	Black males (n = 79) Chagnon *et al.*, 2000	Caucasian males (n = 178) Chagnon *et al.*, 2000
G	0.69	0.84	0.82	0.904	0.860	0.83	0.82
C	0.31	0.16	0.18	0.096	0.140	0.17	0.18

Table 7.4 Comparison of allele frequencies for LYS656ASN between this study and other published work.

	Observed Genotype Frequency	Expected Genotype frequency	
	No.	%	No.
Heterozygotes	64	42	64
Homozygous G	73	48	73
Homozygous C	15	10	15
Total	152	100	152

Table 7.5 Data for Hardy-Weinberg equilibrium and χ^2 analysis of LYS656ASN in this population of postmenopausal women.

	Genotype			P value
	GG	GC	CC	
BMI (kg/m^2) n = 137	27.7 ± 3.4	27.1 ± 3.7	26.6 ± 6.8	NS
Fat mass (g) n = 76	25302 ± 4081	27626 ± 5116	24761 ± 9523	NS
Lean mass (g) n = 76	36246 ± 5846	35775 ± 6225	36589 ± 14703	NS
Serum leptin concentration (ng/ml) n = 72	18.0 ± 2.9	22.3 ± 4.3	15.3 ± 5.9	NS
Serum LBA (%) n = 57	10.6 ± 1.9	10.4 ± 2.2	9.9 ± 5.0	NS

Table 7.6 Mean levels of BMI, fat and lean mass, leptin and serum LBA for each genotype of LYS656ASN. P value is calculated by comparing those homozygous for the G allele with the other genotypes using the appropriate t test.

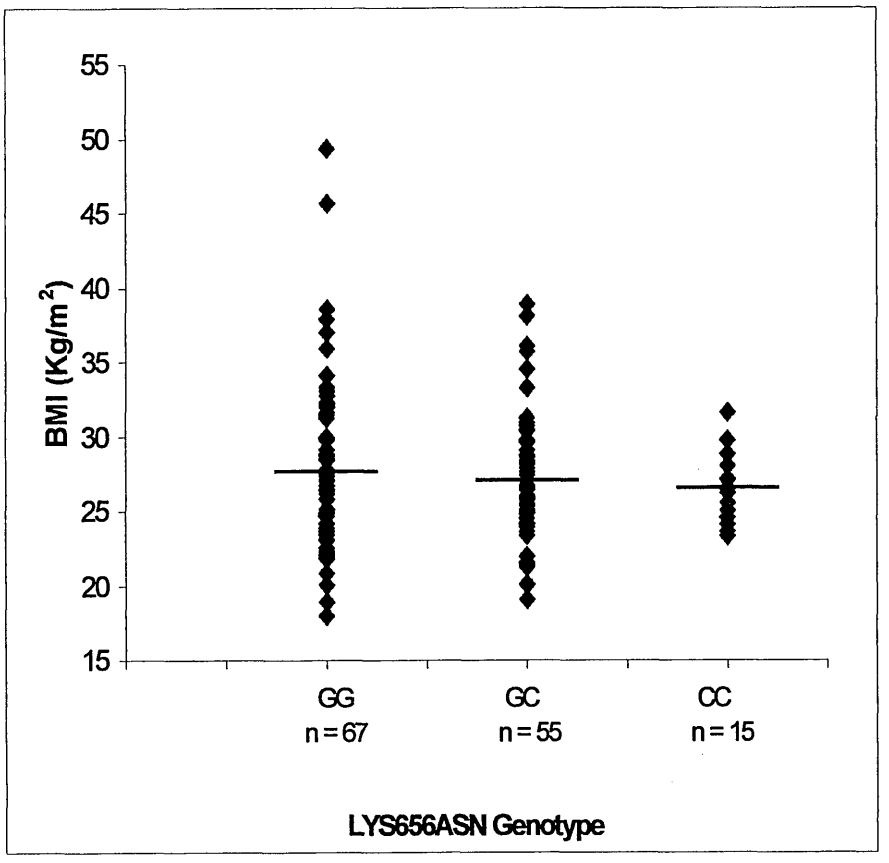
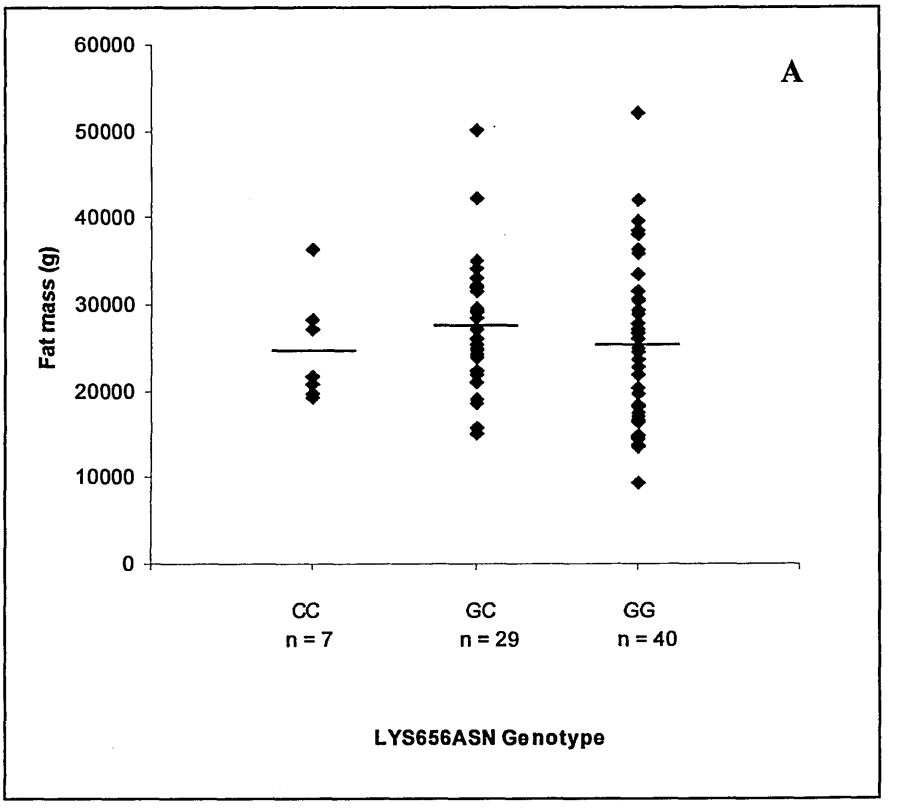


Figure 7.10 BMI and LYS656ASN genotype: there is no significant difference in BMI between the genotypes.



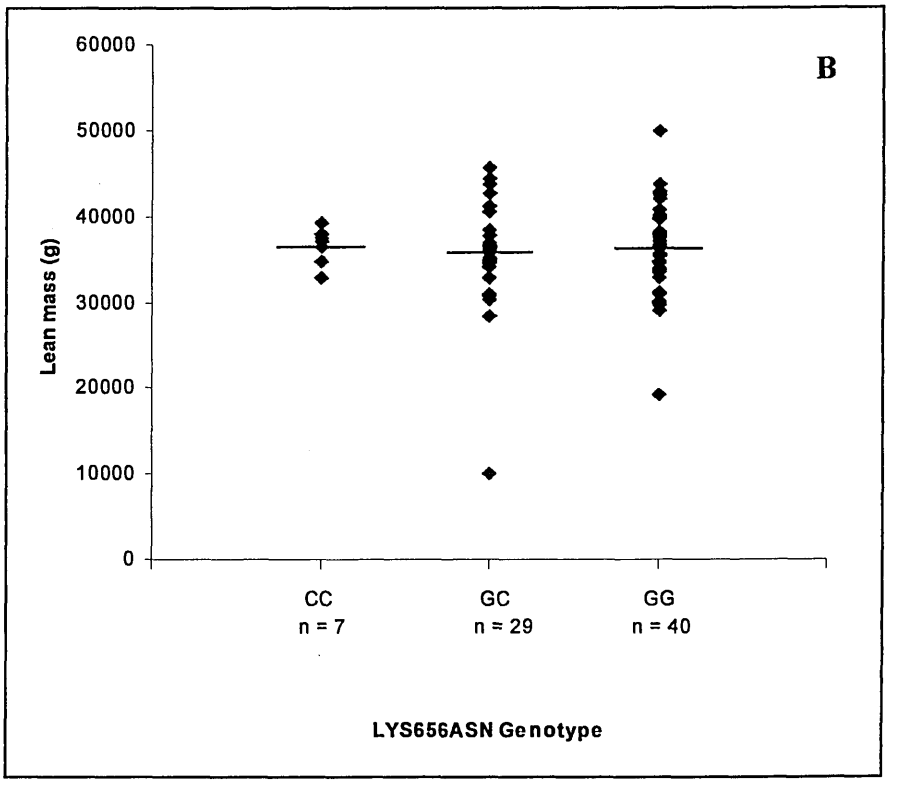


Figure 7.11 Levels of fat mass (A), lean mass (B) and LYS656ASN genotype: there is no significant difference between the genotypes.

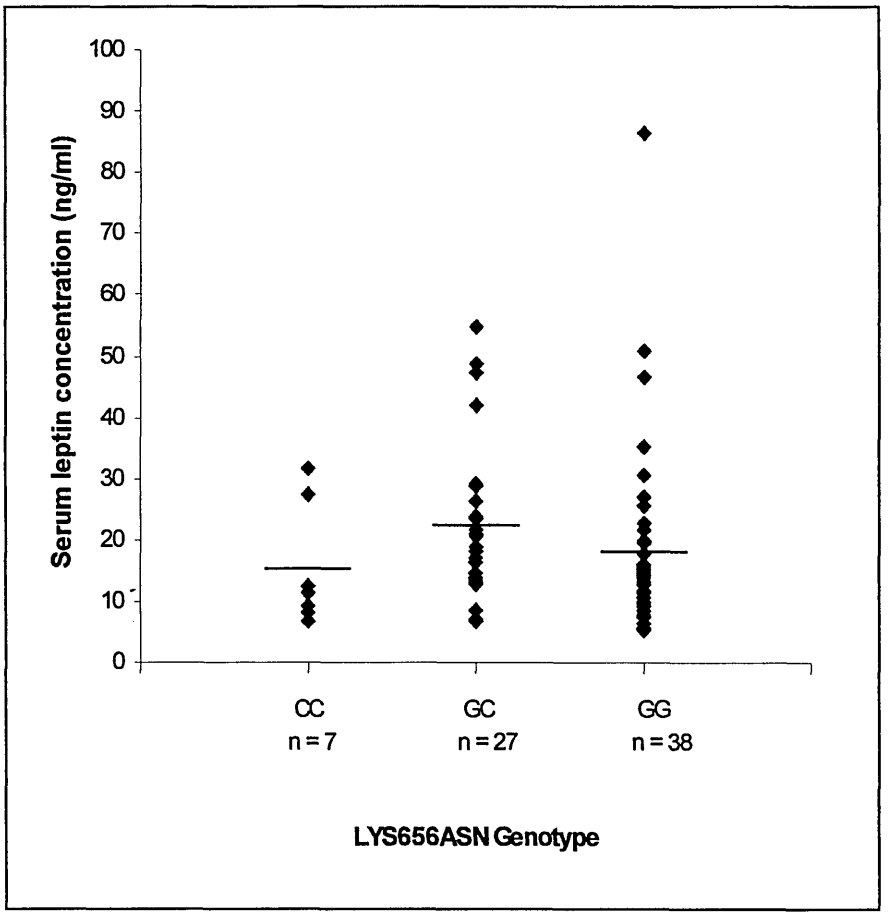


Figure 7.12 Serum leptin levels and LYS656ASN genotype: there is no significant difference between the genotypes.

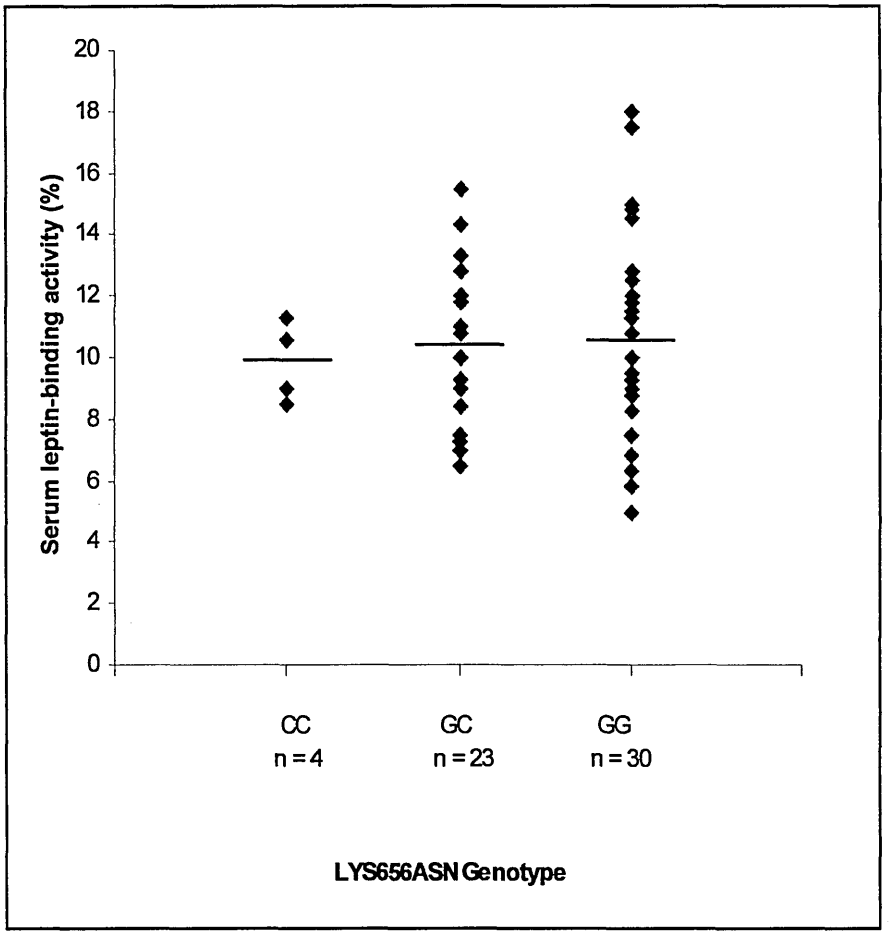


Figure 7.13 Serum LBA and LYS656ASN genotype: there is no significant difference in LBA between the genotypes.

7.3.3 LYS204ARG

This SNP at codon 204 is very close to the polymorphism GLN223ARG. Other workers report that one allele of this SNP is extremely rare (Echwald *et al.*, 1997), this is in agreement with the findings in this population of postmenopausal women. After genotyping 60 subjects, only 1 subject was heterozygous for this polymorphism. As variation with this polymorphism was so rare, it would be uninformative when assessing variation in BMI, serum leptin levels or serum LBA and genotype in a cohort of this size, therefore, the genotyping was discontinued.

7.4 Haplotyping studies of GLN223ARG and LYS656ASN

Previous studies have reported that polymorphisms in the leptin receptor are in linkage disequilibrium (Thompson *et al.*, 1997, Gotoda *et al* 1997). The possibility of the SNPs GLN223ARG and LYS656ASN, studied in this chapter, being in linkage disequilibrium in this population was investigated. Analysis was carried out on 175 subjects. Phase was assigned to all the subjects who were not heterozygous for both of the SNPs (n = 133). A χ^2 test, to assess if carriage of the A allele of GLN223ARG was associated with carriage of the G allele of LYS656ASN, was performed.

		GLN223ARG	
		Carriage of A allele	No carriage of A allele
LYS656ASN	Carriage of G allele	127	71
	No carriage of G allele	41	27

Table 7.7 χ^2 contingency table to investigate the carriage of the common alleles of GLN223ARG and LYS656ASN.

$\chi^2 = 0.32$

p = NS

The χ^2 analysis showed that the A allele of GLN223ARG and the G allele of LYS656ASN were carried independently of each other. These SNPs do not appear to be in linkage disequilibrium in this study.

Further analysis was carried out in order to look at the possible associations between the leptin receptor haplotype and measures of body mass, leptin and LBA. ANOVA with Bonferroni correction was used to observe any differences between each of the haplotypes and the heterozygotes. Significant associations between the leptin receptor haplotype and BMI (Figure 7.13), fat mass (Figure 7.14), lean mass (Figure 7.15), serum leptin levels (Figure 7.16) and LBA (Figure 7.17) were not observed.

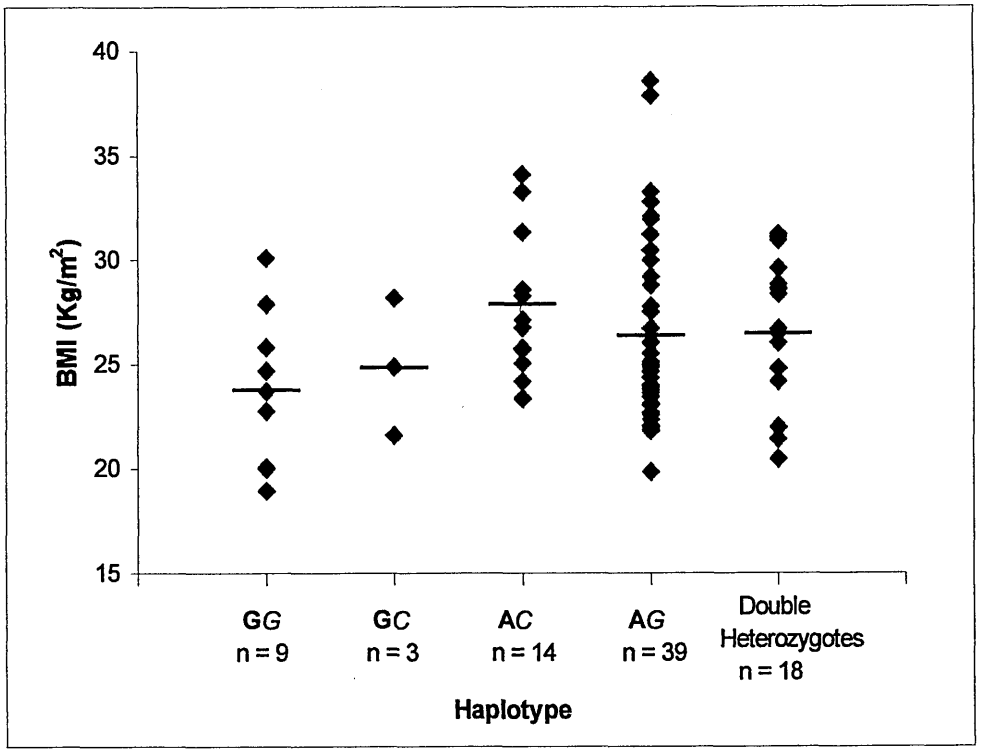


Figure 7.13 Leptin receptor haplotypes and BMI: no significant difference was observed (GLN223ARG allele shown in **bold**, LYS656ASN allele shown in *Italics*).

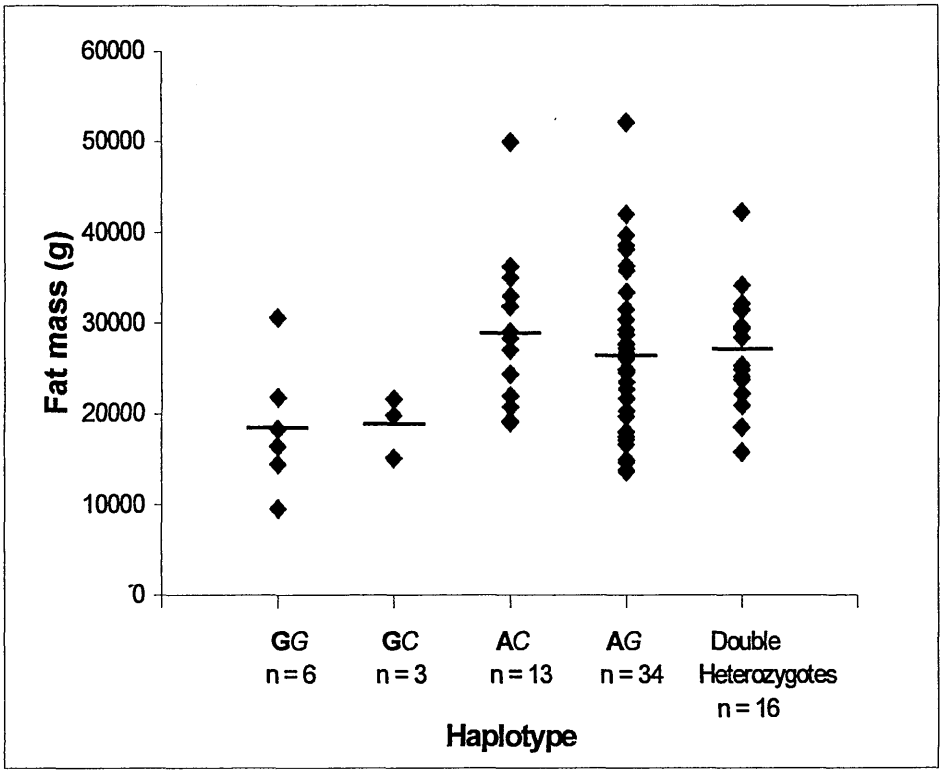


Figure 7.14 Leptin receptor haplotypes and fat mass: no significant associations were observed (GLN223ARG allele shown in **bold**, LYS656ASN allele shown in *Italics*).

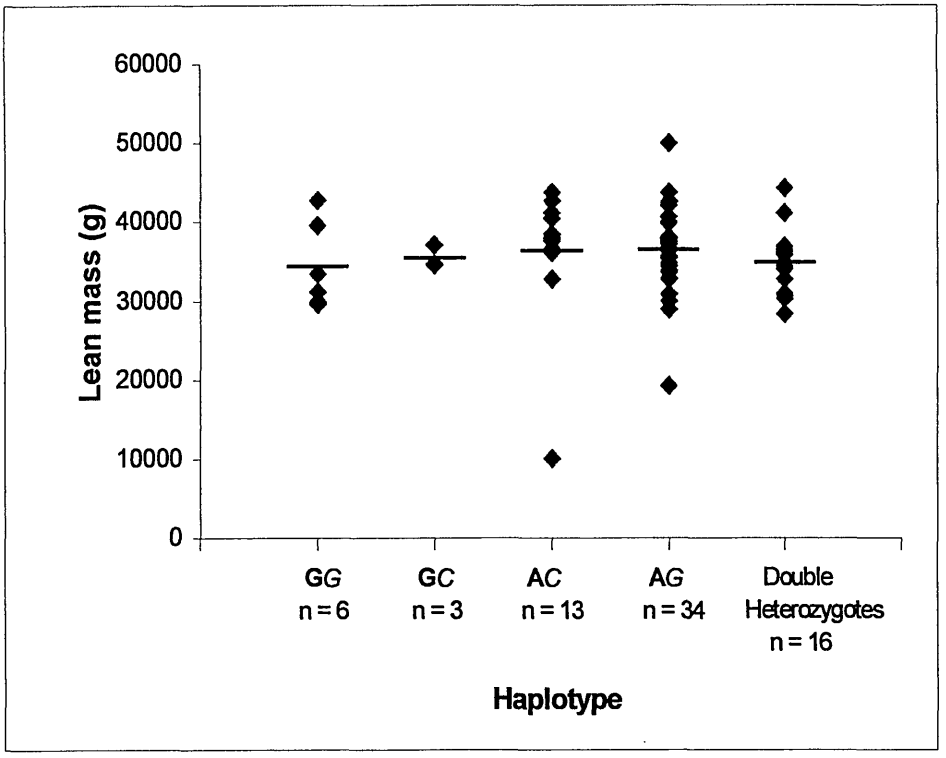


Figure 7.15 Leptin receptor haplotypes and lean mass: no significant associations were observed (GLN223ARG allele shown in **bold**, LYS656ASN allele shown in *Italics*).

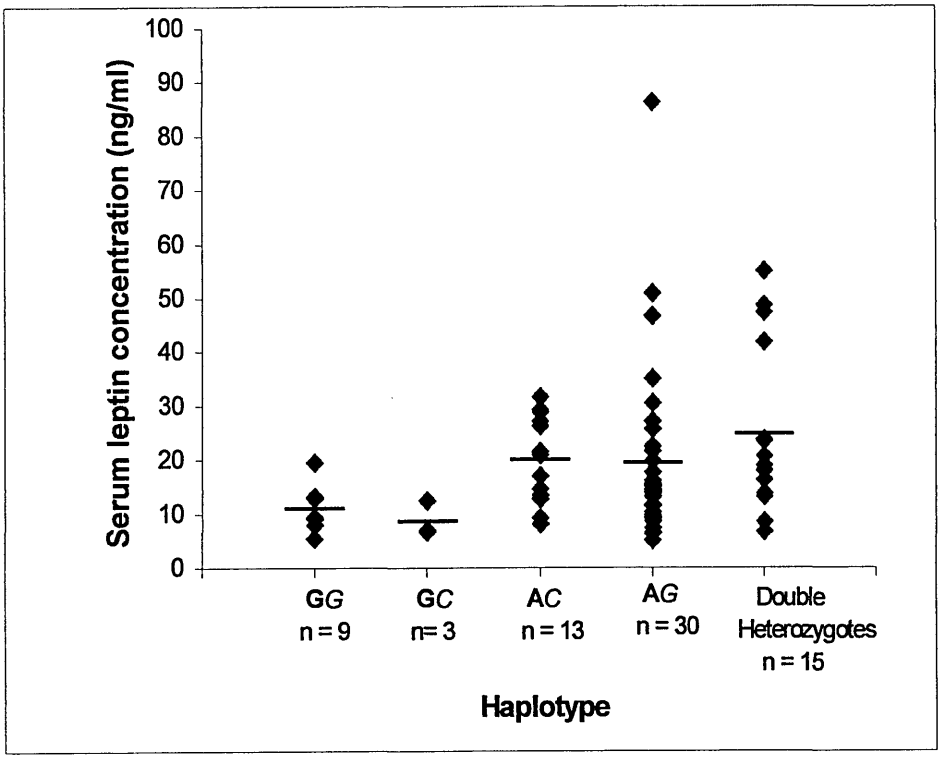


Figure 7.16 Leptin receptor haplotypes and serum leptin concentrations: no significant associations were observed (GLN223ARG allele shown in **bold**, LYS656ASN allele shown in *Italics*).

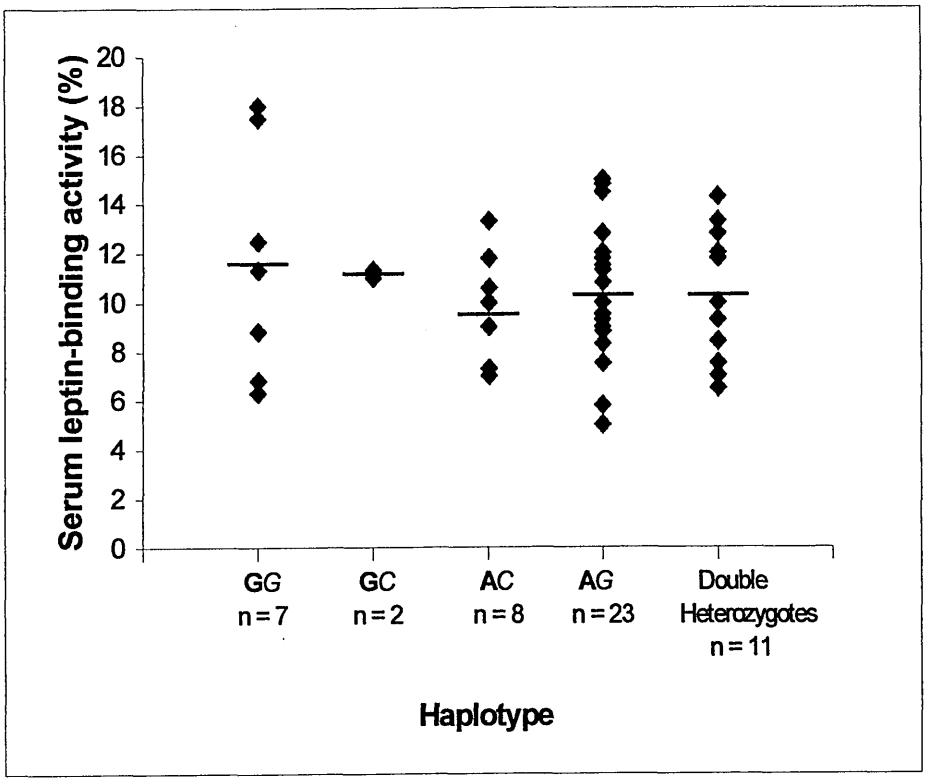


Figure 7.17 Leptin receptor haplotypes and LBA: no significant associations were observed (GLN223ARG allele shown in **bold**, LYS656ASN allele shown in *Italics*).

7.5 Conclusion

This data indicates that homozygosity of the G allele of the GLN223ARG polymorphism in the leptin receptor is associated with differences in BMI, fat mass and serum leptin levels in the Caucasian population. Homozygosity for the G allele is associated with serum leptin levels and associations between homozygosity for the G allele, BMI, fat mass and serum leptin-binding activity have been demonstrated, at least in postmenopausal women. There was no demonstrable difference in levels of lean mass. This data also shows the absence of associations between the LYS656ASN polymorphism and BMI, fat mass and serum leptin levels or serum LBA. This may be due to the small numbers of subjects who were homozygous for the C allele. Investigations of the SNP LYS204ARG demonstrated, as previously described that this polymorphism is extremely rare and as such is unlikely to have a role in the variation of BMI or serum leptin levels. These studies lend weight to the hypothesis that SNPs in or around the leptin receptor gene are important genetic factors in regulation of BMI and fat mass and agrees with the work of Thompson *et al.* (1997) who reported an association between GLN223ARG genotype and BMI in a population of Pima Indians.

Other workers have reported lack of association of the SNP GLN223ARG with indicators of obesity. A recent abstract by Wauters *et al.* (1999) describes investigation of GLN223ARG and other polymorphisms in obese female subjects aged 18-45 and 45-60 years. They did not find any associations between the GLN223ARG polymorphism and leptin level or BMI in either of their two cohorts. Other polymorphisms in the receptor, however, appeared to be associated with hip circumference (LYS109ARG) and waist measurements (LYS656ASN) in premenopausal, but not postmenopausal women. In two studies of obese males, however, associations between polymorphisms in the leptin receptor and BMI and leptin levels were also not detected. Echwald, *et al.*(1997) investigated leptin receptor polymorphisms in males with juvenile onset obesity (BMI $\geq$ 31Kg/m^2) (n=156). They found no association between GLN223ARG or other markers and BMI at any age, and no significant difference in allele frequency between their control group and those with juvenile onset obesity. In another study of a British male population the frequency of the variant allele and the distribution of the different genotypes for GLN223ARG were not significantly different between the obese (n = 190) and control (n = 132) groups. The major difference in this study and the others

in the literature is the fact that all the other studies have reported changes in obese populations. In contrast, a population of normal postmenopausal Caucasian women has been studied. Since homozygosity of the G allele of the GLN223ARG SNP is associated with lower BMI and lower fat mass, rather than with indicators of obesity, it may simply have not been evident in the cohorts examined. In addition, it is possible that the association that we have demonstrated in GLN223ARG, between homozygosity of the G allele and leptin levels, fat mass and BMI, are found only in postmenopausal women: other studies have used premenopausal and perimenopausal women. A recent study has confirmed the associations reported here. Chagnon *et al.* (2000) have reported similar findings of associations between body mass index and fat mass and the SNP GLN223ARG in their cohort of middle-aged men.

Serum leptin-binding activity was measured to determine whether the SNPs in the leptin receptor were associated with changes in the ligand binding. Thompson *et al.*(1997) reported that GLN223ARG is in linkage disequilibrium with three other leptin receptor polymorphisms. Gotoda *et al.*, (1997) also report alleles of GLN223ARG to be in linkage disequilibrium with other leptin receptor SNPs at codons; 109, 343, 656 and 1019. In this cohort, higher serum LBA levels were observed in subjects homozygous for the G allele than for those homozygous for the A allele and heterozygotes for GLN223ARG. No significant difference in serum LBA was observed in any genotypes of the SNP LYS656ASN, although the numbers of those who were homozygous for the C allele of this SNP was small. The possibility that other SNPs within the leptin receptor may affect this data cannot be excluded. This data may indicate a difference in the levels of receptor expression or changed ligand-binding capacity of the leptin receptor. The leptin receptor has two cytokine binding domains (C domain). These C domains represent putative binding domains for leptin to bind to its receptor. A mutation that occurs in the Zucker rat is located in the first C domain of the leptin receptor and this affects the functionality of the receptor (White *et al.*, 1997). The SNP, GLN223ARG, is also located within the first C domain (Chagnon *et al.*, 2000). It can be hypothesised that this polymorphism, which creates an amino acid change, may result in altered binding and therefore, change the signalling capacity of the leptin receptor.

Haplotyping studies were performed on a subset of subjects where the phase of the alleles could be assigned. Those who were heterozygous for both the GLN223ARG and LYS656ASN SNPs were excluded, as phase cannot be assigned. This data suggested

that GLN223ARG and LYS656ASN were not in linkage disequilibrium. Other workers have shown these polymorphisms to be in linkage disequilibrium, this was demonstrated using haplotypes from at least four polymorphisms. There may be a number of reasons as to why these polymorphisms are not observed to be in linkage disequilibrium. The cohort may not be large enough to see the association. Secondly, as the genotyping method used was SSCP, there may be some amount of error. SSCP relies upon the visualisation of the conformation made by single stranded DNA. Although every care was taken when assessing the genotypes; the genotyping was confirmed by sequence analysis and the numbers of genotypes did not differ from Hardy-Weinberg equilibrium at the given allele frequencies, it is possible that some genotypes were incorrectly assigned. This is further suggested by the difference in allele frequencies reported in this study and the others in the literature. In future studies, other methods of genotyping subjects for LYS656SASN, such as mismatch primer generation of an artificial restriction enzyme site, will be used to compare the genotyping performed in these studies. Association studies performed on the leptin receptor haplotypes did not reveal any significant difference in BMI, fat mass, lean mass, serum leptin level or serum LBA. The associations observed in these studies are only seen in the GLN223ARG SNP. It is possible that similar associations may be observed in other SNP in the leptin receptor gene or may reflect another polymorphism in an alternative gene nearby.

These studies indicate that the polymorphic variations in the leptin receptor gene are associated with variation in ligand binding and, as such, inter-individual differences in leptin levels and BMI may reflect altered functional parameters of the leptin receptor, as serum LBA may reflect the binding parameters of cell surface receptors as well. It is, therefore, possible that variant forms of the leptin receptor will become targets for new tailored, therapeutic interventions in the regulation of BMI, reproduction and other systems affected by leptin.

8.1 Discussion

The present studies were performed in order to increase the body of knowledge concerning the role of leptin and the leptin receptor. The studies were in two parts; firstly to study leptin and LBA in human reproductive function, specifically in puberty, normal female reproductive function and the endometrium, and, secondly, to study the effect of leptin receptor polymorphisms on body mass regulation.

Leptin is a peripheral signal produced by the adipose tissue to inform the brain of the state of the body's adiposity. In humans, the amount of leptin present is correlated with BMI and adiposity (Hickey *et al.*, 1996); leptin production is determined by the adipose tissue, both in the numbers of adipocytes present and their ability to express leptin (Jequier and Tappy, 1999). The signalling form of the leptin receptor is present in the hypothalamus, the body's homeostatic centre, where food intake and body mass regulation are controlled. A decrease in body fat leads to a decrease in serum leptin concentration and this in turn, stimulates an increase in food intake (Friedman, 2000). In addition to regulation of BMI, leptin has a well-recognised role in human and animal reproduction, although the exact mechanisms of either system are not fully understood. On administration of recombinant leptin, both male and female *ob/ob* mice have their fertility restored (Chehab *et al.*, 1996). Again in *ob/ob* mice, administration of leptin causes successful progression into puberty (Ahima *et al.*, 1997). As well as these biological effects, leptin appears to be important in regulating sexual behaviour in hamsters. Wade *et al.* (1997) report that leptin administration to *ad libertum* fed female hamsters facilitated sexual behaviour, but decreased this behaviour in starved animals. The leptin system is also important in reproductive function in humans. The hypothesis that Frisch put forward in the 1970s, that a "pre-set" level of fat mass is required for female puberty to begin and ovulation and menstruation to persist is now well accepted and is rationally explained by the leptin system. Several studies have shown a link between serum leptin levels and pubertal development in both males and females. The leptin system is known to be important in the regulation of body mass. Leptin levels correlate well with BMI and also with adiposity.

Many cytokines and hormones are bound to proteins in serum. These proteins can be specific binding proteins or the extracellular domain of the appropriate receptor. Two methods are known to exist for the creation of soluble receptors, either the extracellular domain can be cleaved off of the cell surface by proteolysis or it may occur by expression of an alternatively spliced form of the receptor by cells. It is known that leptin is bound to proteins in serum, with a portion of this protein attributable to the soluble leptin receptor (Diamond *et al.*, 1997, Houseknecht *et al.*, 1996). At the time of writing, the mechanism by which the soluble leptin receptor is created is not known. Using the method of Amit *et* al., (1990) for the measurement of GH-BP, an assay to measure leptin binding activity in serum was developed. Experiments to assess the appropriate conditions for sample preparation and for the assay themselves were performed. The most appropriate conditions were used subsequently for the measurement of samples for LBA. The samples required the endogenous leptin to be removed prior to the assay being performed, to prevent interference from the serum leptin in the assay. This was achieved using a dextran-coated charcoal slurry followed by separation by centrifugation. The assay was specific for only leptin and the system was tested for specificity with growth hormone (as it has a similar structure to leptin) and IL-6 (as the leptin receptor is similar to the IL-6 family of receptors). Scatchard analysis revealed a high affinity ($1.0 - 1.4 \times 10^{-9}$ M^{-1}) binding protein which would be compatible with the expected affinity of the soluble receptor (Liu *et al.*, 1997). This assay measures the activity at which leptin binds to proteins in serum. These proteins, of as yet unknown origin, bind leptin specifically and with high affinity: a proportion of this binding can be attributed to the soluble form of the leptin receptor, although it is possible that novel binding proteins exist for leptin. This assay does not measure low-affinity binding protein interactions, such as that which may exist with α-2-macroglobulin. This assay represents an important tool to investigate the action of leptin in serum.

This assay was employed in measuring serum LBA in subjects, both male and female, at different ages. Emphasis was placed on the measurement of LBA in children at various stages of puberty, as it is believed leptin may be a permissive signal to the initiation of puberty (Mantzoros *et al.*, 1997, Clayton *et al*, 1997). These studies showed that serum LBA was low at birth, high in early childhood, fell during puberty and remained at the post pubertal level throughout adult life. Serum LBA was negatively correlated with age,

pubertal stage, weight and BMI. In females it is known that leptin levels increase throughout puberty, in response to increasing fat mass (Clayton *et al.*, 1997). In boys, leptin appears to increase until Tanner stage 2 of puberty when the testosterone surge occurs, leptin then decreases to the low adult levels seen (Clayton *et al.*, 1997). These levels correspond with adiposity and BMI. This finding, of the direct inverse relationship between LBA and pubertal status or age, with a fall in LBA seen at the earliest stages of puberty, suggests that this may be a link from leptin to the initiation and maintenance of puberty. A decrease in LBA could be explained by a change in leptin receptor expression, which may be the link between the leptin system and puberty. There is still some controversy as to whether leptin merely reflects the pubertal state at any given time or whether it is necessary for the initiation and maintenance of puberty. At a recent meeting, Farooqi *et al.*, (2000) suggested that leptin is a requirement for pubertal development. They report data from an extremely obese female child who has a congenital leptin deficiency. At present the child is receiving treatment in the form of recombinant human leptin and is responding well with weight loss and the amelioration of her hyperphagia. It was not clear if this mutation would result in hypogonadism observed with other mutations in the leptin and leptin receptor genes as the children were not of pubertal age. After a normal peripubertal response the child's pubertal development ceased and there was a return to the hyperphagic state seen previously. This was reversed when the dose of leptin being received was increased, indicating that a certain level of leptin is a requirement for the onset and maintenance puberty. The change in LBA during puberty, described in this study, may be the mechanism by which a greater level of leptin is made biologically available to initiate full reproductive function.

As leptin and LBA appear to be important factors in the regulation and initiation of puberty, and leptin has a known role in female reproduction, studies of the leptin system in normal fertile women were undertaken. Serum leptin levels were found to increase in the luteal phase of the menstrual cycle in two cohorts of normal, regularly cycling women. This information is of importance in two ways; firstly menstrual date must now be controlled for in studies of leptin in female subjects as this may affect study results and secondly, this adds to the known role of leptin in reproduction. Leptin may have a direct and local role in the reproductive process, as well as being a neural signal of bodily adiposity to support a pregnancy to term. In the small study undertaken, serum LBA was not found to vary at any

point in the menstrual cycle. Some other soluble receptors and binding-proteins for related cytokines and hormones do not fluctuate in the menstrual cycle. There is still controversy as to whether soluble IL-6 receptors and IGF-BPs fluctuate during the menstrual cycle. Perhaps, a larger study, with more subjects and more samples per subject will help to elucidate whether LBA varies in the menstrual cycle of normal women.

To complement the studies of the menstrual cycle, studies of the local effect of leptin on the human endometrium were performed. Cytokines have an important role in preparing the endometrium for implantation and subsequent pregnancy (Simon *et al.*, 1995). The receptivity of the endometrium, after ovulation, is orchestrated by cytokines being up and down regulated. The first step in identifying a local role for leptin within the human endometrium was the identification of leptin receptor isoform expression. RT-PCR studies showed that three isoforms of the leptin receptor were present in endometrial cells in culture. Both the short and variant isoforms of the leptin receptor were identified in endometrial epithelial and stromal cells in culture; the long isoform was only detected in the epithelial endometrial cells. To test if these receptors were functioning in human endometrial cells in culture, proliferation assays were performed. In our preliminary study, both stromal and epithelial endometrial cells in culture proliferated in response to leptin. This suggests that another isoform in addition to the long form is capable of signalling. Other workers have suggested that the short isoform is able to signal via the mitogen-activated protein kinase (MAPK) pathway (Yamashita *et al.,* 1998) and is able to induce the early gene c-fos and c-jun (Murakami *et al.*, 1997). As the receptors in the human endometrium appear to be functional, causing an increase in endometrial cell proliferation in response to leptin, it is possible that leptin, as a marker of the cytokine network, also induces effects on cytokine production in the endometrium. Leptin caused a concentration-dependent decrease in TNF-α production in the supernatants of epithelial endometrial cells in culture. Laird *et al* (1996) showed that TNF-α production was lowest in epithelial endometrial cells cultured form biopsies taken after ovulation in the luteal phase of the menstrual cycle. This suggests that leptin has a local effect in the endometrium, possibly in creating the receptivity of the endometrium prior to implantation. Cytokines are important in preparing the endometrium for implantation (Simon *et al.*, 1995). Leptin interacts with both TNF-α and IL-1 in other

human and rodent tissues (Fawcett *et al.*, 1999, Luheshi *et al.*, 1999). It can now be speculated that leptin, as part of the cytokine network, has an important function in preparing the endometrium for embryo implantation.

Studies of leptin during pregnancy have been performed. These studies have shown that levels of leptin are increased during pregnancy and fall to normal levels after birth (Butte *et al.*, 1997). There is some evidence that leptin is higher in the post-partum period, in women who are breastfeeding compared to women who are not (Pickavance *et al.*, 1998). Sattar *et al* (1998) report that maternal fat stores increase to a peak in the late second trimester, before declining towards term. The fat stores are mobilised to support the rapidly growing foetus. Leptin levels also decrease in the third trimester of pregnancy, this may be due to the decrease in maternal fat stores. Studies of leptin in pregnant human subjects with known pregnancy outcomes were carried out in samples taken from 2 time points in the pregnancy, 16 and 28 weeks. These studies showed that leptin levels were higher in subjects with a normal pregnancy outcome than in those subjects who had a poor pregnancy outcome. Poor pregnancy outcome was defined as the infant being light for gestational age or being a pre-term birth. The effect of smoking status on leptin levels in the women was also investigated. Previous studies have shown that leptin levels are reduced in non-pregnant smokers (Wei *et al.*,1997), this may, however, be an effect of a smokers lower BMI (Nicklas *et al.*, 1999). In this cohort of pregnant subjects, leptin levels were lower in smokers than non-smokers. The smokers also had a lower BMI than the non-smokers. When pregnancy outcome and smoking status were both used together to determine leptin levels, normal outcome non-smokers had higher leptin levels than all the other groups. It was interesting to note a decrease in serum leptin concentrations between 16 and 28 weeks in subjects who were non-smokers but who had a poor pregnancy outcome. Leptin levels during pregnancy are complicated by the placental production of leptin. It is possible that in these subjects, fat stores were being mobilised earlier than normal in the pregnancy to support the foetus or that some placental dysfunction was occurring. These studies add to the knowledge of the leptin system during pregnancy, however, the exact function that leptin has in either perpetuating a pregnancy, controlling maternal fat regulation or foetal growth has not yet been fully elucidated.

Inter-individual differences in LBA may reflect genetic changes within the leptin receptor gene. Investigations of leptin receptor SNPs were carried out to assess whether the genetic changes present in the gene are associated with differences in phenotype or effect ligand binding. A cohort of post-menopausal Caucasian women was used to minimise the hormonal effects on serum leptin concentrations and LBA. Two SNPs, GLN223ARG and LYS656ASN, both present in the extracellular domain of the leptin receptor, were investigated. Homozygosity of the G allele of GLN223ARG is reported to be associated with lower serum leptin levels in a population of Pima Indians (Thompson *et al.*, 1997). The studies undertaken, indicated that homozygosity of the G allele was associated with lower BMI, fat mass and serum leptin levels but, not with lean mass. As this SNP is in the extracellular domain of the receptor, it may affect serum LBA levels, and in turn reflect the binding parameters of cell-surface leptin receptors. Serum LBA was significantly higher in those subjects who were homozygous for the G allele. This evidence suggests that GLN223ARG in the leptin receptor gene is an important factor in the regulation of adiposity and consequently BMI. None of the associations seen were independently observed with the SNP LYS656ASN. Haplotyping studies showed that these polymorphisms were not in linkage disequilibrium, and significant differences in BMI, fat mass, serum leptin levels or serum LBA between the haplotypes were not observed.

8.2 Further Work

As leptin is a relatively new cytokine and its full function is still being elucidated, there is much to investigate. To complement the work carried out in the present study, further experimental practises to discover the full role of leptin in the human endometrium should be undertaken. Workers have identified expression of leptin in the bovine uterus (Takahashi *et al.*, 1998), so investigations into the possible production of leptin by the human endometrium should be carried out. From this point, measurement of leptin in endometrial flushings throughout the menstrual cycle may confirm that the increase in serum leptin, observed in the luteal phase of the cycle, occurs at the level of the endometrium and therefore may have a direct effect on cytokine production, as seen in endometrial cells in culture. It would be of use to investigate which signalling pathway is activated by leptin in

both the stromal and epithelial endometrial cells. This could be assessed by investigating mRNA expression after treatment with leptin in endometrial cells in culture. A commercially prepared cDNA array treated with probes made from the cellular mRNA could then be utilised. In addition, investigations of the effects of leptin on endometrial cell lines, such as HEC-1B an endometrial carcinoma cell line, would provide interesting comparisons to the studies of primary endometrial cells.

There is evidence to suggest that the effect of leptin binding to its receptor influences an ATP-dependent potassium channel in pancreatic β-cells (Kieffer *et al.*, 1997 , Harvey *et al.*, 1997). It is known that potassium channels are present in many tissues and cells where leptin has a role, for example T cells (Jensen *et al.*, 1999) and the reproductive tissues (Chien *et al.*, 1999). Further investigations could be carried out to identify if potassium channels were associated with the effect leptin has on the endometrium.

Further work on the remaining polymorphisms in the leptin receptor gene, specifically at codon 109 and in the leptin gene itself should be undertaken. By identifying functional genetic changes, this will aid in the understanding of body weight regulation. This may lead to future production of drugs tailored specifically to the genetic changes in individuals in the leptin and leptin receptor genes.

These polymorphisms could be used to examine the role of the leptin system in a number of situations that are associated with extremes of body weight, for example, in anorexia nervosa and cachexia or in obesity related disorders, such as, hypertension and heart disease. It would be of importance to study these polymorphisms in other population groups, such as, Afro-Caribbeans, Orientals and Polynesians, to see if the same associations with body mass index and adiposity were observed. This would allow understanding of the full effect the leptin receptor polymorphisms have on body mass regulation.

These polymorphisms would also be useful tools to investigate the genetic changes associated with disorders of female reproduction such as unexplained recurrent miscarriage and endometriosis. Unexplained recurrent miscarriage is characterised as three consecutive miscarriages for which there is no known cause. The most common known reason for

miscarriage is chromosomal abnormalities of the foetus, with other explanations being the presence of anti-cardiolipin antibodies or abnormal uterine morphology. The polymorphic changes within the leptin receptor gene could also be used to investigate possible associations between these changes and recurrent miscarriage, as leptin and the leptin receptor are fundamental in female reproduction.

Another possible route of investigation of leptin and leptin receptor polymorphisms could be that of endometriosis. There are several theories regarding the pathogenesis of endometriosis. The first theory is that of "retrograde-menstruation" or "trans-tubal migration". This hypothesis suggests that during menstruation, endometrial tissue becomes loosened and the cells migrate, via the fallopian tubes, out into the pelvic and abdominal cavity where they implant into other tissues, such as the ovaries or the bowel and grow. This theory has now been superceded as it known that this migration of endometrial cells into the abdominal cavity occurs in women without endometriosis. The second theory is that there is a dysfunction in the immune system of women with endometriosis, this allows tissue that has migrated out of the uterine cavity to implant and grow. There is also a genetic predisposition to developing the disorder. Endometriosis is a heritable disorder; the risk of developing endometriosis increases seven-fold for women with an affected female relative. Cytokines are important regulators of endometrial function. It is likely that leptin, which has a role both in endometrial function and in the immune response, has a function in the development of endometriosis. By investigating, the genetic changes in the leptin and leptin receptor genes and in turn, identifying possible associations between certain genotypes and disease characteristics will enable the further elucidation of the genetic aspects of endometriosis.

This thesis has clearly shown that the leptin system has important roles in reproduction, specifically in the onset and maintenance of puberty and in female reproduction, and in body mass regulation. There is now distinct evidence for the leptin receptor to be directly involved in the differences observed in adiposity and BMI. There are great prospects for a further rapid increase in the understanding of reproductive function and body mass regulation, and the role which the cytokine leptin plays in these processes.

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Appendix 1

Primers used to determine the polymorphic variations in the leptin receptor

Primer Name	Sequence	Polymorphism	Product size (bp)
Poly223-F Poly223-R	5' tcc tct tta aaa gcc tat cca gta ttt 3' 5' agc tac caa ata ttt ttg taa gca at 3'	GLN223ARG	416
Poly656-F Poly656-R	5' gca taa gtg tgt gct tca aat atg g 3' 5' cga aga tta ata tca gga tta acc 3'	LYS656ASN	244
Poly204-F Poly204-R	5' tcc tct tta aaa gcc tat cca gta ttt 3' 5' agc tac caa ata ttt ttg taa gca at 3'	LYS204ARG	416

Appendix 2

RT-PCR primer sequences for the various isoforms of the leptin receptor.

Primer Name	Receptor Isoform	Sequence	Position (Base No)	Product size (bp)
132F	Ob-Rb	5' gaa gat gtt ccg aac ccc aag aat tg 3'	2742	
132R	(Long)	5' cta gag cac ttg gtg act gaa c 3'	3170	427
64F	Ob-Ra	5' cca ttg aga agt acc agt tca gtc ttt acc 3'	2600	
64R	(Short)	5' ggg aag ttg gca cat tgg gtt ca 3'	2930	330
121F	Ob-Rvar	As 132F	2742	
121R	(variant)	5' gga ttt ggc agg gtc ata gga caa 3'	3053	234
7B6F	7B6	5' agc cgt aga cgg aac ttc gc 3'		
7B6R		5' cta aaa cag cgg aag agg t 3'		434

Appendix 3

Sequencing of LYS656ASN PCR products to confirm genotyping by SSCP

LYS LYS GLU

5' 3'

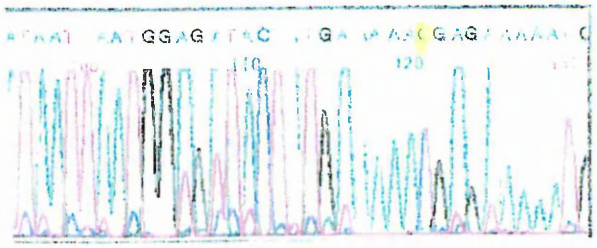
AAA AAG GAG

AAA AAC GAG

LYS ASN ALA

Homozygous for the C allele

Forward



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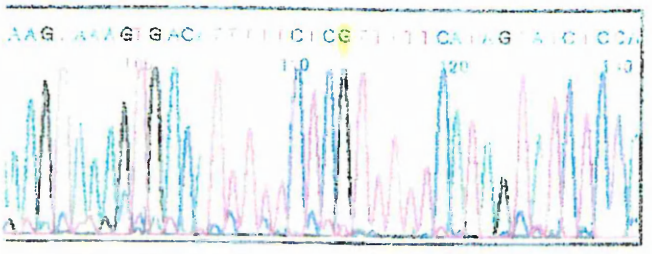
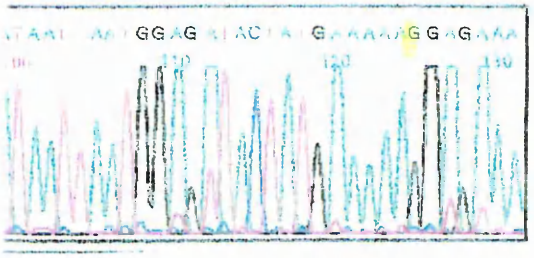


Homozygous for G allele

Forward



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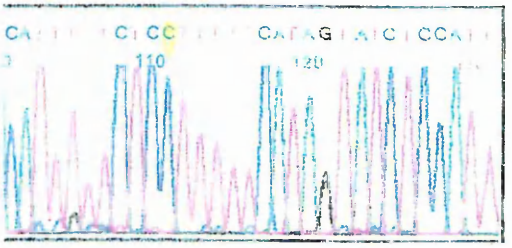
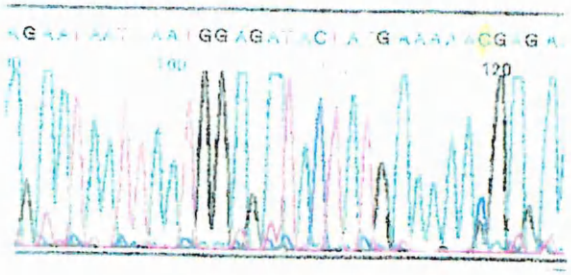


Heterozygous

Forward



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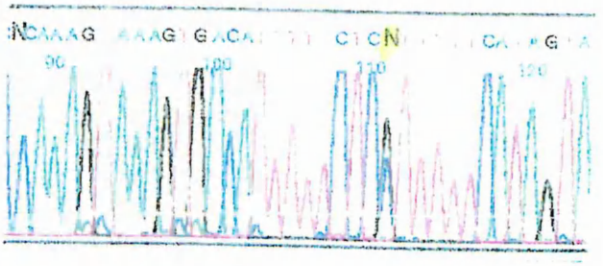

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Serum leptin levels during the menstrual cycle of healthy fertile women

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Abstract: Leptin is a protein, produced by adipose tissue, which has cytokine and hormonal properties. Serum leptin levels can be considered as a measure of body fat mass, and are involved in regulation of body weight. Previous studies suggest that leptin may have an additional role in reproduction, and there is also evidence for involvement in the hypothalamic–pituitary–gonadal axis. In this study, we investigate the possible changes in serum leptin concentration throughout the menstrual cycle. Samples were collected from apparently healthy, fertile women at different stages in their menstrual cycle, timed precisely according to the luteinising hormone (LH) surge. Mean serum leptin levels were significantly higher in the luteal phase (median 11.4 ng/mL) than in the follicular phase (median 10.0 ng/mL) (P <0.001). In addition, mean serum leptin levels correlated with body mass index (r = 0.54, P <0.05), but showed no correlation with luteal-phase progesterone levels. Results showed that levels of serum leptin vary during the menstrual cycle, and add to the mounting evidence that leptin has a role in reproduction. These fluctuations should be taken into account whenever studies are performed using female subjects.

Key words: Body mass index. Leptin. Menstrual cycle. Reproduction.

Introduction

Leptin, the product of the *ob* gene, is a 16 kDa adipose tissue-derived protein which appears to have the properties of both hormone and cytokine. The effects of leptin appear to be pleiotropic; various studies have suggested a role for it in obesity, thermogenesis, body mass regulation[1] and haematopoiesis.[2] More recent studies have suggested that leptin has a role in female reproduction, although the exact nature of the role is not understood.[2–4] Circulating leptin concentrations are higher in females than males, even when corrected for differences in body mass index (BMI).[5,6]

Further evidence for a role for leptin in reproduction is that pregnancy causes changes in the serum concentration of leptin, with levels at 36 weeks gestation higher than at three and six months postpartum.[3] Other studies have suggested that this increase in leptin may be brought about by the progressive increases in plasma progesterone, oestrogen and human placental lactogen in pregnancy.[2,4]

A number of studies have indicated that leptin may interact with the hypothalamic–pituitary–ovarian axis.[7] In women undergoing pituitary down-regulation and ovarian stimulation for *in vitro* fertilisation (IVF), serum leptin levels have been shown to correlate with oestradiol and follicle-stimulating hormone (FSH),[8] and other studies have suggested that leptin may be involved in bringing about the ovarian response to gonadotrophin stimulation.[9] This hypothesis is supported by the presence of leptin receptors in ovarian tissue[2] and the presence of leptin within the preovulatory follicle and mature oocyte.[10]

The majority of previous investigations on the role

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of leptin in female reproduction have been carried out in pregnant women or those undergoing IVF treatment using stimulated cycles. Less is known about the variation in serum leptin levels throughout the menstrual cycle in normal fertile women. In this study, the levels of leptin were measured in serum obtained from 17 women, of proven fertility, throughout the menstrual cycle to test the hypothesis that levels of leptin alter throughout the cycle.

Materials and methods

Serum samples were obtained from 17 normal fertile women with BMI between 19.8 and 27.9 kg/m^2. Blood samples were collected at times in the menstrual cycle that were convenient to the subjects involved, who were not required to fast before sampling took place. The number of serum samples collected from each subject ranged from one to three in the follicular phase and two to three in the luteal phase. The women were aged between 30 and 39 years, with normal menstrual cycles confirmed by detection of luteinising hormone (LH) surge and luteal-phase progesterone levels. Each woman had had at least one successful pregnancy. The samples obtained were timed precisely according to the LH surge. From day nine of the cycle, early morning urine or daily plasma samples were collected from each subject. An established enzyme immunoassay (EIA) (Serono Diagnostics Ltd, Surrey, UK) was used to measure LH in each sample. The day on which the LH surge occurred was designated LH+0.[11]

Each sample was prepared from 10 mL of venous blood. After collection, the blood sample was allowed to clot and then centrifuged at $3000 \times g$ for 10 min. The serum layer was removed and stored in 1 mL amounts at −80°C. In all, 79 samples were collected. Human leptin concentrations were measured in duplicate using a specific human leptin radioimmunoassay (RIA) kit (Linco Research Co, St Louis, MO, USA) with an inter-batch variation between 3% and 6%, and a detection limit of 0.5 ng/mL.

Serum progesterone was measured in samples taken 10 days after the LH surge (LH+10), at the point where progesterone is maximal, in 14 of the women. The progesterone was measured using a progesterone EIA kit (Serozyme, Welwyn Garden City, Herts, UK).

Differences between levels of leptin in the luteal phase and the follicular phase were analysed using non-parametric analysis (Mann–Whitney *U* test). Correlations between BMI and leptin level were analysed using Spearman ranking.

The study had the approval of the local ethical committee (South Sheffield Research Ethics Committee) and all subjects gave informed consent.

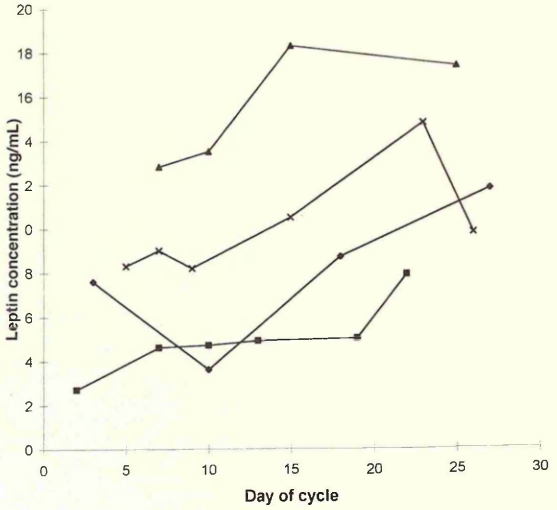

Fig. 1. Concentrations of leptin in serum collected from four subjects throughout the menstrual cycle.

Results

Fig. 1 shows the leptin levels in the serum collected from four representative subjects. The results for these four subjects are shown as they had varying BMI and leptin levels within the normal range (2–20 ng/mL) for women of normal weight. Levels of leptin gradually increased throughout the cycle and were maximal in the luteal phase. In 14 of the 17 subjects, mean luteal leptin levels were arithmetically higher than mean follicular leptin levels. The mean follicular and luteal leptin levels in the remaining three subjects were unchanged.

The median serum follicular and luteal leptin levels

Table 1. Median leptin levels for each subject in the follicular and luteal phases

Subject	Mean leptin level (ng/mL)	
	Follicular phase	Luteal phase
1	12.3	15.9
2	7.4	11.4
3	10.0	19.4
4	36.0	61.1
5	4.7	5.0
6	13.3	27.3
7	4.3	5.7
8	5.7	7.6
9	5.6	10.3
10	11.4	10.8
11	13.2	17.9
12	12.4	14.3
13	6.6	7.4
14	16.4	16.9
15	8.3	10.5
16	7.0	8.2
17	10.7	13.7

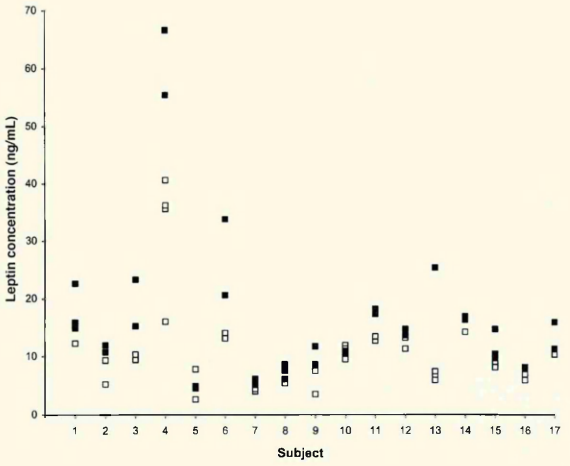

Fig. 2. Levels of leptin for each subject in the follicular (open square) and luteal (black square) phases.

for each woman are shown in Table 1, and all levels plotted in Fig. 2. Leptin levels were significantly higher (P <0.01) in serum obtained during the luteal phase than in serum collected during the follicular phase. The overall median serum leptin levels during the follicular and luteal phases were 10.0 ng/mL (range: 4.3–32.2 ng/mL) and 11.4 ng/mL (range: 4.9–66.7 ng/mL) respectively.

BMI measurements were not obtained for subjects 2 and 10. For the 15 subjects whose values were obtained, significant correlation was seen between leptin level and BMI (r = 0.54, P <0.05, Fig. 3). Luteal-phase progesterone levels were obtained in 14 of the 17 subjects; however, no correlation was seen between leptin and progesterone at the peak progesterone level, 10 days after the LH surge (r = –0.34, P <0.370, data not shown).

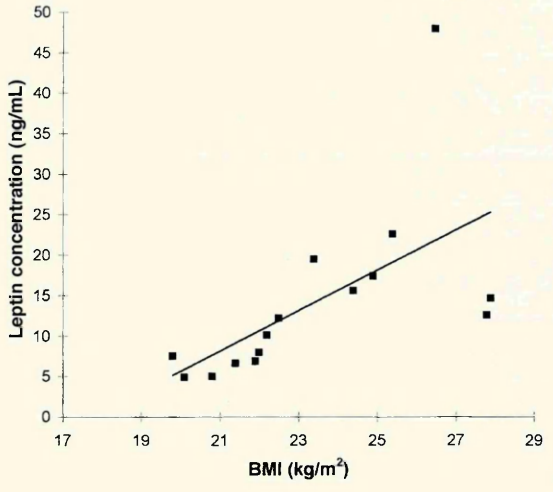

Fig. 3. The relationship between mean serum leptin levels and BMI. A significant correlation was seen (r = 0.54, P <0.05).

Discussion

The results indicate that circulating leptin concentration increases after ovulation in the luteal phase of the menstrual cycle, and this was seen in 14 of the 17 women included in the study. Levels of leptin showed no change in three women. The reason for this is unknown, but it may be due to differences in levels of reproductive hormones. This work confirmed and extended the work of Hardie *et al.*[12] who also found that leptin levels increased during the luteal phase of the menstrual cycle. Their study, however, was limited by the fact that it included only six regularly cycling women. In the present study, measurements were obtained in 17 women. In addition to similar changes seen throughout the menstrual cycle in both studies, the absolute levels of leptin in serum were similar in the present study and that of Hardie *et al.*[12]

Observation that leptin levels are higher during the luteal phase of the cycle would agree with suggestions for a role in reproduction[13] and possibly implantation.[10] The presence of leptin receptors in the ovary[10] also suggests that it may play a role in ovarian function, and the rise in leptin level seen during the luteal phase supports the hypothesis that it interacts with the hypothalamic–pituitary–ovarian axis to help maintain the corpus luteum.

Several studies have shown that serum leptin level correlates well with BMI.[3] In this study, significant correlation of BMI with serum leptin level was also seen. The relationship between BMI and reproductive ability is well established. Gymnasts, marathon runners and others with low-percentage body fat often have amenorrhoea, and puberty is delayed in females with low-percentage body fat mass.[14] In addition, it has been shown that a critical fat mass (>10th centile) is required for ovulation in women,[15] and it has been suggested that leptin may serve as a signal to the reproductive system to indicate that stores are sufficient to sustain reproduction.[16]

Some interpersonal variation in the leptin levels measured was noted. The reason for this is unknown, although a study has shown that leptin levels in women are highly variable, even when normalised for BMI. However, subject 4 had a far higher serum leptin level than other subjects, with only a moderately raised BMI of 26.5 kg/m^2. A possible explanation for this anomaly is that she had eaten prior to having the sample taken and, hence, had a short-term change in metabolism. Alternative explanations may be the presence of a leptin receptor defect or the presence of a much greater adiposity than in the other subjects. BMI is not a measure of adiposity but of total body weight compared with height, and leptin levels have been shown to correlate better with measures of adiposity than with BMI.[4] In addition, other

factors such as exercise[17] which may affect serum leptin concentrations were not controlled for in this study. A number of other factors including diurnal rhythms, food intake, cytokines and hormones are known to influence leptin levels. We can offer no clear explanation of the raised levels of leptin in subject 4, which were consistent in all her samples.

In contrast to the study by Hardie *et al.*,[12] no correlation was seen between luteal-phase progesterone levels and mean luteal-phase leptin levels. In our study, progesterone measurements were only obtained at one point in the cycle, whereas in the study by Hardie *et al.*[12] they were obtained throughout the cycle. Other studies, in women undergoing IVF, have suggested that levels of leptin correlate with oestradiol and FSH. We did not measure these in this study; however, leptin may correlate better with these reproductive hormones than it does with progesterone.

In summary, we have shown that serum leptin levels increase during the luteal phase of the menstrual cycle, supporting the contention that leptin may have a role in reproduction. In addition, this fluctuation should be taken into account when studies involving the measurement of leptin are performed in female subjects.

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Leptin Binding Activity Changes with Age: The Link between Leptin and Puberty*

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ABSTRACT

The timing of the physical transition from child to adult is determined by a biological clock that switches off the pituitary gonadal axis during infancy until puberty. Body composition (and in particular, fat mass), through leptin, are critical signals to this clock. However, no direct relationship between leptin and puberty has been demonstrated. Leptin is bound in the circulation by a high-affinity binding protein, which has been identified as a soluble leptin receptor. We found circulating levels of leptin binding activity (LBA) to be low at birth, to be high in the prepubertal years, to fall through puberty, and then to remain stable during adult life. LBA correlated with pubertal status in both boys and girls. We postulate that the fall in LBA, associated with increasing age and puberty, reflects a reduction in expression of truncated leptin receptors, and leptin is then available to the full-length receptor, which transmits the biological signal for leptin. The high levels of LBA occur during the years when the pituitary gonadal axis is quiescent. Thus, the change in LBA could explain how leptin regulates puberty. (*J Clin Endocrinol Metab* **84:** 2336–2341, 1999)

PUBERTY, the transition from child to adult is the result of reactivation of the pituitary gonadal axis. Sex steroids reach adult levels in late gestation, driving sexual differentiation in the fetus, then (shortly after birth) the pituitary gonadal axis switches off and lies dormant until the onset of puberty. We are now beginning to define some of the metabolic switches that program this biological clock. The importance of body composition has been recognized since the 1970s, when Frisch (1) suggested that it was necessary for women to maintain a specific percentage of body fat to achieve menarche and fertility. The recent cloning of the obesity gene and characterization of its product leptin, which is secreted from fat cells, has provided us with a signaling system whereby energy stores are sensed by physiological systems. It is now clear that leptin has a much wider role in human metabolism than just the regulation of body fat mass (2, 3).

Leptin acts through a class 1 cytokine receptor, the leptin receptor, which is highly expressed in the hypothalamus (4, 5). The biological actions of leptin include the regulation of appetite and energy expenditure, as well as involvement in the control of the hypothalamic-pituitary-gonadal axis (2, 3). In rodents, leptin administration accelerates the onset of reproductive function and restores fertility in the mutant ob/ob mouse (6, 7). Two recent reports of humans with mutations in the leptin receptor and leptin gene, who failed to progress through puberty, provide important evidence for the role of leptin in facilitating pubertal development (8, 9). However, the link between puberty and leptin is not fully established. Cross-sectional and longitudinal studies of leptin levels before and during puberty indicate that leptin levels follow changes in fat mass (10–12), although a brief pulse of leptin may precede the onset of puberty in males (13). In children, age is also an independent determinant of leptin levels, and leptin may act as a permissive signal to puberty over time (12).

Binding proteins for circulating leptin have been described in both the human and rodents (14–16). Varying sizes of the binding protein have been reported but would be compatible with the size of the extracellular domain of the leptin receptor (14, 15). In humans, the leptin-binding protein can be precipitated by a leptin receptor antibody (14). In the pregnant mouse, the binding protein has been sequenced and confirmed as the extracellular domain of the leptin receptor (17), and expression of the extracellular domain of the human leptin receptor in COS7 cells results in the production of a binding protein in the medium (6). These results are consistent with observations made for other members of the class I cytokine family of receptors, a number of which produce soluble receptors that represent the extracellular domain of the receptor (18).

Leptin-binding protein [or leptin binding activity (LBA)]

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has only been measured in limited clinical situations. In the studies performed, LBA was lower in obese patients, compared with lean, suggesting that the obese have higher free levels of leptin (14–16). We have modified an assay for GH-binding protein (GHBP) to measure LBA (19). The assay specifically measures a high-affinity binding protein; and using this assay, we have measured levels of LBA through the ages from birth to old age. The results show that the major change in LBA occurs at puberty.

Materials and Methods

LBA assay

Stripping of leptin from serum sample. To 200 μL of serum, 500 μL of prechilled 2% Norit A charcoal (American Norit Co., Atlanta, GA) 0.2% Dextran T70 (Amersham Life Science, Buckinghamshire, UK) in assay buffer was added at room temperature, vortex mixed, and immediately placed on ice for 5 min. The sample was centrifuged at 4 C for 12 min at 10,000 × *g*. The supernatant (stripped of free leptin) was removed by pipette and placed in a new tube.

Measurement of LBA. Fifty microliters of stripped serum was incubated with 150 μL assay buffer (0.01 mol/L phosphate buffer (pH 7.4), 0.18 mol/L $MgCl_2$, 1% BSA), 100 μL [^{125}I]Leptin [135 μCi/μg (Linco Research, Inc., St. Louis, MO)], in the presence (nonspecific) or absence (total binding) of 1 μg unlabeled leptin (R&D Systems, Abingdon, Oxford, UK). For separation of bound from unbound, 1 mL prechilled 2% charcoal-0.2% Dextran T70 slurry in assay buffer was added to the overnight incubation, vortexed, placed on ice for 10 min, and centrifuged at 4 C for 12 min at 10,000 × *g*. One milliliter of the supernatant was aspirated by pipette and counted in an automatic γ-counter. The specific binding (total binding minus nonspecific binding) obtained was expressed as a percentage of the total [^{125}I]Leptin counts per minute incubated in 50 μL of serum and called the LBA. All samples were measured in duplicate.

Serum samples with high and low levels of LBA were used as control serum and were run in each assay at the beginning and end of the assay.

High-performance liquid chromatography (HPLC) analysis

The HPLC method was developed from a method used to measure GH-BP by Tar *et al.* (20). HPLC separations were performed using a liquid chromatograph (model 600, Waters Corp., Milford, MA) equipped with a sample injector (model U6K) fitted with a 250-μL loop and an analytical Protein Pak 300sw column (Waters; 0.75 × 30 cm). Absorbance at 280 nm was monitored with an LC spectrophotometer (Waters), and radioactivity was recorded on-line using a Bertold LB 504 γ-detector (E G and G, Evry, France) connected to a Compaq computer.

Serum (150 μL) was incubated overnight at 4 C with 30 μL assay buffer and 20 μL [^{125}I]Leptin. A parallel incubation was carried out in the presence of an excess of unlabeled leptin (2 μg). After filtration through a 0.45-μm minifilter Millipore Corp. (Watford, UK), the entire incubation was placed onto an HPLC Protein Pak 300sw column. Elution was performed autocratically using a degassed buffer (0.1 mol/L Na_2SO_4 and 0.1 mol/L potassium phosphate, pH 7.0) pumped at a rate of 0.5 mL/min.

Leptin measurement

Human leptin concentrations were measured in duplicate using a specific Human Leptin RIA kit (Linco Research, Inc.) with an interbatch variation of between 3.0 and 6.0% and a detection limit of 0.5 ng/mL.

Statistics

Statistical analysis was performed using the software package SPSS. Pearson product-moment correlations were calculated to test association among variables. One-way ANOVA was used to investigate the effect of pubertal status on LBA. The *t* test, with Bonferroni adjusted *P* values, was used to look for differences between groups. A stepwise multiple-regression analysis with a *P* value less than 0.05 for entry and less than

TABLE 1. Clinical details

	Umbilical cord	Pre-pubertal		Pubertal		Young men median (range)	Young women median (range)	Old men median (range)	Old women median (range)
		Boys median (range)	Girls median (range)	Boys median (range)	Girls median (range)				
Number	10	26	26	39	41	18	17	10	9
Age (years)	NM	7.8 (5–11.8)	8.0 (5.3–11.6)	13.9 (11.1–18.2)	12.6 (8.91–17.2)	22 (20.0–23.5)	21.5 (21.0–25.0)	68 (67.0–76.0)	67.5 (60.0–81.0)
BMI (kg/m^2)	NM	16.1 (13.9–22.9)	15.8 (11.8–23.1)	19.3 (15.0–27.0)	18.9 (15.52–30.24)	24.6 (19.0–26.4)	22.8 (19.0–26.4)	26 (23.1–30.5)	25.8 (20.0–30.0)
Weight (kg)	3.6 (1.7–4.2)	26.0 (17.6–46.8)	26.6 (17.1–49.8)	54.7 (31.4–89.4)	45.5 (27.6–72.4)	NM	NM	77 (62.1–90.2)	60.55 (50.3–77.5)
Leptin (ng/mL)	34.3 (8.4–100)	3.5 (1.7–14.2)	3.3 (1.8–13.9)	2.84 (1.3–24.3)	9.6 (2.0–30.8)	1.3 (0.1–25.2)	9.1 (1.5–26.5)	8.2 (2.5–17.0)	16.0 (3.9–105.0)

NM, not measured.

0.10 for removal was used to determine which of the following possible explanatory variables [age, puberty, body mass index (BMI), and leptin] could be used to predict LBA in boys and girls.

Serum samples

All children and adults gave informed written consent or assent, and the studies had the approval of the local research ethics committees. Serum samples were taken between 0900 and 1000 h, and neonatal samples were from umbilical cord blood. Samples were separated and stored at −20 C until analysis. Pubertal staging was performed by an experienced pediatrician using the Tanner stages of puberty. Clinical details are given in Table 1.

Results

Assay validation

Competition experiments. Displacement curves with serial dilutions of neat stripped serum showed parallel displacement. Specificity for leptin was confirmed by incubation with unlabeled human GH and interleukin-6 (Fig. 1A), which showed no displacement. Scatchard analysis of competition experiments with human leptin showed a linear plot with a binding affinity of 1.0–1.4 × 10^9 mol/L^{-1} (Fig. 1B).

Serum was stripped of endogenous leptin by preincubation with dextran-coated charcoal. To establish the effect of the preincubation with dextran-coated charcoal, serum samples were incubated for up to 1 h, and then the leptin and LBA were measured. Leptin was reduced to undetectable levels with preincubation (n = 19), confirming that the dextran-coated charcoal was removing all endogenous free leptin.

The assay had a sensitivity of 0.6% specific binding (mean + 2 SD of 10 repeated measurements with zero sample). At 12 and 6% specific binding, the intraassay coefficient of variation was 3.2 and 4.1%, and the interassay coefficient of variation was 6.4 and 4.8%, respectively.

Results from this assay were compared with those obtained by HPLC. A single profile is shown in Fig. 1C. HPLC revealed a peak at the appropriate size for a soluble receptor (~85 kDa). Results of specific binding were comparable, considering the different methodolgy for the eight samples analyzed; mean ± SEM for LBA 10.5 ± 1.07% *vs.* HPLC 9.4 ± 0.78%. A comparison of samples analyzed is summarized in Table 2; these have been corrected to allow for the difference in sample volume used in the two assays.

LBA through the ages

Figure 2 shows the median LBA at the different ages of life. LBA was lowest at birth, highest in prepubertal children, and fell during puberty to a mean level of between 7 and 9% in young men and women and remained at this level through to old age. There were no differences in LBA between males and females at any age.

In adults, there was no evidence of a relationship between LBA and age, BMI, or leptin levels (Table 3). In contrast, in children there was an inverse relationship between LBA and age, pubertal status, weight, and BMI (Table 3 and Fig. 3).

Multivariate stepwise regression analysis, with LBA as the dependent variable and age, puberty, BMI, and leptin as the independent variables, was performed. In boys, the model explained 41% of LBA, with only age significantly associated with LBA. In girls, the whole model explained 51% of LBA,

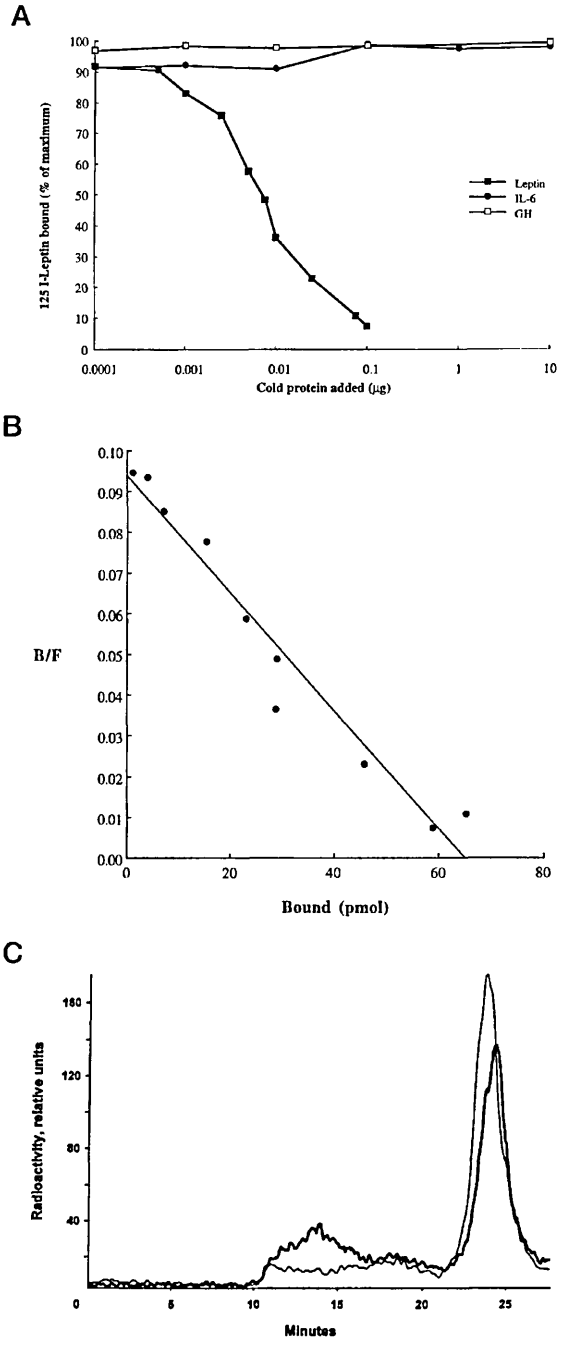

FIG. 1. A, Hormonal specificity of binding of [^{125}I]Leptin to normal human serum. The specific binding of [^{125}I]Leptin is expressed as a percentage of the maximal binding. Values are the mean of duplicate determinations from a representative experiment. IL-6, Interleukin-6. B, Scatchard analysis of [^{125}I]Leptin to normal human serum. B/F, Bound/Free. C, Elution profile of [^{125}I]Leptin incubated with human serum. [^{125}I]Leptin (4 × 10^4 cpm) was incubated with 150 μL serum without (*thick line*) and with (*thin line*) excess of native leptin. Binding was expressed as the radioactivity in the first peak over the radioactivity in peak 1 and peak 2 (free [^{125}I]Leptin. For the profile shown, total binding was 29.0%, and nonspecific 13.1%, radioactivity.

with age (46%) and BMI (5%) significantly associated with LBA. ANOVA showed a significant effect of pubertal status on LBA ($F = 21.2$, $P < 0.0001$) with LBA at all stages of puberty being significantly lower than levels at stage 1 of puberty, and stage 2 being different from all stages except 3. Pearson correlations between LBA and age, BMI, or leptin at each pubertal stage were performed. In girls, LBA was negatively correlated with age during Tanner stage 1.

TABLE 2. Comparison of results from LBA and HPLC method

Samples	Specific binding (%)	
	LBA	HPLC
1	8.1	6.3
2	9.7	8.1
3	6.5	11.7
4	10.5	8.8
5	9.3	7.2
6	12.5	10.4
7	16.5	12.6
8	10.5	10.3
Mean ± SEM	10.5 ± 1.07	9.4 ± 0.78

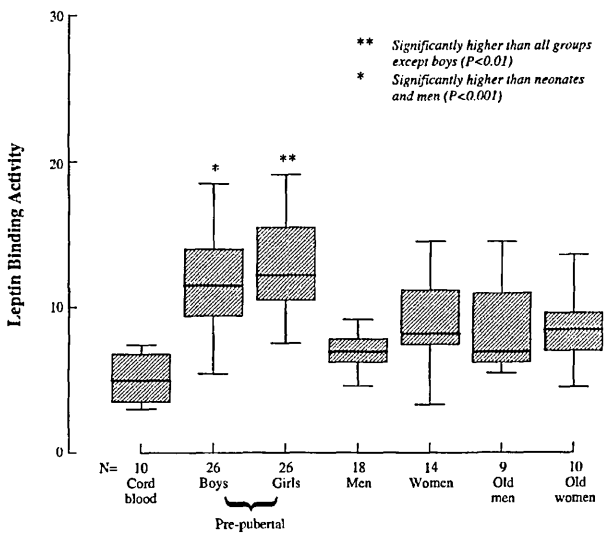

FIG. 2. LBA through the ages from birth to the elderly.

In boys, LBA was inversely correlated with serum leptin during stage 4.

Discussion

Circulating soluble receptors are a feature of a number of members of the cytokine family of receptors (18). The most widely studied, with respect to the endocrine system, is the GHBP, which is derived (by proteolytic cleavage) from the extracellular domain of the GH receptor (21). In most studies, GHBP levels reflect the tissue-specific expression of the GH receptor; and recently, a truncated form of the receptor has been identified that generates large amounts of GHBP and acts as a dominant negative antagonist of GH signaling (22, 23). The leptin receptor gene is expressed as a number of alternatively spliced isoforms (Ob-Ra to Ob-Re) (24, 25) of which Ob-Ra, b, and e are the major splice forms (26). Ob-Rb contains all elements for signaling, Ob-Ra has a truncated cytoplasmic domain, and Ob-Re is truncated at the extracellular domain, which, when expressed in transfection studies, generates an LBA (6). The finding of a fall in high-affinity LBA may reflect a change in expression of truncated leptin receptors, and this may result in more free leptin being available at the hypothalamus, which expresses Ob-Rb, the full-length signaling leptin receptor.

We have validated an assay for measuring LBA in human serum. The assay is based on a method previously described for the measurement of GH-binding protein activity (19). The LBA assay was specific, reproducible, and sensitive. It detected a high-affinity binding protein, Ka 1.0–1.4 $\times$ 10^9 M^{-1}, which would be compatible with the expected affinity of the soluble receptor (6). Results from this assay were comparable with HPLC analysis of leptin-binding protein. Scatchard analysis suggested that the assay detected a single species of binding protein, although there may be lower-affinity leptin-binding proteins that were not detected by this assay (14). LBA has been reported to relate inversely to leptin levels; however, this may have been an artefact caused by interference in the assay by endogenous leptin (16). In the present study, endogenous leptin was removed before assaying LBA, and no statistical evidence of a relationship was found between leptin levels and binding activity except in girls. In girls, leptin increases with age and puberty as LBA is decreasing. The negative correlation would therefore be expected.

LBA was low at birth, high in early childhood, fell during

TABLE 3. Correlation analysis between different variables and LBA

Variable	Umbilical cord	Boys	Girls	Men	Women
Number of subjects	10	65	67	18	23
Age	NM	$r = -0.64$ $P = 0.0001$	$r = -0.66$ $P = 0.0001$	$r = -0.09$ $P = 0.713$	$r = -0.35$ $P = 0.226$
Pubertal status	NM	$r = -0.62$ $P = 0.0001$	$r = -0.61$ $P = 0.0001$	NM	NM
Testicular volume	NM	$r = -0.60$ $P = 0.0001$	NA	NM	NA
BMI	NM	$r = -0.38$ $P = 0.002$	$r = -0.51$ $P = 0.0001$	$r = -0.19$ $P = 0.47$	$r = -0.35$ $P = 0.28$
Leptin	$r = -0.31$ $P = 0.45$	$r = -0.03$ $P = 0.80$	$r = -0.42$ $P = 0.001$	$r = -0.21$ $P = 0.41$	$r = -0.06$ $P = 0.83$

NM, Not measured; NA, not applicable.

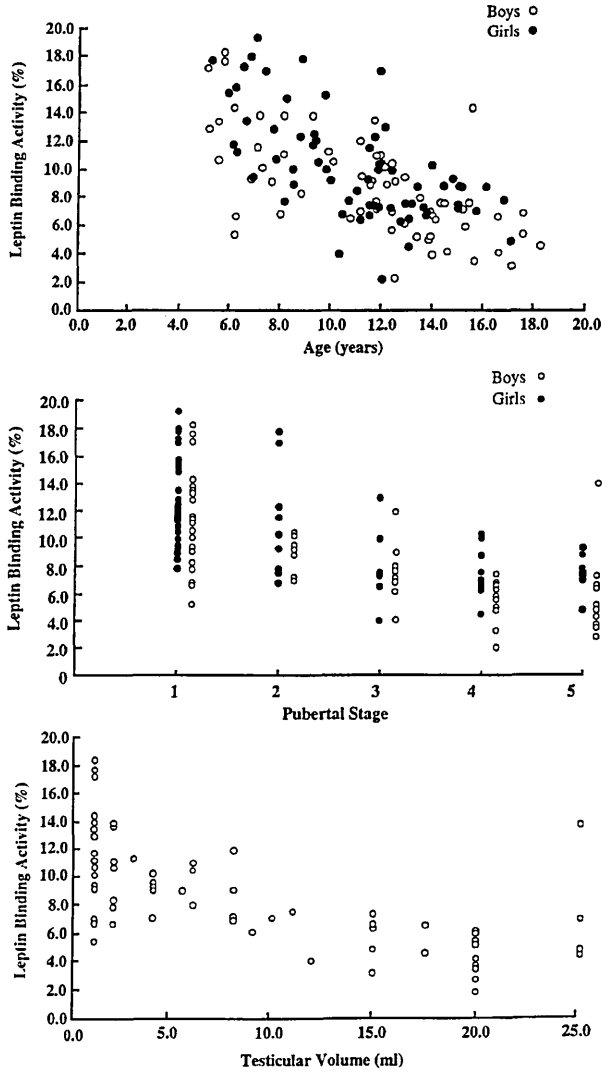

FIG. 3. LBA against: age (A), pubertal status (B), and testicular volume (C).

puberty, and remained at the postpubertal level throughout adult life. These changes in LBA parallel the known changes in activity of the pituitary gonadal axis, with high levels of LBA being present during the childhood years, when the pituitary gonadal axis is quiescent. There was no difference between boys and girls or men and women in LBA. In pubertal children, LBA showed a significant relationship with age, pubertal status, BMI, and testicular volume in boys. Multivariate analysis suggested that the most important factor was age. There is an absolute requirement for leptin for the initiation and progression of puberty, as demonstrated by the hypogonadal state of leptin-resistant or -deficient human subjects (8, 9). However, leptin may only provide a tonic background signal on which other initiators act, because leptin levels in puberty primarily relate to fat mass (2). Our finding, of the direct inverse relationship between LBA and pubertal status or age, with a fall in LBA seen at the earliest stages of puberty, suggests that this is a link from leptin to the progression of puberty.

In adult subjects, there were no differences in LBA between the sexes. This is in sharp contrast to the well-recognized sexual dimorphism in serum leptin levels (3). If the change in LBA is related to a change in leptin receptor subtype, then this may be an alteration that occurs at puberty and remains unaltered through adult life. We found no relationship between BMI and LBA in adults, although our studies were confined to a normal population. Previous studies have suggested that LBA is reduced in obesity (14–16).

In conclusion, we have developed a simple and specific assay for the measurement of LBA. This assay demonstrates an inverse relationship between age and LBA and a strong correlation between puberty and a fall in LBA. The fall in the earliest stages of puberty suggests that this is a primary event, not driven by changes in sex steroids. The fall in LBA could be explained by a change in leptin receptor expression, which may be the link between leptin and puberty.

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