ADIPOSE TISSUE DERIVED FACTORS IN OBESITY, INFLAMMATION & ENERGY HOMEOSTASIS

By

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DECLARATION

I declare that this thesis is a record of results obtained by myself and, is composed by myself, unless otherwise stated in the text in the acknowledgments. None of the work has been previously submitted for a higher degree.

All sources have been specifically acknowledged by means of reference.

SUMMARY

Obesity is the foremost contributory factor in the progression to type 2 diabetes mellitus (T2DM). Moreover, chronic inflammation, through activation of innate immunity is proposed to link obesity, insulin resistance and T2DM. Adipose tissue, traditionally considered a storage compartment for triglycerides, also functions as an active endocrine organ. Adipocyte-secreted products, termed adipokines, may link obesity-associated inflammation and insulin resistance. Adipokines exert multiple effects on insulin sensitisation, glucose homeostasis, inflammatory processes or central systems mediating energy expenditure. This thesis principally examined two adipokines; resistin and adiponectin. Resistin and components of innate immunity were assessed in human obesity. *In-vitro* analysis established that resistin was expressed and secreted by human adipocytes. Furthermore, key factors in the innate immune pathway were highly expressed in obese and T2DM adipose tissue. This thesis further explored the pro-inflammatory actions of resistin in adipocytes. Resistin stimulated the secretion of inflammatory cytokines from adipocytes and, the expression of key intermediates of the innate immune and insulin signalling pathways. Clinical studies entailed examination of resistin as a marker of inflammation in childhood obesity. Serum analysis revealed gender-differences in resistin levels in obese children. Furthermore, bacterial endotoxin correlated with several markers of inflammation and cardiovascular disease; suggesting endotoxin as a contributor to inflammation in childhood obesity. This thesis subsequently examined another adipokine, adiponectin; considered to have a 'ying-and-yang' relationship with resistin. Studies explored a central role for adiponectin in energy homeostasis. Gel-filtration liquid chromatography established that the adiponectin trimer was predominant in human cerebrospinal fluid. Such identification of trimeric adiponectin *in vivo* implicates the pharmacologically generated globular adiponectin in central regulation of energy expenditure. In conclusion, resistin may serve as a pathogenic pro-inflammatory factor, exacerbating inflammation within adipose tissue; potentially contributing to the progression of obesity-driven T2DM. Alternatively, adiponectin may have favourable central actions, influencing energy expenditure through its basic trimeric form. Collectively, this thesis suggests that resistin and adiponectin, with a range of opposing properties, may substantially affect whole-body metabolism.

ABBREVIATIONS

AbSc	Abdominal Subcutaneous
AbSc AT	Abdominal Subcutaneous Adipose Tissue
Ad	Adipocyte
ADIPOR	Adiponectin Receptor
ADSF	Adipocyte-specific Secretory Factor
AGT	Angiotensinogen
ALLN	N-Acetyl-Leu-Leu-Nle-CHO (Calpain Inhibitor I)
AMPK	AMP-activated Protein Kinase
ANCOVA	Analysis of Covariance
ANG-II	Angiotensin-II
AP-1	Activator Protein-1
ap2	Adipocyte Fatty Acid Binding-protein
ASP	Acylation-stimulating Protein
AT	Adipose Tissue
BAT	Brown Adipose Tissue
BBB	Blood-brain Barrier
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CAC	Coronary Artery Calcification
$CaCl_2$	Calcium Chloride
CART	Cocaine-and Amphetamine-Regulated Transcript
cDNA	Complementary (to mRNA) Deoxyribonucleic acid
CNS	Central Nervous System
CO_2	Carbon Dioxide
CRH	Corticotrophin-releasing Hormone
CRP	C-reactive Protein
CSF	Cerebrospinal Fluid
Ct	Cycle Threshold
CV	Coefficient of Variance
CVD	Cardiovascular Disease
Cys	Cysteine Residue
Cys-39Ser	Transgenic 'Trimeric' Adiponectin Mutant Mice

Da	Daltons
db/db	Leptin Receptor-deficient Mouse
DC	Detergent Compatible
ΔCt	Delta Cycle Threshold
dH_2O	Distilled Water
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DN*ase*	Deoxyribonuclease
dNTPs	Deoxynucleotides Triphosphates
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
ECSIT	Evolutionarily Conserved Signalling Intermediate in Toll Pathways
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbant Assay
EMBL-EBI	European Bioinformatics Institute
ER	Endoplasmic Reticulum
FABP-2	Fatty Acid Binding-protein-2
FABPs	Fatty Acid Binding-proteins
fa/fa	Obese Zucker Rat
FAM	RT-PCR Reporter Fluorochrome/Dye Label
FFAs	Free Fatty Acids
FGF	Fibroblast Growth Factor
FIZZ	Found in Inflammatory Zone
FPLC	Fast Protein Liquid Chromatography
g	Gram
g	Force of Gravity
GI	Gastrointestinal
GLUT-4	Glucose-transporter-4
GLUTs	Glucose Transporters
G6Pase	Glucose-6-phosphatase
GSK-3	Glycogen Synthase Kinase-3
HBSS	Hank's Balanced Salt Solution

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HMW	Higher Molecular Weight
H_2O	Water
HOMA	Homeostasis Model Assessment
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
hr	Hour
HRP	Horseradish Peroxidase
ICAM-1	Intercellular Adhesion Molecule-1
i.c.v	Intracerebroventricular
IGF-1	Insulin-like Growth Factor-1
IGF-1BP	IGF-1 Binding protein
IGT	Impaired Glucose Tolerance
IκB	Inhibitor of NF-κB
IKK	Inhibitor of NF-κB Kinase
IKKα	Inhibitor of NF-κB Kinase-α
IKKβ	Inhibitor of NF-κB Kinase-β
IKKγ/NEMO	Inhibitor of NF-κB Kinase-γ
IL	Interleukin
IR	Insulin Receptor
IRAK	IL-1 Receptor-associated Kinase
IRS	Insulin Receptor Substrate
JNK	c-Jun N-terminal Kinase
kDa	Kilodaltons
L	Litre
LMW	Low Molecular Weight
LPS	Lipopolysaccharide
LXR	Liver X Receptor
M	Molar
M_r	Molecular Weight
mal1	Adipocyte/macrophage Fatty Acid-binding Protein-5
MALP-2	Macrophage-activating Lipopeptide-2
MAPK	Mitogen-activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1
MEKK1	Mitogen-activated Kinase Kinase-1

$MgCl_2$	Magnesium Chloride
MS	Metabolic Syndrome
μg	Microgram
mg	Milligram
min	Minute (time)
μl	Microlitre
ml	Millilitre
mM	Millimolar
MMPs	Matrix Metalloproteinases
MONICA	Multinational Monitoring of Trends and Determinants in CVD
MPO	Myeloperoxidase
mQH_2O	Milli Q water (ultra-filtered water)
mRNA	Messenger Ribonucleic acid
MyD88	Myeloid Differentiation Primary Response Gene-88
NaCl	Sodium Chloride
NF-κB	Nuclear Factor-κB
NEFA	Non-esterified Fatty Acid
NIK	NF-κB-inducing Kinase
ng	Nanogram
nm	Nanometre
NPY	Neuropeptide Y
N.S	Non-significant
ob/ob	Leptin-deficient Mouse
OB-Rb	Leptin Receptor
OD	Optical Density
Om	Omental
Om Ad	Omental Adipocytes
Om AT	Omental Adipose Tissue
p	Phosphorylated
PAI-1	Plasminogen Activator Inhibitor-1
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered Saline
PBS-T	Phosphate-buffered Saline containing 0.1% Tween 20

PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate Carboxylase
PDK-1	3-phosphoinositide-dependent Protein Kinase-1
PI3K	Phosphoinositide-3 Kinase
PIP2/PtdIns(4,5)P_2	Phosphatidylinositol-4,5-bisphosphate
PIP3/PtdIns(3,4,5)P_3	Phosphatidylinositol-3,4,5-trisphosphate
PKB/Akt	Protein Kinase B
PKC	Protein Kinase C
POMC	Proopiomelanocortin
PPAR-γ	Peroxisome Proliferator Activated Receptor-γ
PPRE2	PPAR-γ Response Element-2
PPREs	PPAR-γ Response Elements
psi	Pounds Per Square Inch
PVDF	Polyvinylidene-fluoride
PVN	Paraventricular Nucleus
RAGE	Receptor for Advanced Glycation End-products
RELM	Resistin-like Molecule
RELMs	Resistin-like Molecules
RETN	Human Resistin Gene
Retn	Mouse Resistin Gene
RIA	Radioimmunoassay
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RSG	Rosiglitazone
RT	Room Temperature
RTn	Reverse Transcriptase
RT-PCR	Real-time PCR
s	Second (time)
S_A	Adiponectin Sensitivity Index
SAPE	Streptavidin-Phycoerythrin
Sc	Subcutaneous
Sc Ad	Subcutaneous Adipocytes
Sc AT	Subcutaneous Adipose Tissue

Ser	Serine Residue
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SH2	Src Homology 2
sICAM-1	Soluble Intercellular Adhesion Molecule-1
S-Resistin	Short Resistin
SNPs	Single Nucleotide Polymorphisms
SOCS	Suppressor of Cytokine Signalling
SOCS-3	Suppressor of Cytokine Signalling-3
sTNF-R2	Soluble TNF-receptor 2
3T3-L1	Mouse Embryonic Fibroblast Cell Line
TAK1	TGF-β-activated Kinase-1
Taq	Thermus Aquaticus (DNA polymerase)
TBS	Tris-buffered Saline
TBS-T	Tris-buffered Saline containing 0.1% Tween 20
TCA	Trichloroacetic Acid
T2DM	Type 2 Diabetes Mellitus
TEMED	N, N, N', N'-Tetramethylethelenediamine
TG	Triglyceride
TGF-β	Transforming Growth Factor-β
TIR	Toll-interleukin 1 Receptor-resistance
TLR	Toll-like Receptor
TLRs	Toll-like Receptors
TNF-α	Tumour Necrosis Factor-α
TNFR	TNF-α Receptor
TNF-R2	TNF-α Receptor-2
TRAF	TNF Receptor-associated Factor
TRAF-6	TNF Receptor-associated Factor-6
Tris	Tris (hydroxymethyl) Aminomethane
Tris-HCl	Tris Hydrochloride
TZD	Thiozoladinedione
TZDs	Thiozoladinediones

U	Units
UV	Ultraviolet
V	Volts
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VIC	RT-PCR Fluorochrome/Dye Label
v/v	Ratio of Volume per Volume
w/v	Ratio of Weight per Volume
WAT	White Adipose Tissue
WHO	World Health Organisation
yrs	Years

Chapter 1

Introduction

1.1. Obesity: The Epidemic.

The development of type 2 diabetes mellitus (T2DM) occurs as a consequence of several metabolic disorders and is now considered a 'polygenic disease'. Obesity is rapidly becoming a worldwide epidemic and is acknowledged as one of the single most contributory factors in the pathogenesis of T2DM and insulin resistance. Epidemiological data show that the prevalence of obesity has significantly increased over the past 25 years by almost 400% (D'Arcy 2006) and continues to do so at an alarming rate (Wang *et al.* 2005). More specifically, within the UK, approximately 50% of the adult population are either overweight or obese, with more than a million people affected with T2DM (Canoy D *et al.* 2007). Moreover, a global epidemic of paediatric obesity has increased in recent years and is continuing to do so (Chinn *et al.* 2001; Els *et* al. 2005; Reilly 2006). In particular, there is a rising incidence of T2DM in children within the UK (Haines *et al.* 2007). The worldwide spread of the obesity epidemic has been highlighted by the MONICA World Health Organisation (WHO) project; detailing increased rates of obesity in 48 different countries (Berrios *et al.* 1997; Heseker *et al.* 2000).

The epidemiological link between obesity and diabetes is largely apparent in western societies. Whilst the incidence of obesity is clearly increasing in many ethnic populations, synergistically is the prevalence of T2DM. Globally, the prevalence of diabetes for all age-groups was estimated to be 2.8% in 2000 and 4.4% in 2030 (Wild *et al.* 2004); the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Engelgau *et al.* 2007). As such, the obesity epidemic is now termed the 'diabesity' epidemic. The epidemiological association between obesity and T2DM has been acknowledged by numerous studies; in

particular, the Nurses Health Study and Health Professionals Follow up Study (Chan *et al.* 1994; Colditz *et al.* 1995). This study specified that, as body mass index (BMI) increases, the risk of developing T2DM also increases, to such an extent that women with a BMI of 35 kg/m^2 or above have a 40-fold increased risk of developing T2DM. Similar findings were reported for men, whereby a BMI of 35 kg/m^2 or above, increased the risk of TD2M by 60-fold. 'Moderate' weight gain has additionally been shown to enhance diabetic risk. Recent studies have highlighted that for each kilogram in body weight gained, the relative population risk of developing T2DM increases by 4.5% (Ford *et al.* 1997) and, in some cases, 9% per kilogram (Mokdad *et al.* 2000). The increased diabetic risk following obesity is, to an extent, reversible with weight reduction. A near 50% reduction in diabetic risk was observed following a weight loss of 5-11 kg (Chan *et al.* 1994). Moreover, the study reported that the risk of developing T2DM was almost eradicated in subjects having lost 20 kg or with a BMI below 20 kg/m^2 (Chan *et al.* 1994). Similar observations were described in the Swedish Obese Subjects Study; one of the largest long-term evaluations for the use of surgery for weight reduction in obese patients (Sjostrom *et al.* 1997). Following gastric bypass surgery, 69% of subjects diagnosed T2DM subjects were no longer classified as diabetic (Sjostrom *et al.* 1997). Moreover, only 0.5% developed T2DM over the following two years (Sjostrom *et al.* 1997). It has therefore been proposed that the effect of sustained weight loss, achieved through surgical intervention, results in a reduced risk in the development of T2DM (Ballantyne *et* al. 2006; Ferchak *et al.* 2004).

To understand the importance of adipose tissue mass within T2DM, this introduction will highlight the impact of obesity on the pathogenesis of T2DM; detailing the role of the adipose-secreted factors and several pathogenic mechanisms that increase diabetic risk. Within this context, our current understanding of the emerging link between obesity-induced T2DM and inflammation will be described.

1.1.1 Obesity in Childhood & Adolescents: The Emerging Epidemic.

In the developed world, obesity is now the most common disease in children and adolescence. This striking global epidemic of paediatric obesity has occurred in recent years, with its prevalence dramatically continuing to increase. Using international definitions, at least 10% of children are overweight or obese worldwide (Libman *et al.* 2007; Lobstein *et al.* 2004); with the highest percentages in American (32%), European (20%) and Middle Eastern (16%) populations. An important concern is the onset of cardiovascular and metabolic risk factors that are consequently associated with paediatric obesity; the impact of such detrimental consequences have, to date been underestimated (Reilly 2006).

1.1.2 The Definition of Obesity.

Obesity is an excess of whole body fat (adipose tissue) mass; a level of adipose tissue mass that is considered harmful. Over recent years, obesity has been classified using body mass index (BMI). The BMI is the most routinely used indicator of obesity, due to its ease of determination. BMI is defined as the weight in kilograms of a subject divided by the square of their height in metres (kg/m^2). The WHO classified a BMI between 25-29.9 kg/m^2 as overweight; values above this are categorised as clinically

obese. Within the defined 'obese' category there is further sub-division, as demonstrated in **Table 1.1.2.1**.

WHO classification for Europids	**BMI (kg/m^2)**
Underweight	<18.5
Healthy/Lean	18.5-24.9
Overweight	25-29.9
Obese I	30-34.9
Obese II	35-39.9
Morbid Obesity	>40

Table 1.1.2.1 Table of the BMI scale, highlighting the classified limits (Weisell 2002).

BMI is further considered a good correlate of body-fat (Webster *et al.* 1984) and associated mortality/morbidity (Willett *et al.* 1999). However, BMI does not take into consideration differences in extremities; for example, subjects with high-fat mass or high-muscle mass. It has been proposed that obesity is best diagnosed using BMI centile charts; such centile charts take into account BMI alterations with differences in age and sex (Reilly 2006).

Although BMI definitions were generated for international use, it is now acknowledged that the prevalence and risk of T2DM varies amongst ethnic minorities. For instance, Japanese Americans have a higher rate of incidence of T2DM in comparison to those of European origin (Seidell *et al.* 2001). Furthermore, in certain areas of Asia, some populations with an average BMI of below 25 kg/m^2 have remarkably high rates of T2DM and cardiovascular risk. Additionally, when matched

for BMI, certain Asian populations have considerably higher percentage body-fat than Caucasian Europeans (Deurenberg *et al.* 1998; Swinburn *et al.* 1999; He *et al.* 2001). In particular, Pakistani, Indian and Bangladeshi men have relatively high levels of central obesity, with 41% of Indian men classified as centrally obese compared to 28% of men in the general population (Joint Health Surveys Unit 2000). It was therefore apparent that the original BMI limits designated for 'at risk groups' did not account for variation caused by difference in ethnicity; to address this issue, the BMI scale was adjusted for several Asian populations. The results of such a BMI scale adjustment are highlighted in **Table 1.1.2.2**. As an alternative to setting BMI limits for each nationality, the WHO established new BMI limits for people of Asian origin. New upper boundaries for the BMI scale were 23.0, 27.5, 32.5 and 37.5 kg/m^2 for lean, overweight, obese and morbidly obese respectively (WHO Expert Consultation (2004)). Such new boundaries for BMIs reflect the increased risk of metabolic syndrome in Asian populations with proportionately lower levels of body fat.

Ethnic Origin	Overweight		Obesity	
	Point Analysis	ANCOVA	Point Analysis	ANCOVA
China	24	25	29	30
China (Hong Kong)	23	22	27	27
Indonesia	24	22	26	27
Japan	25	24	30	29
Singapore	22	23	27	27
Thailand (Urban)	25	23	30	28
Thailand (Rural)	27	25	31	30

Table 1.1.2.2 Established thresholds (kg/m^2) calculated for overweight and obese groups of different Asian origins. Point analyses are calculated by the assumption that BMI limits for Caucasians equate to the same percentage body-fat in each Asian group. ANCOVA values are based on the analysis of co-variance with BMI as one variable, the ethnic group as a grouping variable and age, sex and percentage body fat as co-variates (WHO Expert Consultation (2004)).

1.1.3 Gender, Ethnicity & Specific Adipose Tissue Distribution.

The distribution of adipose tissue is altered by gender and ethnicity. Male subjects have a tendency to accumulate surplus adipose tissue around the abdominal area of the body, whereas female subjects accumulate adipose tissue predominantly around the hips and thighs. This was originally described by Vague, who observed differences between male 'android' obesity and female 'gynoid' obesity (Vague 1956). A stereotype of 'apple' *versus* 'pear' shaped fat-distribution has been described **(Figure 1.1.3)**; highlighting that men and women with a comparable BMI have significantly different percentages of body-fat. Interestingly, subjects with a gynoid 'pear-shaped' fat-distribution are at a lower risk of developing disease in comparison to BMI matched subjects with an 'apple-shaped' fat-distribution (Vague 1956).

Furthermore, only women with a similar fat-distribution to men seemed to develop diabetes at a high-rate of incidence (Vague 1956).

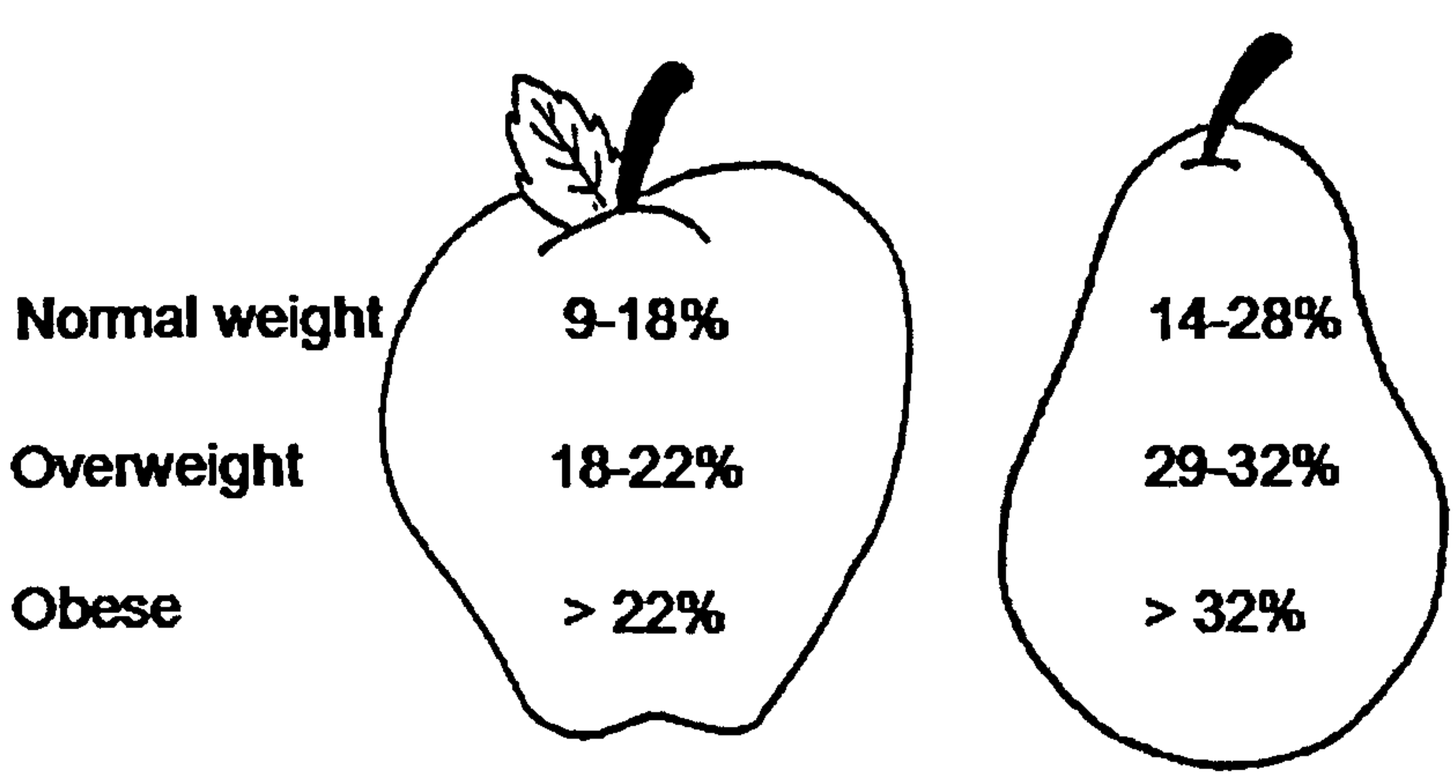

Figure 1.1.3 Diagrammatic representation highlighting the differences in body-fat percentage in each BMI category between males with an 'android' fat-distribtution and females with 'gynoid' fat-distribution. Figure adapted from (Vague 1956).

In terms of ethnicity, it has been reported that Asian populations gain excess fat more centrally, therefore have a high degree of central obesity (Pi-Sunyer FX 2004). Asian populations are therefore likely to adopt an 'apple shaped' rather than a 'pear shaped' body-fat distribution; as such, are more pre-disposed to abdominal obesity than people of European origin, which confers higher risks of T2DM and cardiovascular disease (CVD). It has further been reported that waist circumference is highly correlated with metabolic syndrome components and health risks than percentage body-fat and BMI (Shen *et al.* 2006).

Post-menopausal women also accumulate fat more centrally; this consequently conferes a higher risk of developing T2DM (Ruderman *et al.* 1998). Although speculative, androgens are postulated to regulate adipose tissue distribution. Post-

menopausal women have reduced levels of oestrogen. As a result, adipose tissue becomes the major source of oestrogen (Bjorntorp 1995); this may be responsible for re-distribtuion of adipose stores, causing central adiposity. Central adiposity has been shown to be re-distributed by Hormone Replacement Therapy (Haarbo *et al.* 1991), therefore implying oestrogen treatment to positively influence fat-distribution.

1.1.4 Types of Abdominal Adipose Tissue.

Abdominal adipose tissue is compartmentalised into two distinct anatomical regions/depots. These are 'subcutaneous' adipose tissue, surrounding the abdominal cavity beneath the skin, or intra-abdominal 'visceral' adipose tissue. Visceral adipose tissue consists of mesenteric omental adipose tissue mass; comprising of approximately <10% of total adipose tissue mass in lean subjects, which can reach up to twice the size in obese subjects (Wajchenberg 2000; Gasteyger and Tremblay 2002). Magnetic resonance imaging has shown that visceral fat accumulation confers a higher risk of T2DM than subcutaneous fat (Despres *et al.* 1995; Albu *et al.* 1997; Albu *et al.* 2000). Conversely, other studies have proposed that subcutaneous fat accumulation is the predominant contributor to the increased risk of T2DM, rather than visceral fat accumulation (Abate *et al.* 1996). Such controversy may arise from the relative proportions of visceral and subcutaneous fat. Visceral fat accounts for up to 20% of an obese subject's total fat-mass; whereas subcutaneous fat comprises the remaining 80% of total fat-mass (Montague *et al.* 1997), therefore implying that subcutaneous fat may have more of an impact on an obese subjects' metabolic profile.

1.2 The Composition of Adipose Tissue & the Development of Obesity.

In humans, white adipose tissue is the major storage site for excess energy; this in itself is stored in the form of lipids or triglycerides. More specifically, lipid composition consists primarily of myristic, palmitoleic, stearic, palmitic, linoleic and oleic fatty acids. Water (5-30%) and proteins (2-3%) contained within adipocytes form the remaining fraction, which is subsequently bound by a framework of collagen fibres.

For normal tissue function, adipose tissue mass is comprised of many cell types; these include adipocytes, pre-adipocytes, leukocytes, macrophages and endothelial-vascular cells. Adipocytes are uniocular cells, ranging in diameter from 25-200 microns and have a variance in diameter of up to 20-fold; this consequently allows the adipocyte cells to increase in volume of up to a 1000-fold (Fruhbeck *et al.* 2001). Adipocytes are therefore the predominant cell-type in adipose tissue. Pre-adipocytes are adipocyte precursor cells, serving as a store for adipocyte recruitment. Upon activation of hyperplastic growth, pre-adipocytes differentiate into fully mature adipocyte cells. Endothelial cells function to supply adipose tissue with blood, transporting proteins to and from the periphery, thus forming the tissue vasculature. Leukocytes, in particular macrophages, reside within adipose tissue, prompted to a site of inflammation to eliminate infected cells.

Obesity occurs when energy intake exceeds energy expenditure; such a notion is termed 'positive energy balance' and can occur as a result of overnutrition and lack of physical activity. This excess energy is stored in white adipose tissue (Frayn *et al.* 1995; Gregoire *et al.* 1998) and can, consequently, acutely modify the tissues

architecture and framework. For instance, one such modification of adipose tissue is to rapidly expand in size through hyperplastic and hypertrophic growth of adipocytes. Hyperplastic growth increases adipocyte number, via cell proliferation and differentiation. Whereas hypertrophic growth increases the adipocytes cell size, due to an uptake of lipids by the cell. Both processes are regulated at hormonal and genetic levels. Furthermore, key genes activate the 'formation of triglyceride' (TG) (lipogenesis) and the 'breakdown of TG' (lipolysis) (Gregoire *et al.* 1998).

1.2.1 Hyperplastic Growth of Adipocytes.

Shortly after birth, adipose tissue is formed by an increase in hyperplastic and hypertrophic growth of adipocytes (Gregoire *et al.* 1998). This early accumulation of adipocytes occurs through 'clonal expansion' of pre-adipocytes, resulting in the formation of new populations of precursor cells (Prins *et al.* 1997); such a process is termed 'proliferation'. Pre-adipocytes are derived from pluripotent stem cells, of which can develop into several different cell-types, including adipocytes, chondrocytes, osteoblasts or myocytes; such a process is known as cellular 'differentiation' or 'maturation'. Hyperplastic growth is regulated through both pre-adipocyte proliferation followed by differentiation; collectively, these processes are termed 'adipogenesis' **(Figure 1.2.1)**.

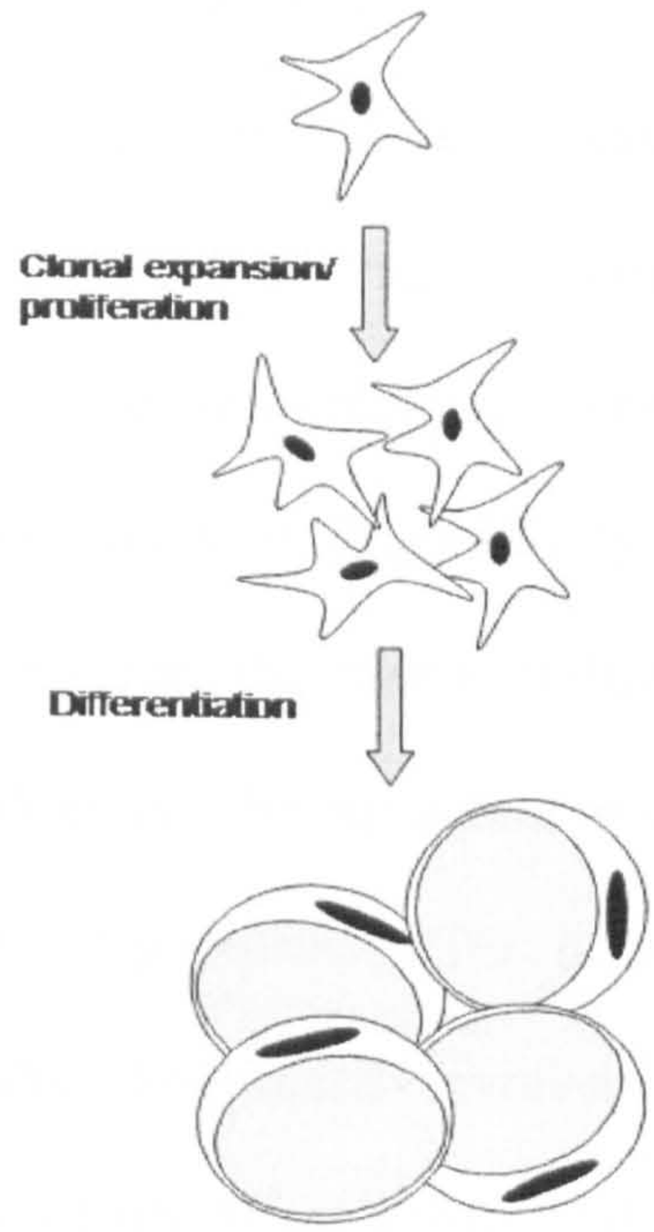

Figure 1.2.1 The stages in adipogenesis. Clonal expansion or proliferation of pre-adipocytes is followed by differentiation of the fibroblast-like precursor cells into mature adipocyte cells.

During differentiation, pre-adipocytes undergo drastic morphological changes, transforming from fibroblast-like structures to spherical cells. These alterations are coupled with changes in the surrounding extracellular matrix and cytoskeleton (Hansen *et al.* 1999). Differentiating pre-adipocytes are further subject to external stimuli; this determines whether the pre-adipocyte remains quiescent, proliferates, or commits to differentiation. Factors that stimulate adipogenesis include 'insulin-like growth factor-1 receptors' that signal through insulin receptor substrate (IRS) molecules (Miki *et al.* 2001) and free fatty acids (FFAs). Alternatively, factors that inhibit adipogenesis promote hypotrophic growth of existing mature adipocytes, thus hindering hyperplastic growth; such factors include inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) (Knowler *et al.* 1981; O'Rahilly 1997) and interferon-γ.

1.2.2 Hypertrophic Growth of Adipocytes.

Adipocytes store post-prandial triglycerides, thus increasing the adipocytes size; such a process is termed 'adipocyte hypertrophy'. Triglycerides, cholesterol and phospholipids circulate as lipoprotein complexes; these include very low density lipoproteins, low density lipoproteins and high-density lipoproteins. However, non-esterified free fatty acids (NEFAs) are the most abundant molecules in adipose tissue, circulating complexed with albumin. The regulation of triglyceride concentrations in circulation is maintained by 'lipogenesis' (TG formation) and 'lipolysis' (TG breakdown). More specifically, lipogenesis involves the hydrolysis of systemic triglycerides to form NEFAs, of which are transported into mature adipocytes; here the NEFAs are re-esterified to form triglycerides and stored intracellularly in the lipid droplet. Lipolysis involves the hydrolysis of intracellular triglycerides back into NEFAs, then released back into circulation; this reduces the amount of stored intracellular triglycerides and thus reduces the adipocytes' size; such a process is termed 'adipocyte hypotrophy'. The balance between lipolysis and lipogenesis has profound effects on systemic levels of NEFAs; of which elevated levels are a characteristic of obesity and T2DM and consequently, alter the mass and distribution of adipose tissue.

1.3 Obesity & Insulin Resistance.

The processes of adipogenesis, lipolysis and lipogenesis all alter the dynamic morphology of adipose tissue in response to nutrient intake. T2DM develops progressively within obese subjects; initially, subjects become resistant to insulin (Gulli *et al.* 1992; Jackson *et al.* 2000; Tripathy *et al.* 2000) due to an increase in systemic insulin levels, referred to as 'hyperinsulinemia'. Such an increase in

systemic insulin counteracts insulin resistance by maintaining hepatic glucose production and the response to glycemic load, thus overcoming impaired glucose uptake in peripheral tissues (Vauhkonen *et al.* 1997; Weyer *et al.* 1999). Once hyperinsulinemia cannot overcome whole body insulin resistance, the condition progresses to a state of Impaired Glucose Tolerance (IGT). The progression to T2DM is therefore not associated with an increase of insulin resistance, rather the gradual inability of the pancreas to maintain high levels of insulin production (Bogardus *et al.* 1984; Saad *et al.* 1989; Polonsky *et al.* 1996). As insulin production deceases, the progression from IGT to overt T2DM occurs. The processes whereby insulin regulates whole body metabolism and the key stages that influence insulin resistance and glucose intolerance are briefly outlined.

1.3.1. Insulin Signalling & Glucose Uptake.

To overcome periods of fasting, nutrients, for instance glucose are stored in tissues. Normal fasting glucose levels range from 4-7 mM (Saltiel *et al.* 2001). However, when systemic levels of glucose are high, for instance, >10 mM, the secretion of insulin is enhanced. Insulin serves to maintain glucose homeostasis by increasing glucose absorption in the intestine and glucose uptake in skeletal muscle and adipose tissues, whilst further inhibiting hepatic glucose production (Klip *et al.* 1990).

Insulin signalling is initiated through binding of insulin to its receptor. The insulin receptor (IR) is comprised of two identical α and β subunits; insulin binds to the α subunit, causing autophosphorylation hence activation of the two adjacent β subunits. Activation of the IR initiates phosphorylation of tyrosine residues on insulin receptor substrate (IRS) molecules (Sesti *et al.* 2001). Such phosphotyrosine residues serve as

docking sites for proteins with a Src homology 2 (SH2) domain. For example, binding of the SH2 domain of phosphoinositide 3-kinase (PI3K) to IRS, catalyses the formation of phosphatidylinositol 3,4,5-triphosphate (PIP3/PtdIns(3,4,5)P_3) from phosphatidylinositol-4,5-bisphosphate (PIP2/PtdIns(4,5)P_2), which then activates 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (Rameh *et al.* 1999), consequently activating protein kinase B (PKB)/Akt, which in turn mediates glucose transporter-4 (GLUT-4) translocation to the cell membrane (Kotani *et al.* 1995; Ono *et al.* 2001). The key sequential stages of insulin signalling are highlighted below in **Figure 1.3.1**.

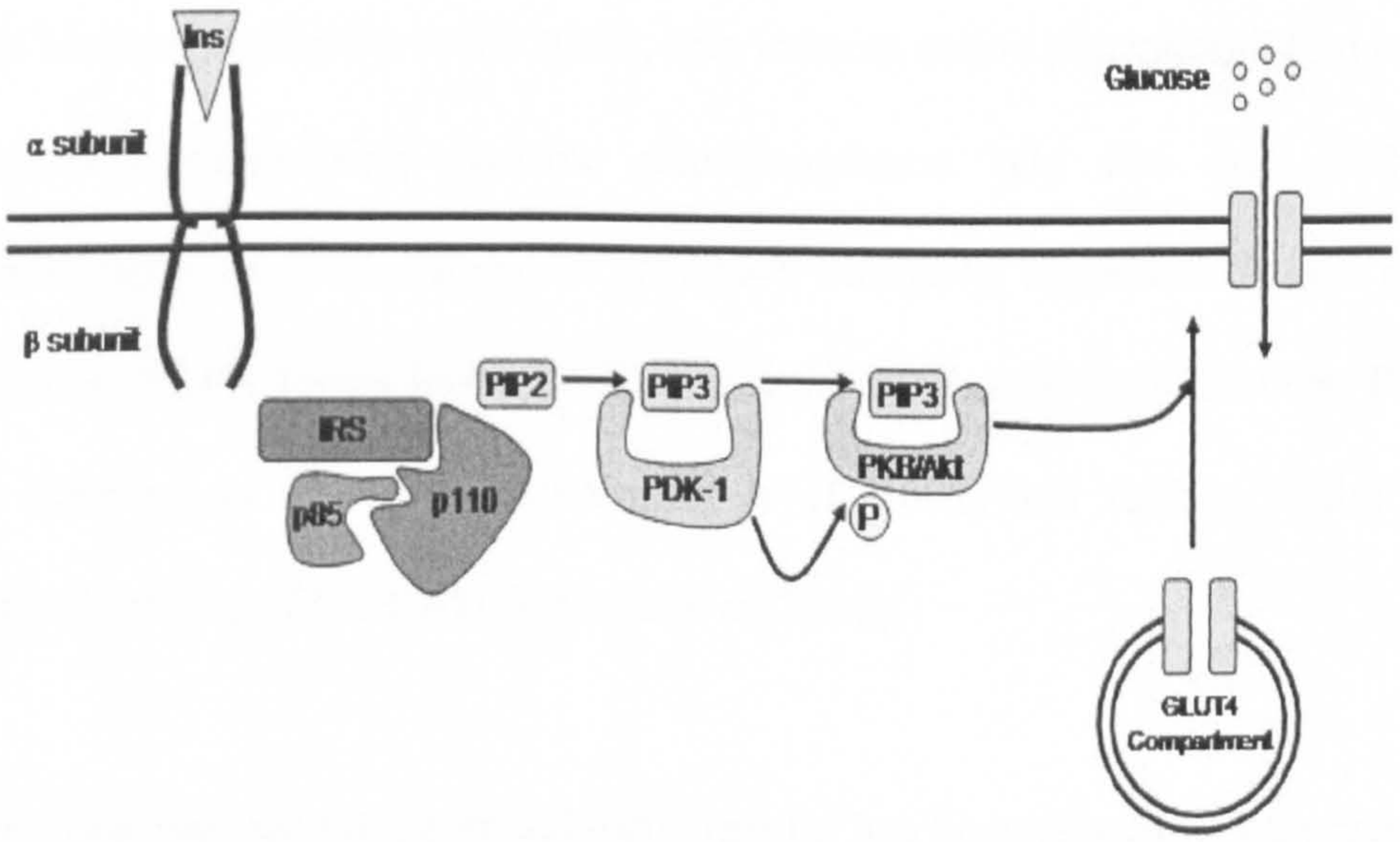

Figure 1.3.1 Insulin signalling. This diagram outlines the interaction between IRS and the catalytic and regulatory p110 and p85 subunits, respectively, of phosphoinositide 3-kinase (PI3K); such binding eventually causes the translocation of GLUT-4 from intracelluar sites to the cell membrane, for cellular glucose uptake (adapted from (Bevan 2001)). Ins, insulin; IRS, insulin receptor substrate; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PKB/Akt, protein kinase B; P, phosphorylation; GLUT-4, glucose transporter-4.

Insulin signalling enhances the storage of glucose as glycogen, thus reducing hepatic glucose production. Such enhanced glycogen synthesis is mediated through

deactivation of a key enzyme in glycogen synthesis, glycogen synthase kinase-3 (GSK-3), by PKB/Akt (Miron *et al.* 2001). Hepatic glucose output is regulated by the rate of gluconeogenesis and glycogenolysis; insulin directly inhibits both processes (Michael *et al.* 2000). Insulin signalling further regulates the activity and expression of several key hepatic enzymes (Pilkis *et al.* 1992); these include phosphoenolpyruvate carboxylase (PEPCK), an enzyme involved in the rate-limiting step in gluconeogenisis and, the glycolytic enzymes glucokinase and pyruvate kinase (Sutherland *et al.* 1996). The development of insulin resistance therefore involves defects in insulin signalling. For instance, elevated NEFA levels cause insulin resistance through inhibition of IRS-1 signalling (Shulman 2000), by activating protein kinase-Cθ (Kelley *et al.* 2000), this induces serine phosphorylation of IRS-1, consequently suppressing tyrosine phosphorylation (De Fea and Roth 1997; Ravichandran *et al.* 2001; Greene *et al.* 2004). Similarly, acute and chronic elevation of systemic NEFA levels has been shown to inhibit interactions between PI3K and IRS-1 (Dresner *et al.* 1999; Kruszynska *et al.* 2002) and further inhibit insulin-stimulated activity of PKB/Akt (Chavez *et al.* 2003).

In obese and glucose intolerant subjects, insulin has been shown to suppress hepatic glucose output, which further correlates with elevated NEFA levels and enhanced lipid oxidation (Bogardus *et al.* 1984; Seppala-Lindroos *et al.* 2002). The relationship between plasma NEFA levels, NEFA oxidation and hepatic glucose output in T2DM and obesity can be demonstrated by the following (Bays *et al.* 2004): increased plasma NEFA increases NEFA uptake by hepatocytes, thus causing accelerated lipid oxidation. Accelerated lipid oxidation stimulates rate-limiting enzymes in gluconeogenesis and glucose-6-phosphatase (G6Pase), a rate limiting enzyme in

glucose release (Williamson 1966; Massillon *et al.* 1997). Additionally, elevated plasma NEFA levels induce hepatic insulin resistance by inhibiting insulin signalling (De Fea and Roth 1997; Dresner *et al.* 1999; Ravichandran *et al.* 2001).

1.4 The Endocrinology of Adipose Tissue.

Traditionally, it was considered that the major role of adipose tissue was to merely serve as an inert energy storage compartment for triglycerides. However, it is now recognised that adipose tissue functions as an active endocrine organ (Ahima and Flier 2000; Scherer 2006); integrating and responding to inflammatory, metabolic and neuronal stimuli by secreting a vast array of cytokines and hormones (Fruhbeck *et al.* 2001; Rajala and Scherer 2003; Faraj *et al.* 2004), collectively referred to as 'adipokines'. Such adipose-derived factors include leptin, TNF-α, IL-6, adiponectin, resistin and visfatin (Tilg *et al.* 2006) (**Figure 1.4**). Several adipokines are capable of exerting multiple endocrine and immunological effects; simultaneously influencing a variety processes, such as glucose homeostasis, insulin sensitivity, energy expenditure, inflammation and lipid metabolism (Fasshauer *et al.* 2003).

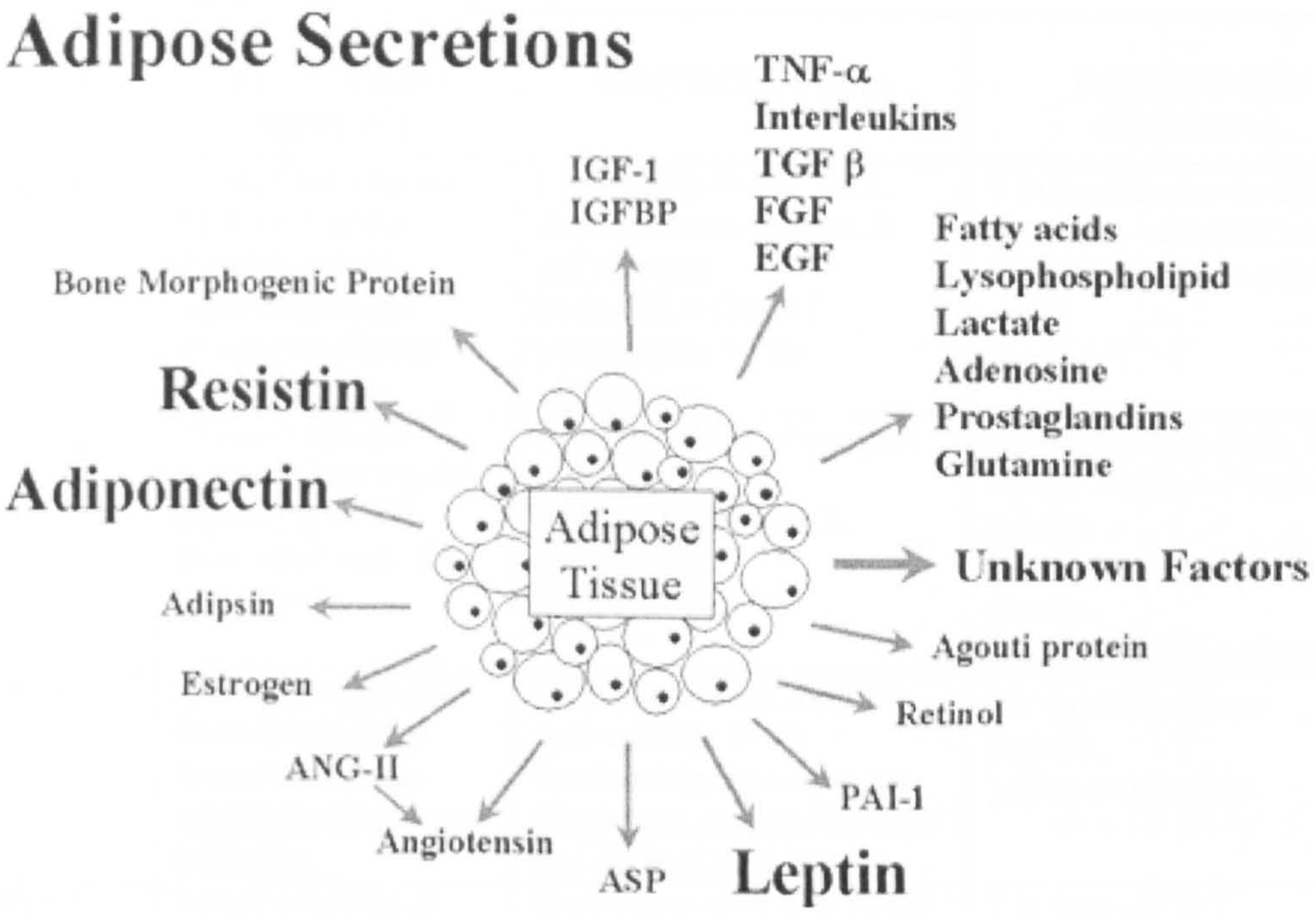

Figure 1.4 Adipose tissue secretes multiple factors that can alter lipid metabolism, glucose homeostasis and inflammatory processes. IGF-1, Insulin-like growth factor-1; IGFBP, IGF-1 binding protein; ANG-II, Angiotensin-II; ASP, Acylation-stimulating protein; PAI-1, Plasminogen Activator Inhibitor-1; TGF-β, Transforming Growth Factor-β; FGF, Fibroblast Growth Factor; EGF, Epidermal Growth Factor.

Whilst under normal physiological conditions, adipokines play an influencial regulatory role in glucose metabolism and lipid oxidation. With increased adiposity however, these processes can be substantially dysregulated (Mohamed-Ali *et al.* 1998; Shoelson *et al.* 2006). Such abnormal endocrine behaviour of adipose tissue in states of obesity consequently implicates adipokines in obesity-related inflammation, insulin resistance and the progression of T2DM. **Table 1.4** below describes several adipokines in relation to human obesity the pathogenesis of T2DM.

Adipokine	FUNCTION/ EFFECT	DISTRIBUTION	EFFECT OF OBESITY
Leptin	Satiety and appetite. Signals to brain to regulate energy homeostasis and peripheral skeletal muscle effects.	Secreted by WAT, BAT, skeletal muscle, stomach and placenta. Sc (2.5X) > Om AT (Adipocytes > Pre-adipocytes).	↑ in human obesity and correlates with BMI and, ↓ after fasting or weight loss.
TNF-α	Reduces insulin secretion and insulin signalling in AT, pancreatic cells, liver and muscle.	Predominant in adipocytes in WAT. Sc (1.67X) > Om AT.	Correlates with BMI, ↑ in human obesity: Obese 2X > Lean. ↑ in rodent models of obesity. ↓ adipose differentiation.
IL-6	Effects glucose and lipid metabolism. Improves insulin sensitivity & glucose tolerance.	35% of the basal supply is derived from WAT. Produced by macrophages, fibroblasts, endothelial cells and skeletal muscle cells.	↑in morbidly obese patients. ↓ after weight loss.
PAI-1	Potent inhibitor of fibrinolytic pathway.	Correlation with abdominal adiposity; pattern of adipose tissue distribution.	↑ in humans to ↑ thromboembolic complications.
Angioten-sinogen (AGT)	Precursor of ANG-II. Regulates blood supply.	mRNA Om AT > Sc AT.	↑ in humans. Induces differentiation of pre-adipocytes.
Adiponectin	Improves energy homeostasis, insulin sensitivity and glucose uptake. Anti-inflammatory properties.	Secreted exclusively by adipocytes. mRNA and protein > in Sc AT than Om AT.	↓ in mouse models of obesity and insulin resistance (*ob/ob* and *db/db*) ↓ in human obesity and T2DM. ↑ after weight loss.
TGF-β	Varied role in proliferation, differentiation, apoptosis and development	Multifunctional, produced by variety of cells. Inhibitor of differentiation.	↑ *ob/ob* obese and *db/db* mice. ↑ pre-adipocyte cell proliferation, as with ↑ TNF-α.
PPAR-γ	Regulates adipose cell differentiation.	Sc Ad = Om Ad (BMI < 28 kg/m^2).	↑ Sc (2X) WAT > Om WAT (BMI < 30 kg/m^2).
Ghrelin	Implicated in the coordination of energy balance and weight regulation.	Secreted by endocrine cells in the gastrointestinal tract.	↓ in morbidly obese subjects. ↑ circulating levels after chronic weight loss in humans.
Adiponutrin	Possible contribution to energy homeostasis, vesicular targeting and protein transport.	Non-secreted adipocyte protein. Expressed exclusively in inguinal and epididymal WAT and in interscapular BAT.	↑ mRNA (50-fold) in obese *fa/fa* rats. ↓ fasting mRNA levels. Human gene expression may be regulated by changes in energy balance.

Table 1.4 Summary of factors implicated in the pathogenesis of obesity-related T2DM. PAI-1, plasminogen activator inhibitor-1; AGT, angiotensinogen; ANG-II, angiotensin-II; mRNA, messenger ribonucleic acid; TGF-β, transforming growth factor-β; PPAR-γ, peroxisome proliferator-activated receptor-γ; AT, adipose tissue; WAT, white adipose tissue; BAT, brown adipose tissue; Sc AT, Subcutaneous

adipose tissue; Om AT, Omental adipose tissue; Sc Ad, subcutaneous adipocytes; Om Ad, omental adipocytes; *ob/ob*, leptin-deficient mouse; *db/db* leptin receptor-deficient mouse; *fa/fa*, obese Zucker rat (adapted and updated from Frübeck *et al.* (Fruhbeck *et al.* 2001)).

This thesis focuses primarily of two adipokines, resistin and adiponectin. Detailed descriptions of these two adipokines are therefore given. Resistin is firstly detailed in terms of its role in glucose homeostasis and its recently established inflammatory functions. Similarly, adiponectin is described in terms of its peripheral effects on hepatic insulin sensitisation and, its emerging effects central actions on lipid oxidation and energy expenditure. Other adipokines, such as TNF-α, IL-6 and leptin are additionally described, albeit to a lesser extent.

1.4.1 Resistin: The Discovery.

Resistin, the novel adipocyte-derived polypeptide, was originally isolated and cloned by three independent groups, each with different perspectives (Holcomb *et al.* 2000; Kim *et al.* 2001; Steppan *et al.* 2001). Holcomb and co-workers initially screened for secreted proteins implicated in allergic pulmonary inflammation, subsequently identifying the protein 'found in inflammatory zone 1' (FIZZ1); as a result, they discovered the expression of a sequence-tag related to this protein, which they termed 'FIZZ3' (Holcomb *et al.* 2000). Meanwhile, using subtractive screening on 3T3-L1 adipocytes to identify potential targets of peroxisome proliferator activated receptor-γ (PPAR-γ) agonist treatment, Lazar and colleagues discovered a novel adipocyte-specific transcript, induced during adipogenesis and suppressed by thiozolidinedione (TZD) treatment (Steppan *et al.* 2001). This mouse *Retn* gene encoded a 10 kDa secreted protein, which was titled 'resistin' (Steppan *et al.* 2001). Finally, using cDNA microarray analysis to identify novel genes induced during 3T3-L1 pre-

adipocyte differentiation in rats, Kim *et al.* identified a 12.5 kDa protein, termed 'adipocyte-specific secretory factor' (ADSF) (Kim *et al.* 2001). Due to the potential implications resistin had in the pathogenesis of obesity and T2DM, work by Lazar and colleagues brought resistin to much scientific attention.

Initial studies by Steppan *et al.* (Steppan *et al.* 2001) demonstrated that resistin is expressed almost exclusively in white adipose tissue; furthermore, circulating levels of resistin are increased in both high-fat diet-induced and genetic (leptin-deficient, *ob/ob*; leptin receptor-deficient, *db/db*) models of obesity and insulin resistance. Moreover, administration of recombinant resistin has antagonistic effects on glucose tolerance and insulin action in wild-type mice (Steppan *et al.* 2001). Immunoneutralisation of resistin in obese mice using anti-resistin IgG antibodies was shown to reduce hyperglycaemia and improves insulin sensitivity. Additionally, both resistin messenger ribonucleic acid (mRNA) and protein expression was down-regulated by the TZD rosiglitazone (RSG) treatment rodent models of obesity and diabetes (Steppan *et al.* 2001). Kim and colleagues further reported that treatment of 3T3-L1 adipocytes with ADSF impairs adipocyte differentiation (Kim *et al.* 2001). Collectively, these initial observations characterised resistin as a potential molecular link between obesity and diabetes. This discovery not only incited much interest in the field of resistin, but revealed possibilities of mechanistic action for TZDs and their therapeutic applications.

1.4.1.1 The Structure of Resistin.

The resistin gene encodes a 114 amino acid polypeptide (Steppan *et al.* 2001), which contains a 20 amino acid signal sequence and comprises of an unique 10 cysteine residue repeat motif ($CX_{12}CX_8CXCX_3CX_{10}CXCXCX_9CC$) (Kim *et al.* 2001).

Resistin belongs to a family of cysteine-rich C-terminal proteins, known as resistin-like molecules (RELMs). These include RELM-α/FIZZ 1, RELM-β/FIZZ 2 and the recently discovered RELM-γ (Holcomb *et al.* 2000; Gerstmayer *et al.* 2003); all of which are characterised by their conserved cysteine motif (Steppan *et al.* 2001; Gerstmayer *et al.* 2003). Mutagenesis studies further revealed that the N-terminal cysteine (Cys) residue, Cys-26, mediates disulfide-dependent dimerisation (Chen *et al.* 2002).

A significant contribution to the research of resistin was made in 2004 when Patel and colleagues used X-ray crystallography to determine resistins' complex multimeric structure, as highlighted in **Figure 1.4.1.1.1** (Patel *et al.* 2004). The group established that in mice, resistin circulates in two distinct assembly states; the more abundant high-molecular-weight (HMW) hexamer and the substantially more bioactive low-molecular-weight (LMW) monomeric form (Patel *et al.* 2004). The monomeric form of resistin corresponds to a mutant version (Cys26Ser), unable to form intertrimer disulfide bonds and is substantially more bioactive in terms of impairing hepatic insulin action *in vivo* (Patel *et al.* 2004).

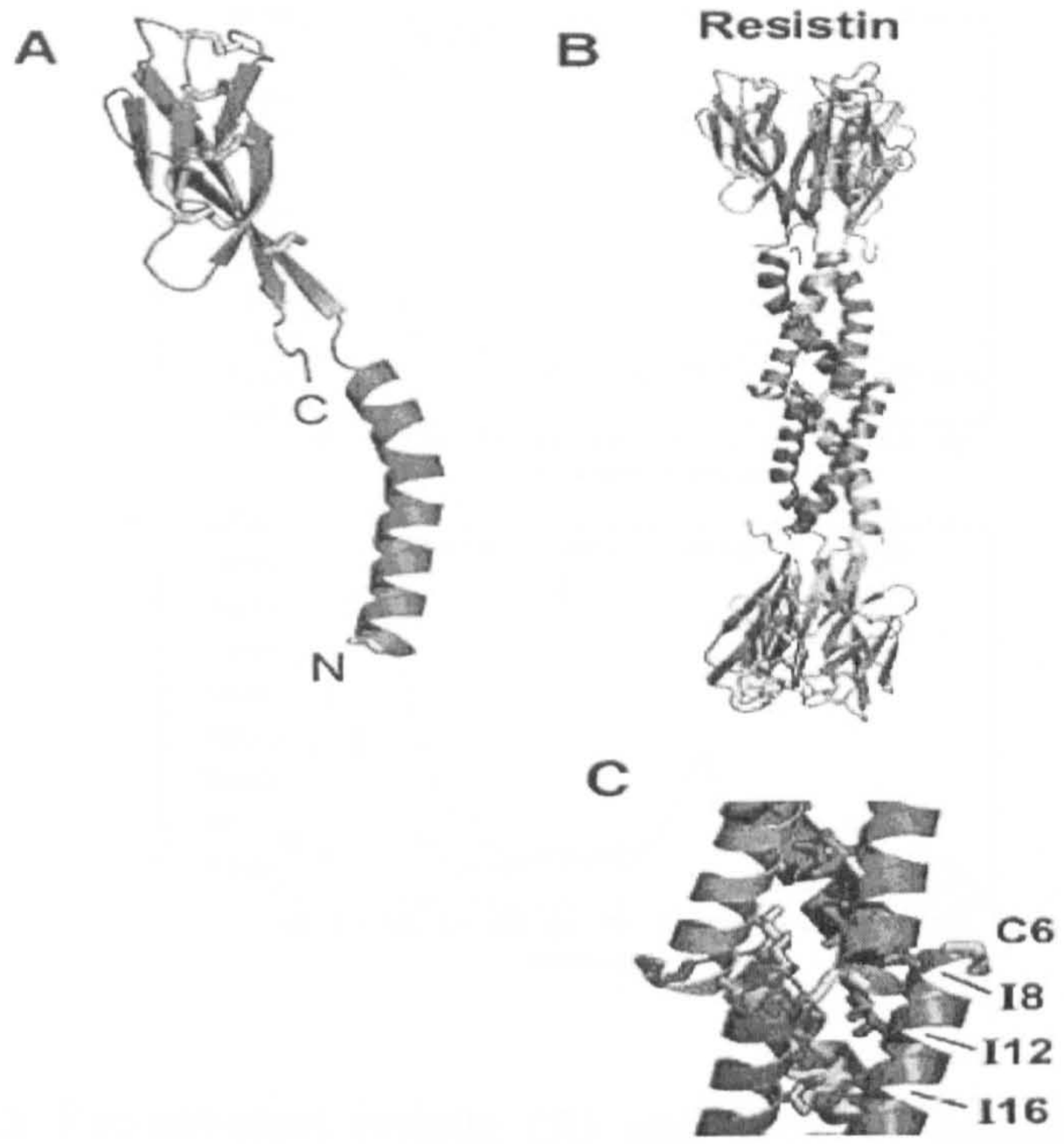

Figure 1.4.1.1.1 Ribbon diagram representations of resistin. (A) The monomeric form of resistin, consisting of a carboxy-terminal disulfide-rich β-sandwich 'head' domain and an amino-terminal α-helical 'tail' segment. (B) The α-helical segments associate to form three-stranded parallel coiled coils; tail-to-tail disulfide-linked trimers form the resistin hexamer. (C) Orientation of highly exposed interchain disulfide linkages binding N-terminal regions in the resistin hexamer; suggesting possible instability of this hexamer. Structures obtained from (Patel *et al.* 2004).

Resistin demonstrates a high degree of structural similarity to RELM-β; as each are covalently linked disulfide-dependent homodimers (Blagoev *et al.* 2002). In contrast, RELM-α, acquires a covalently linked monomeric form, as it lacks the critical cysteine residue (Cys-26) necessary for dimerisation (Banerjee *et al.* 2001). Furthermore, the RELM family members have five cysteine residues featured in the globular head domains that are topologically conserved amongst species (Patel *et al.* 2004). Recently, Gerber *et al.* detected the presence of several HMW isoforms of resistin in human serum (Gerber *et al.* 2005). Using size-exclusion chromatography, major peaks of 660 and 55 kDa were detected (**Figure 1.4.1.1.2**); this implied oligomerisation of resistin (Gerber *et al.* 2005) or aggregation with other RELM members (Chen *et al.* 2002).

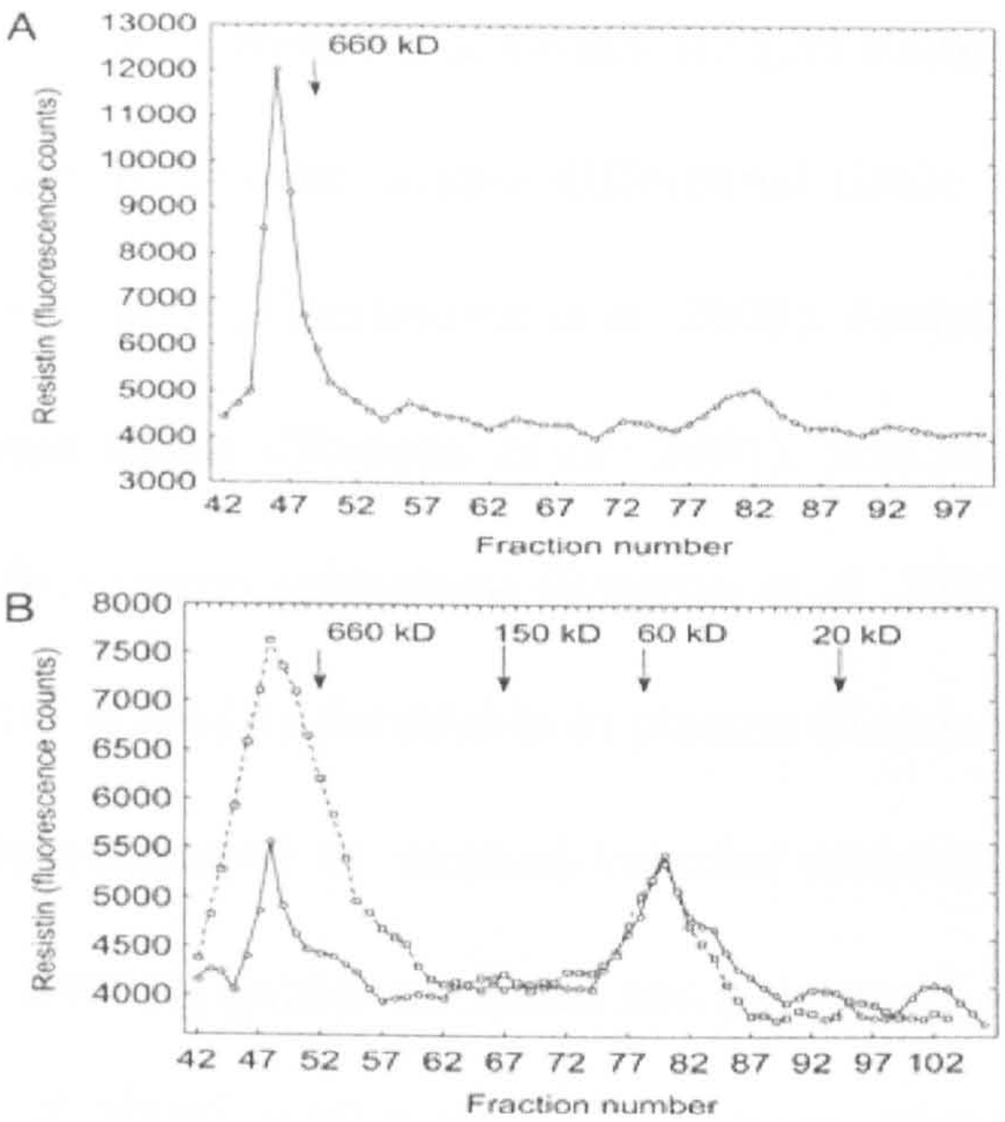

Figure 1.4.1.1.2 Recombinant resistin (A) and pools of human serum (B) were fractionated by size exclusion chromatography. Both figures demonstrate the presence of several HMW isoforms of resistin in human serum (Gerber *et al.* 2005). Major peaks were detected at approximately 660 and 55 kDa, therefore indicating the formation of multimeric structures of resistin.

Graveleau *et al.* further reported that oligomerisation of human and mouse resistin is required to impair insulin-stimulated glucose uptake in mouse cardiomyocytes (Graveleau *et al.* 2005); additionally providing the first evidence that human resistin has properties similar to those of murine resistin. The study further highlighted key fundamental differences between liver and muscle in the response to monomeric and HMW hexameric resistin (Graveleau *et al.* 2005). In contrast to the monomeric form of resistin exerting potent effects in the liver (Patel *et al.* 2004), the biological actions of resistin in cardiac muscle cells required oligomerisation. The HMW form of resistin may therefore undergo processing in the liver to generate the monomeric form (Graveleau *et al.* 2005). Different resistin oligomers may therefore exhibit different bioactivities in muscle and liver tissues (Fruebis *et al.* 2001; Pajvani *et al.* 2003; Tsao *et al.* 2003), especially in models of obesity and insulin resistance.

1.4.1.2 Tissue Distribution of Resistin & Other RELM Family Members.

Each RELM family member has an unique differential tissue distribution (Holcomb *et al.* 2000; Steppan *et al.* 2001; Gerstmayer *et al.* 2003). Resistin expression was first characterised in adipose tissue (Steppan *et al.* 2001). RELM-β is localised within proliferating cells of the colonic epithelium (Steppan *et al.* 2001). Whilst absent from adipose tissue, RELM-β is readily detectable in plasma (Rajala *et al.* 2003). RELM-α on the other hand, is expressed in stromal-vascular constituents of WAT, heart, tongue (Steppan *et al.* 2001; Rajala *et al.* 2002) and pulmonary inflammatory zones in rodents (Holcomb *et al.* 2000), whilst absent in humans. Gerstmayer *et al.* recently identified an additional RELM family member, RELM-γ, which demonstrates a high degree of homology to RELM-α (Gerstmayer *et al.* 2003). Gene expression of RELM-γ is located predominantly in haematopoietic tissues (Gerstmayer *et al.* 2003). **Table 1.4.1.2** details the current knowledge of the distribution of resistin, RELM-α, β and γ in human and rodent tissues.

	Resistin				RELMβ				RELMα		RELMγ	
	Rodent		Human		Rodent		Human		Rodent		Rodent	
Tissue	R	P	R	P	R	P	R	P	R	P	R	P
WAT	X	X	X	X					X		X	
Pre-adipocytes	X		X	X								
Adipocytes	X	X	X	X								
PBMCs				X							X	
Hypothalamus	X		X									
Pituitary gland	X											
Adrenal gland	X		X									
Spleen	X		X								X	
Skeletal muscle	X		X	X								
Pancreas	X		X	X								
Placenta			X						X			
GI-tract	X		X		X		X	X	X		X	
Lung			X					X	X	X	X	

Table 1.4.1.2 The distribution of mRNA and protein expression levels of resistin and RELM-β in rodents and humans and, RELM-α and RELM-γ in rodents. RELM, resistin-like molecule; R, mRNA expression; P, protein expression; WAT, white adipose tissue; PBMCs, peripheral blood mononuclear cells; GI, Gastrointestinal.

In rodents, resistin is expressed primarily in adipocytes (Steppan *et al.* 2001). Unlike the mouse gene, the human homologue of resistin is expressed at low levels in human adipocytes (Nagaev *et al.* 2001). There is controversy as to whether or not human resistin is secreted primarily from adipocytes, pre-adipocytes, or macrophages (Savage *et al.* 2001; Janke *et al.* 2002; Fain *et al.* 2003; Kaser *et al.* 2003; McTernan *et al.* 2003; Patel *et al.* 2003). It has however, been recognised that adipose tissue overproduction is responsible for increased systemic levels of resistin. Several studies have reported that macrophages are the predominant source of systemic resistin in humans (Kaser *et al.* 2003; Patel *et al.* 2003). Adipocytes and pre-adipocytes may also contribute to the circulating levels of resistin in humans, which may become increasingly relevant in states of increased adipose tissue mass. Finally, although recent evidence highlights the similarity in *function* of mouse and human resistin homologues (Graveleau *et al.* 2005); there are still significant quantitative differences of resistin *expression* between the species. Whilst a difference in resistin mRNA levels between adipocytes and macrophages is apparent (Janke *et al.* 2002; Fain *et al.* 2003; Patel *et al.* 2003), further studies will need to fully substantiate whether this difference is observed at protein level.

1.4.1.3 Homology of Human & Rodent Resistin.

Whilst four genes for the family of RELMs have been identified in mice (*Retn, Retn1α, Retn1β* and *Retnlγ*) encoding four distinct proteins (resistin/FIZZ3/ADSF, RELM-α/FIZZ1, RELM-β/FIZZ2 and RELM-γ), only two homologues have been identified in the complete human genome (Steppan *et al.* 2001; Gerstmayer *et al.* 2003). Additionally, an alternatively spliced variant of the *Retn* gene was identified in humans (Resistin delta2) (Nohira *et al.* 2004), whilst another in rats, termed 'S-Resistin' (short resistin) (Del Arco *et al.* 2003).

The human *RETN* gene is located on chromosome 19 in a region syntenic to the mouse *Retn* gene on chromosome 8. One group proposed that orthology exists between human and mouse genes (Steppan *et al.* 2004), whereas another questioned the relevance of resistin, due to the absence of a true homologue for the murine *Retn* gene in humans (Ghosh *et al.* 2003). Contrasting opinions regarding the orthology may relate to how the genomic, transcribed and translated sequence identities are viewed. At the mRNA level, mouse and human resistin display 64.4% sequence homology, whereas at the genomic level, mouse resistin shares 46.7% sequence identity with the human resistin (Ghosh *et al.* 2003). Notably, the mouse genomic sequence is approximately three times bigger than human resistin (Ghosh *et al.* 2003). Furthermore, whilst the same numbers of introns are present in the coding regions, the human sequence lacks a 3'extreme intron (Ghosh *et al.* 2003). This intron, termed 'intron X' has been proposed to contribute to TZD-dependent regulation of mouse resistin, as it contains PPAR-γ response elements (PPREs) (Ghosh *et al.* 2003).

At protein level, human resistin encodes a 108 amino acid polypeptide and is 55% identical to its murine counterpart, as demonstrated in **Figure 1.4.1.3**. Interestingly, the highest degree of protein sequence homology to human resistin sequence is observed between porcine and bovine resistin (76% and 73% respectively). The moderate amino acid sequence homology between mouse and human resistin may explain the diverse expressional and regulatory patterns of this protein, or simply suggest that, murine biology may only moderately relate to humans, and, resistin may not be evolutionary well conserved across species.

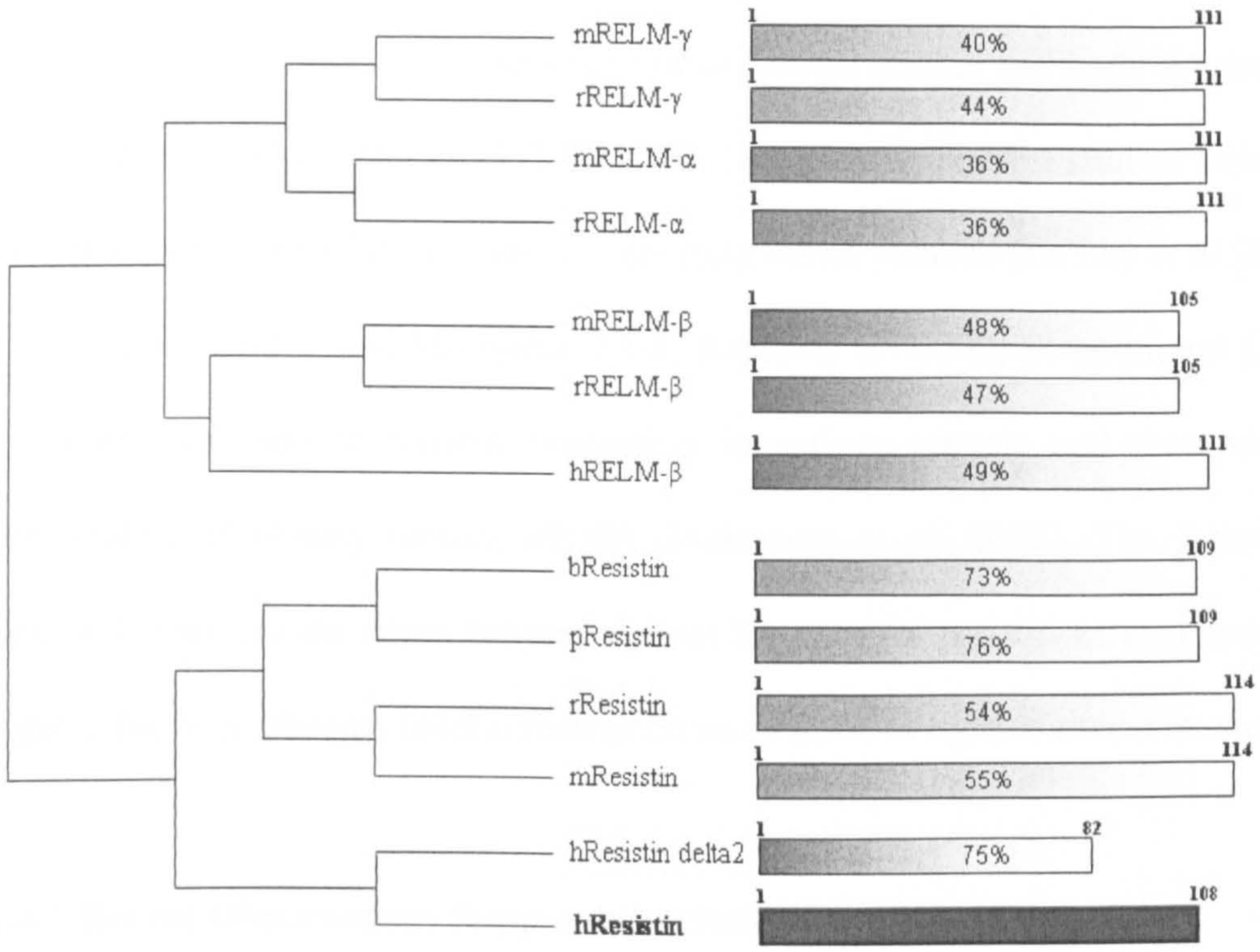

Figure 1.4.1.3 Amino-acid sequence identities of human (h), rodent (r), porcine (p), bovine (b) resistin, other RELM family members and the recently identified human alternatively spliced variant of resistin, resistin delta2. Percentage sequence identities were obtained from the EMBL-EBI (European Bioinformatics Institute) server (http://www.ebi.ac.uk). The different colours represent the different RELM family members.

1.4.1.4 The Metabolic Functions of Resistin in Obesity.

1.4.1.4.1 Initial Observations.

Resistin was initially linked to obesity-mediated insulin resistance based on the observation that serum and plasma levels of resistin were elevated in diet-induced and genetic (*ob/ob and db/db*) murine models of obesity and diabetes (Steppan *et al.* 2001). Comparative increases were further observed between resistin and BMI in Fischer 344 rats (Levy *et al.* 2002). These rodent studies were subsequently reinforced by human analysis showing increased resistin expression in adipose tissue

(Savage *et al.* 2001), particularly abdominal depots (McTernan *et al.* 2002; McTernan *et al.* 2002). Positive associations between human serum resistin and body-fat content were further reported (Zhang *et al.* 2002). On the contrary, several studies failed to demonstrate such correlations in rodents, reporting either reduced (Le Lay *et al.* 2001; Way *et al.* 2001; Fukui and Motojima 2002; Rajala *et al.* 2002; Steppan and Lazar 2002) or no alteration in resistin expression in various genetic and diet-induced murine models of obesity (*ob/ob, db/db*) (Makimura *et al.* 2002). The following sections will thus review cases for and against the role for resistin as an important pathogenic factor in obesity, insulin resistance and T2DM in light of recent studies.

1.4.1.4.2 Recent Observations Supporting a Role of Resistin in Obesity.

Studies by Lee *et al.* showed that various murine models of obesity had higher circulating resistin levels compared to their lean counterparts (Lee *et al.* 2004). Recently, Asensio *et al.* determined that high-fat fed mice had enhanced adipocyte differentiation, as denoted by fatty acid binding protein (ap2) gene expression, a surrogate marker of differentiation; this induction further positively correlated with resistin gene expression (Asensio *et al.* 2004). Subsequently, in view of this and previous studies (Haugen *et al.* 2001; Kim *et al.* 2001), it was suggested that elevated resistin expression was a result of adipocyte differentiation (Asensio *et al.* 2004). Moreover, the increase in adipocyte number may have caused a rise in local resistin production, inhibiting insulin-action on glucose-uptake in adipose tissue and thus preventing further adipocyte differentiation (Asensio *et al.* 2004). Therefore, at least in rodents, a feedback regulatory mechanism for resistin in adipogenesis may occur, acting as an adipose sensor for nutritional status. In accordance with these observations, Kim *et al.* generated transgenic mice over-expressing a dominant

inhibitory form of resistin that functioned to block the inhibition of resistin mediated adipocyte differentiation (Kim *et al.* 2004). These transgenic mice developed obesity, possibly owing to enhanced adipocyte differentiation and adipocyte hypertrophy, indicated by increased circulating levels of adiponectin and leptin (Kim *et al.* 2004).

Recent investigations of human resistin in relation to obesity reported higher serum resistin levels in obese subjects in comparison to lean subjects (Degawa-Yamauchi *et al.* 2003; Schaffler *et al.* 2004; Vendrell *et al.* 2004), which positively correlate with the changes in BMI and visceral fat area (Azuma *et al.* 2003; Degawa-Yamauchi *et al.* 2003; Yannakoulia *et al.* 2003; Vozarova de Courten *et al.* 2004). The implication that resistin is important in human adipose tissue has been corroborated by studies showing increased protein expression with obesity (Degawa-Yamauchi *et al.* 2003) and protein secretion from isolated adipocytes (McTernan *et al.* 2003). A further study has shown a significant reduction in circulating resistin levels following moderate weight loss (Valsamakis *et al.* 2004) and post gastric bypass surgery in morbidly obese patients (Vendrell *et al.* 2004). Collectively, these observations suggest resistin may be subjected to nutritional regulation in humans.

1.4.1.4.3 Observations that Argue Against a Major Role for Resistin in Obesity.

Contrary to the studies suggesting a role for resistin in obesity, Maebuchi *et al.* reported resistin was undetectable in serum of obese mice (Maebuchi *et al.* 2003), with the same study indicating reductions of resistin mRNA and protein expression in obese *db/db* and high-fat fed mice. Similarly, two other groups independently revealed that resistin gene and protein expression levels showed a lack of concordance with increased adiposity in *ob/ob* mice (Asensio *et al.* 2004; Rajala *et al.* 2004). However, it has been suggested that resistin mRNA expression does not

necessarily correlate with protein expression (Rajala *et al.* 2004). Recent human studies have also demonstrated no correlation of serum (Lee *et al.* 2003) or plasma resistin concentrations with any markers of adiposity (Silha *et al.* 2003). Heilbronn *et al.* further reported no relationship between circulating resistin levels and percentage body-fat, visceral adiposity or BMI (Heilbronn *et al.* 2004). However, the authors further suggested the lack of correlation of serum resistin with increased adiposity was possibly due to the confounding variable of age; as non-obese subjects were significantly younger than obese subjects (Heilbronn *et al.* 2004).

Although there is some degree of controversy surrounding resistin, it is worth highlighting the importance of developing highly accurate methods of determining serum resistin concentrations. With reference to the use of commercially available ELISAs, both rodent and human ELISAs may have the potential to cross-react with circulating RELMs. Furthermore, due to recent advances in the understanding of the tertiary and quaternary structure of resistin (Patel *et al.* 2004), further studies are required to establish whether the complex distribution of the individual structural forms of resistin affect the validity of the currently available human and rodent assays. This may thus require development of a human and mouse assay specific to the individual structural forms of resistin to understand the complex distribution of circulating levels of this protein and subsequently its biological functions.

1.4.1.5 The Metabolic Functions of Resistin in Insulin Resistance & T2DM.

It has been established that central obesity is a contributory factor to the pathogenesis of insulin resistance and T2DM. Although it is apparent that inconsistencies remain in

the data for a role of resistin in obesity, there is a growing body of evidence suggesting a role for resistin in the aetiology of insulin resistance and T2DM.

1.4.1.5.1 The Regulation of Resistin in Models of Insulin Resistance & Glucose Intolerance.

Initital rodent studies demonstrated that reduced serum resistin levels are associated with improved insulin sensitivity (Hirosumi *et al.* 2002). Furthermore, resistin mRNA and protein levels are elevated in high-fat fed rats exhibiting insulin resistance (Chen *et al.* 2003). Rajala *et al.* further reported that circulating resistin levels are significantly elevated and positively concordant with rising levels of insulin, glucose, and lipids in *ob/ob* mice (Rajala *et al.* 2004). This study also highlighted the potential interplay between resistin and leptin, with leptin suppressing resistin mRNA and protein levels (Rajala *et al.* 2004). Similarly, Asensio *et al.* highlighted that leptin administration to *ob/ob* mice improves insulin sensitivity, concomitant with a decrease in resistin expression (Asensio *et al.* 2004). Collectively, these studies suggest leptin may exert insulin resistance-ameliorating effects via counter-regulatory interactions and further, exert potential suppressive mechanisms towards resistin. In contrast, Lee and co-workers reported that neither transcriptional regulation of resistin nor circulating resistin levels correlated with serum insulin or glucose levels (Lee *et al.* 2004). Subsequent studies have reported that resistin expression was either suppressed in rat models of insulin resistance (Juan *et al.* 2001) or unchanged following adrenalectomy of *ob/ob* mice (Makimura *et al.* 2002).

In evaluating resistin and its association with insulin sensitivity in humans, several studies have identified positive correlations between resistin levels and insulin

resistance in obese subjects *in vivo* (Silha *et al.* 2003) and *in vitro* (Smith *et al.* 2003). Serum resistin levels are increased by ~20% in T2DM subjects (McTernan *et al.* 2003); such findings were re-affirmed by Fujinami *et al.* (Fujinami *et al.* 2004). In contrast, several studies have reported no associations between serum resistin levels and markers of insulin resistance in T2DM patients (Lee *et al.* 2003; Pfutzner *et al.* 2003; Stejskal *et al.* 2003). Moreover, serum and plasma resistin levels were either reduced in T2DM patients, or increased with no significant correlation with HOMA-IR, waist circumference, BMI or total cholesterol (Yang *et al.* 2003; Youn *et al.* 2004). Consequently, these studies suggest resistin may be unlikely to play a key endocrine role in insulin resistance or energy homeostasis in humans. Nevertheless, a paracrine or autocrine manner of resistin to moderately affecting metabolism cannot be ruled out.

1.4.1.5.2 The Effects of Resistin on Glucose Homeostasis.

Rodent studies highlight that the major target of resistin action *in vivo* is the liver, causing hepatic insulin resistance (Rajala *et al.* 2003; Banerjee *et al.* 2004; Muse *et al.* 2004; Rangwala *et al.* 2004), with secondary peripheral effects on skeletal muscle and adipose tissue (Pravenec *et al.* 2003; Satoh *et al.* 2004). Steppan *et al.* initially demonstrated that administration of recombinant resistin to mice impairs glucose homeostasis and insulin action (Steppan *et al.* 2001). Alternatively, transgenic overexpression of resistin was shown to impair insulin-mediated glucose transport, reducing intrinsic activity of cell-surface glucose transporters (GLUTs) (Moon *et al.* 2003). Euglycemic-hyperinsulinemic pancreatic clamp studies demonstrated that infusion of either resistin or RELM-β into rats decreases hepatic insulin sensitivity (Rajala *et al.* 2003), by impairing insulin-mediated suppression of hepatic gluconeogenesis (Rajala *et al.* 2003). In contrast, resistin null mice exhibit low blood

glucose levels, due to reduced hepatic glucose production (Banerjee *et al.* 2004). Such a glucose-lowering effect was shown to be mediated by an increase in activation of hepatic AMP-activated protein kinase (AMPK) (Banerjee *et al.* 2004), thus reducing expression of key gluconeogenic enzymes, G6Pase and PEPCK (Banerjee *et al.* 2004). Furthermore, treatment of resistin to these knock-out mice enhanced hepatic glucose production and increased glucose levels by ~25% (Banerjee *et al.* 2004). Similarly, high-fat feeding or transgenic overexpression of resistin was shown to enhance glucose levels and hepatic insulin resistance, due to increased levels of PEPCK and G6-Pase (Rangwala *et al.* 2004); treatment of these mice with resistin anti-sense oligonucleotides however, reversed hepatic insulin resistance (Muse *et al.* 2004). Similar metabolic defects have been observed in peripheral tissues; transgenic over-expression of resistin and adenovirus-mediated hyper-resistinemia in rats impaired insulin-stimulated glucose utilisation and dyslipidemia (Pravenec *et al.* 2003; Satoh *et al.* 2004). Additionally, resistin was shown to decrease lipid metabolism in skeletal muscle, by targeting AMPK (Palanivel *et al.* 2005). Resistin may therefore induce hepatic insulin resistance by direct action on the liver or, by altering the effects of skeletal muscle on lipid homeostasis.

Whereas rodent studies demonstrate pro-diabetogenic properties for resistin in glucose metabolism, human studies are somewhat inconsistent; describing the lack of association of resistin with insulin resistance in various diseased states. Although circulating levels of resistin are higher in T2DM patients than in normal glucose-tolerant patients (Zhang *et al.* 2003), studies in Pima Indians report that serum resistin levels are not associated with glucose or insulin levels (Vozarova de Courten *et al.* 2004). However, the first assessment of serum resistin and insulin sensitivity in human subjects using the insulin-clamp technique revealed that resistin levels are

inversely correlated with glucose-disposal rates (Heilbronn *et al.* 2004). Furthermore, HIV-positive patients with insulin resistance have hyper-resistinemia; resistin levels were shown to decrease in concordance with insulin-stimulated glucose-disposal, following RSG treatment (Kamin *et al.* 2005).

In vitro analysis of resistin revealed that treatment of human differentiated pre-adipocytes with recombinant resistin moderately effects glucose-uptake (McTernan *et al.* 2003). Furthermore, resistin impairs insulin signalling in 3T3-L1 adipocytes, by inhibiting factors in the insulin signalling cascade and further, up-regulating suppressor of cytokine signalling-3 (SOCS-3) (Steppan *et al.* 2005), a known inhibitor of insulin signalling (Steppan *et al.* 2005). The underlying mechanisms by which resistin operates in the insulin signalling cascade in humans are however, not fully established.

1.4.1.5.3 Regulation of Resistin by Insulin Sensitisers.

Several studies have reported the downregulation of resistin following RSG, darglitazone (Haugen *et al.* 2001) and troglitazone (Shojima *et al.* 2002) treatment in WAT of *db/db* mice, diabetic fatty rats (Moore *et al.* 2001; Steppan *et al.* 2001) and 3T3-L1 adipocytes (Haugen *et al.* 2001). Conversely, other studies report that pioglitazone (Way *et al.* 2001), troglitazone (Fukui *et al.* 2002) and metformin (Fujita *et al.* 2002) increase resistin expression levels. Similarly, treatment of monocytes with PPAR-γ agonists failed to have any effect on resistin (Savage *et al.* 2001). These initial observations suggest downregulation of resistin may not be crucial for the antidiabetic effect of TZDs in all model systems. However, recent *in vitro* analysis revealed that RSG reduced resistin secretion from human isolated adipocytes

(McTernan *et al.* 2003) and resistin expression in human macrophages (Patel *et al.* 2003; Lehrke *et al.* 2004). Whether the downregulation of resistin expression by RSG occurs via a PPAR-γ-mediated transcriptional mechanism is yet to be determined. However, five putative PPREs in the resistin gene have been identified (Patel *et al.* 2003). One such response element, PPAR-γ response element-2 (PPRE2), was shown to bind PPAR-γ (Patel *et al.* 2003). RSG has also demonstrated anti-inflammatory effects in human macrophages (Ricote *et al.* 1998); reducing inflammatory cytokine production, which may consequently affect resistin production (Hong *et al.* 2003). Collectively, these human studies indicate that suppression of resistin expression may contribute to the insulin sensitising and glucose-lowering actions of the TZDs. Furthermore, the potential anti-inflammatory effects of TZDs on adipocytokine mediation may be of equal importance in the prevention of T2DM.

1.4.1.5.4 Hormone and Cytokine Modulators of Resistin Expression.

Various hormones and cytokines associated with insulin resistance and diabetes have been investigated in concordance with resistin. Such studies are thus detailed below in **Table 1.4.1.5.4**.

Hormone/Cytokine	Effect on Resistin
Pituitary hormones	Growth hormone (1 mg/kg/day) ↑ resistin gene expression (720-950%) in WAT of spontaneous dwarf rats. ↑ gene expression levels in response to hyperprolactinemia in mice. ↓ mRNA and protein expression (30-50%) in 3T3-L1 adipocytes.
Steroid hormones	Dexamethasone ↑ mRNA and protein levels (2.5 to 3.5-fold) in 3T3-L1 adipocytes and ~ 70% mouse WAT.
Sex hormones	↑ in mice with elevated androgen levels. ↑ by hyperprolactinemia and testosterone. Administration of dehydroepiandrosterone ↑ gene expression in WAT of male Wistar rats. Oestrogen ↓ adipose gene expression in ovariectomized rats and in isolated rat adipocytes. Testosterone ↑ adipose tissue mRNA levels in male rats.
Thyroid hormone	Severely ↓ expression in hyperthyroid rats.
Somatotrophin	Moderate inhibitory effects on resistin mRNA and protein (30-50%) levels in 3T3-L1 adipocytes.
Epinephrine	↓ mRNA and protein levels by 30-50% in 3T3-L1 adipocytes.
Neuropeptide Y (NPY)	Intraceberoventricular administration of NPY ↑ gene expression in mice WAT.
β_3-Adrenoreceptors	β_3-agonist isoproterenol ↓ gene expression levels by 20% in 3T3-L1 adipocytes; reversible by the β_3-antagonist propranolol.
Endothelin-1	100 nM significantly ↓ basal resistin secretion by 59%.
Insulin	↓ gene expression (~50%) in 3T3-L1 adipocytes. ↑ secretion from 3T3-L1 adipocytes. ↑ mRNA synthesis (23-fold) in streptozotocin-diabetic mice or Zucker diabetic fatty rats. Gene expression and protein concentration ↑ in fasted mice. ↑ resistin protein secretion in a concentration-dependent manner in human subcutaneous adipocytes.

Table 1.4.1.5.4 The effects of hormones and cytokines on the level of resistin expression and secretion. WAT, white adipose tissue; NPY, neuropeptide Y; mRNA, messenger ribonucleic acid.

1.4.1.5.5 Gender & Resistin Regulation.

In both white and brown adipose tissues, resistin mRNA expression is higher in male than female rats (Nogueiras *et al.* 2003; Nogueiras *et al.* 2003); albeit contrasting a another study whereby resistin mRNA levels were significantly higher in female than male mice (Gui *et al.* 2004). In humans, several studies reported that resistin concentrations are significantly higher in female subjects in comparison to male

subjects (Lee *et al.* 2003; Silha *et al.* 2003; Yannakoulia *et al.* 2003). Gender-related differences on the level of resistin expression in rodents and humans therefore, remain to be fully determined.

1.4.1.6 Resistin & Inflammation.

Initial *in vitro* investigations into the role of resistin in relation to inflammatory processes revealed that the bacterial endotoxin, lipopolysaccharide (LPS), in addition to IL-1, TNF-α and IL-6 upregulated resistin gene expression in 3T3-L1 adipocytes (Lu *et al.* 2002) and human peripheral blood mononuclear cells (PBMCs) (Kaser *et al.* 2003); this was however, attenuated by aspirin and RSG (Lehrke *et al.* 2004). Subsequent clinical data revealed that resistin correlates with IL-6 and intercellular adhesion molecule-1 in obese patients with obstructive sleep apnoea syndrome (Harsch *et al.* 2004). Similarly, resistin was shown to correlate with the leptin, soluble TNF-receptor 2 (sTNF-R2) and C-reactive protein (CRP) in patients with severe inflammatory diseases, obesity and T2DM (McTernan *et al.* 2003; Stejskal *et al.* 2003; Vendrell *et al.* 2004). Resistin gene expression has been demonstrated to be high in PBMCs (Kaser *et al.* 2003; Lehrke *et al.* 2004).

Recent studies have further provided evidence of the potential immuno-modulatory functions of resistin. Silswal *et al.* recently documented that human resistin stimulates the secretion of pro-inflammatory cytokines, TNF-α and IL-12 in macrophages via a nuclear factor-κB (NF-κB)-dependent pathway (Silswal *et al.* 2005). Similarly, the potent pro-inflammatory nature of resistin was further evident in a study showing an association between resistin and inflammation in rheumatoid arthritis (Bokarewa *et al.* 2005). The group further reported that resistin may be an important regulatory cytokine, triggering the release of other pro-inflammatory cytokines, such as TNF-α,

IL-1β and IL-6; these pro-inflammatory effects of resistin were shown to be mediated through the NF-κB signalling pathway (Bokarewa *et al.* 2005).

New questions are therefore being asked regarding the functional role of resistin; can resistin act in concordance with inflammatory molecules? If so, deducing whether resistin has mechanistic action within inflammatory pathways may prove beneficial. This may further subsequently lead us to ask the question, does resistin have the potential to be a therapeutic target?

1.4.1.7 Human Genetics of Resistin.

Obesity is not the only contributory factor of insulin resistance, often defects in key genes that dictate glucose homeostasis can also contribute to the development of insulin resistance. As such, relationships between single nucleotide polymorphisms (SNPs) and insulin resistance may reflect such associations. Several SNPs have been identified in the *RETN* gene; however, only few have minor allele frequencies over 5% and are associated with disease risk (Engert *et al.* 2002; Mattevi *et al.* 2004; Osawa *et al.* 2004). In a study of non-diabetic French Canadians in Quebec, two *RETN* 5′-flanking SNPs (-537 and -420) were associated with increased BMI (Engert *et al.* 2002). Furthermore, a resistin genotype at nucleotide +299 (IVS2 + 181G→A) and obesity was a significant determinant of T2DM risk among type 2 diabetic Caucasians in Boston, Mass., USA (Ma *et al.* 2002). Additionally, the –420 C>G SNP (–180 relative to putative transcription start site) was associated with higher resistin mRNA levels in abdominal fat of obese subjects (Smith *et al.* 2003). Conversely, Mattevi *et al.* showed an association between the –420 C>G polymorphism with lower BMI, in non-diabetic individuals from a Brazilian

population of European descent (Mattevi *et al.* 2004). Whilst amongst non-diabetic Caucasians in Sicily and Gargano, Italy, an ATG triplet repeat in the 3'-untranslated region of the resistin gene was associated with a decreased risk of insulin resistance (Pizzuti *et al.* 2002).

Genetic analysis of resistin using a Japanese population demonstrated that a -420G/G genotype was associated with human T2DM and could accelerate the onset of disease by 4.9 years (Osawa *et al.* 2004), moreover, the genotype itself was a primary variant determining T2DM susceptibility (Osawa *et al.* 2004). Consistent with these findings, elevated levels of serum resistin were reported in T2DM subjects carrying the -420G/G genotype (Azuma *et al.* 2004). In contrast, studies in a Japanese obese population reported the -638G>A, -420C>G, and -358G>A SNPs, which whilst associated with serum resistin, did not confer any association with obesity or insulin resistance (Osawa *et al.* 2002; Ochi *et al.* 2003). These genetic studies highlight the discrepancies amongst resistin SNP analysis examining the association with obesity related insulin resistance; these may partly be explained by different genetic backgrounds or environmental conditions of the populations studied.

1.4.2 Adiponectin: Structure & Function.

Adiponectin, in its most basic form, is a 30 kDa protein secreted exclusively from adipocytes (Scherer *et al.* 1995). In comparison to other adipokines, adiponectin is extrememly abundant in circulation, with levels reaching up to 30 μg/ml in healthy subjects (Matsubara *et al.* 2002). Adiponectin was identified and isolated during comparative analysis of mRNAs from a differentiating a 3T3-L1 adipocyte cell-line (Scherer *et al.* 1995). Structural analysis revealed that adiponectin consists of an

amino-terminal signal sequence, a variable region, a collagenous-tail domain and a C-terminal globular head domain (Pajvani *et al.* 2003) (**Figure 1.4.2.1**).

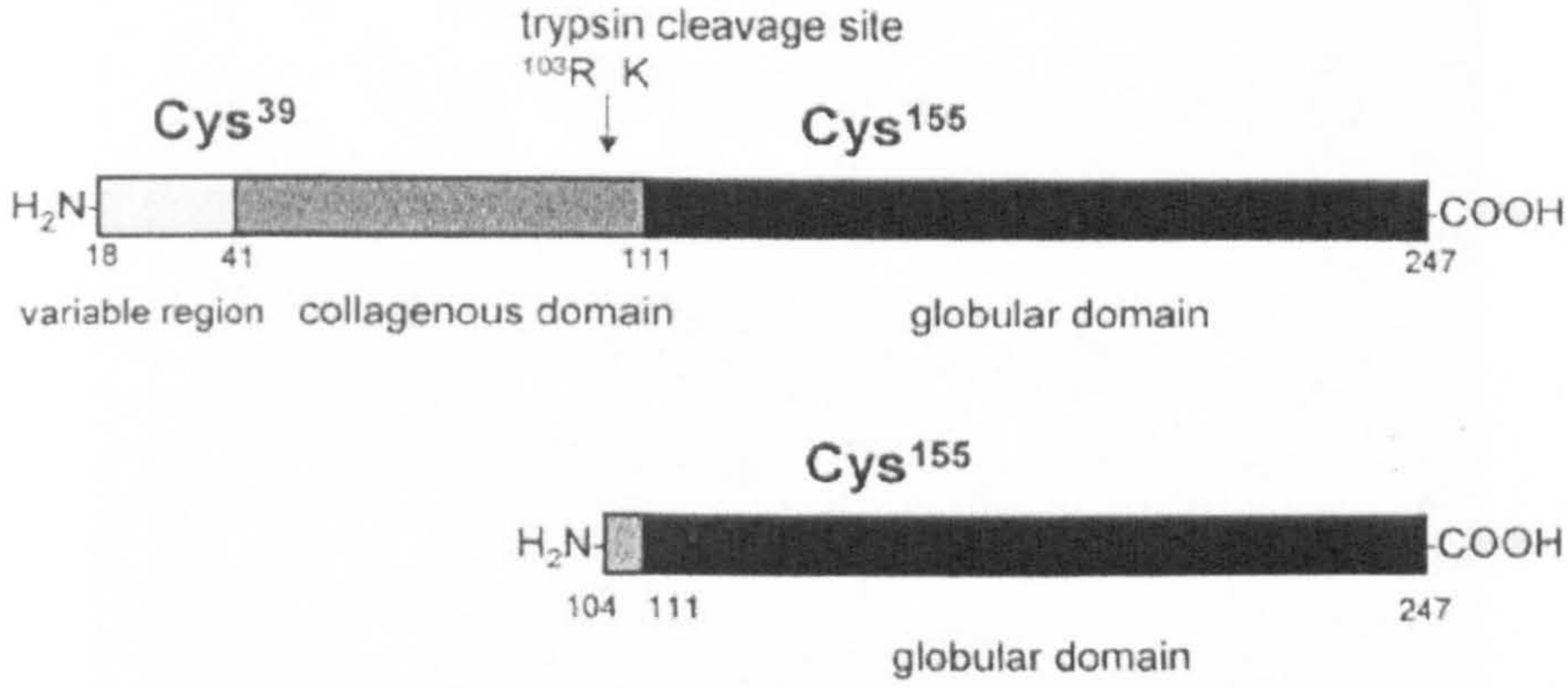

Figure 1.4.2.1 Diagrammatic representation of adiponectin, highlighting the structural domains. The residue cysteine (Cys) 39 is critical to HMW formation, whereas cysteine 155 is maintains the integrity of the HMW species of adiponectin (Scherer *et al.* 1995).

The globular domain of adiponectin shares homology with collagens VIII and X, complement factor C1q and, the hibination protein hib27 (Tsao *et al.* 2002; Pajvani *et al.* 2003). The 3D crystalline structure of the globular head unit shares striking homology with TNF-α (Matsubara *et al.* 2002); suggesting an evolutionary link between the function of these proteins. Intermolecular bonding between globular head domains causes the formation of a tightly bound trimer (**Figure 1.4.2.2**), the smallest unit of this protein observed in circulation.

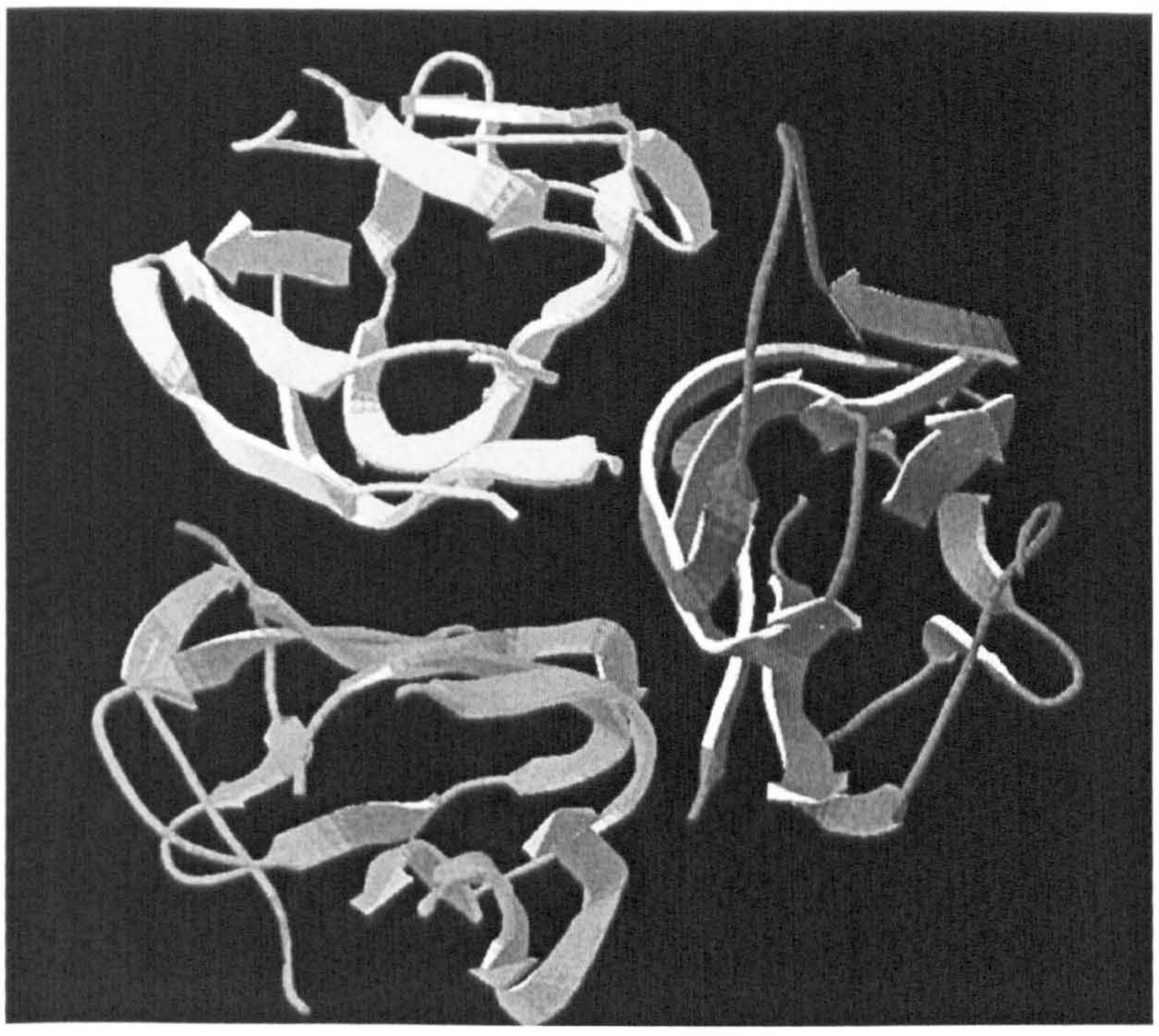

Figure 1.4.2.2 3D crystalline structure of the globular head domain of adiponectin. Diagram demonstrates three single units bound in a trimeric formation (Shapiro *et al.* 1998).

Adiponectin trimers associate through disulphide bonds within the collagenous domains of single monomers to form 'bouquet-like' higher-order structures (Pajvani *et al.* 2003). Such higher-order structures include LMW hexamers of 180 kDa and, HMW structures, consisting of up to 18 units of the protein (**Figure 1.4.2.3**), approximately >500 kDa in size and, by far the most prominent form of adipnectin in serum (Trujillo *et al.* 2005). Oligomerisation of adiponectin depends on disulphide-formation, mediated by the residue cysteine at position 39 (**Figure 1.4.2.1**); as demonstrated by the failure to produce HMW species when this residue is mutated (Pajvani *et al.* 2003). Furthermore, bonding between cysteine residues at position 155 is critical to the integrity of the HMW structures (Pajvani *et al.* 2003; Waki *et al.* 2003).

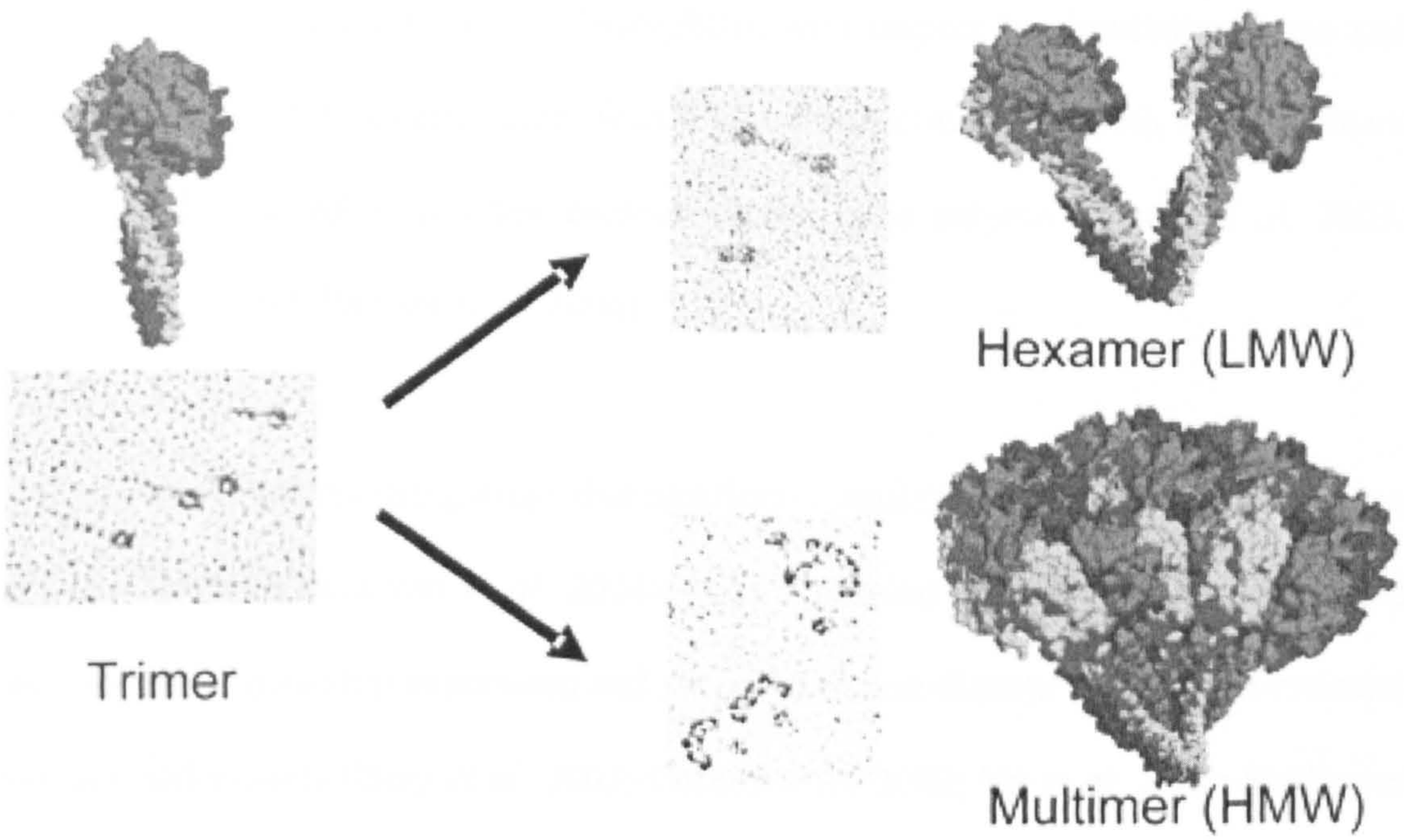

Figure 1.4.2.3 Model of assembly of adiponectin. Three monomers of adiponectin form a trimer (30 kDa) through the globular domains. Trimers then associate through interactions within the collagneous domain to form low-molecular-weight (LMW) hexamers (180 kDa) and high-molecular-weight (HMW) subunits (>500 kDa) (Pajvani *et al.* 2003).

Adiponectin mRNA and serum levels are lower in obese subjects in comparison to lean subjects (Arita *et al.* 1999) and, are linked to decreased insulin sensitivity and reduced thrombogenic risk. Furthermore, weight reduction in morbidly obese patients results in a dramatic increase in plasma adiponectin levels, following by substantial improvement in whole-body insulin sensitivity (Yang *et al.* 2001). Similarly, circulating adiponectin levels are significantly lower in patients with T2DM, with a further reduction associated in subjects with coronary artery disease (Hotta *et al.* 2000; Hirose *et al.* 2002; Lihn *et al.* 2003). Interestingly, low adiponectin serum levels have been shown to independently predict the future risk of developing T2DM (Lindsay *et al.* 2002). Such a protective role of adiponectin was further highlighted in a study on rhesus monkeys, in which adiponectin levels decreased with the onset of obesity in primates, prior to the development of insulin resistance (Hotta *et al.* 2001).

Adiponectin also exhibits sexual dimorphism with respect to circulating levels and percentage of HMW species rather than total adiponectin levels; both are significant higher in female subjects when compared with male subjects (Cnop *et al.* 2003; Combs *et al.* 2003; Pajvani *et al.* 2004).

TZDs are insulin-sensitising drugs that ameliorate insulin resistance and lower plasma glucose levels (Vasudevan *et al.* 2004). Administration of TZDs results in marked increases in adiponectin expression and secretion in non-diabetic and insulin-resistant humans and rodents (Berg *et al.* 2001; Combs *et al.* 2002; Yu *et al.* 2002; Phillips *et al.* 2003; Ye *et al.* 2003). As adiponectin levels are associated with improved insulin sensitivity in TZD-treated subjects, adiponectin may therefore be partially responsible for such an improvement in health (Phillips *et al.* 2003).

Genetic studies that have reported mutations in the proximal promoter regions of the adiponectin gene that are associated with the development of T2DM (Vasseur *et al.* 2002; Gu *et al.* 2004). Similarly, several SNPs identified in the adiponectin locus are associated with many of the complications linked with insulin resistance (Menzaghi *et al.* 2002; Hu *et al.* 2004). Furthermore, the human adiponectin gene has been mapped to genomic locus 3q27; a region previously linked to the susceptibility of T2DM and the metabolic syndrome (Kissebah *et al.* 2000).

1.4.2.1 Adiponectin Bioactivity: Adiponectin Oligomers in Hepatic Glucose Metabolism & Skeletal Muscle Lipid Oxidation.

The production of recombinant adiponectin in both mammalian and bacterial expression systems has allowed detailed studies concerning the effects of adiponectin

on *in vivo* and *in vitro* systems. Several laboratories have generated bacterially produced full-length adiponectin (Berg *et al.* 2001) or a proteolytic cleavage product, encapsulating only the 'globular head' domain of adiponectin (Fruebis *et al.* 2001; Yamauchi *et al.* 2001); the latter was initially recognised as the functionally active portion of adiponectin. Treatment of mice with the globular adiponectin was shown to reduce the postprandial rise in glucose, triglycerides and NEFAs (Fruebis *et al.* 2001; Yamauchi *et al.* 2001). Further *in vitro* analysis revealed that such metabolic improvements were attributable to globular adiponectin increasing the clearance of NEFAs in skeletal muscle, by enhancing β-oxidation (Fruebis *et al.* 2001). Another group generated a recombinant mutant form of adiponectin (Cys-39), whereby the Cys-39 residue crucial for adiponectin oligomerisation was substituted with a serine residue, thus generating only the trimeric form of adiponectin (Pajvani *et al.* 2003). This Cys-39 mutant trimeric form of adiponectin demonstrated a high level of bioactivity (Pajvani *et al.* 2003).

Conversely, diabetic mice injected with full-length recombinant adiponectin exhibit low circulating levels of glucose (Berg *et al.* 2001); suggesting that the native full-length adiponectin acts primarily on the liver, to reduce glucose output (Berg *et al.* 2001). Interestingly, full-length adiponectin was also shown to inhibit both the expression of hepatic gluconeogenic enzymes and, the rate of endogenous glucose production (Combs *et al.* 2001). *In vitro* analysis of full-length adiponectin action on isolated hepatocytes further revealed that glycosylation and hydroxylation of the collagenous domain was critical to its bioactivity (Wang *et al.* 2002). Understanding of the bioactivity of this molecule and its mode of action was further achieved by examination of the function of both the globular head domain and full-length

adiponectin in hepatocytes and myocytes (Yamauchi *et al.* 2002). This study focused on the effect of both forms of adiponectin on 5'-AMPK to elucidate whether this enzyme featured downstream of adiponectin signalling. In the study, globular adiponectin was shown to enhance AMPK activity in myocytes, whereas full length adiponectin stimulated AMPK in the liver. Interestingly, the globular head domain was identified to bind to muscle membranes with a higher binding affinity than full-length adiponectin (Yamauchi *et al.* 2002). Although initial studies highlight different functions for full-length adiponectin and globular adiponectin, more recent studies have demonstrated an overlap in peripheral and hepatic functions of full-length adiponectin (Waki *et al.* 2003; Tonelli *et al.* 2004; Wang *et al.* 2005), as highlighted in **Figure 1.4.2.1** below.

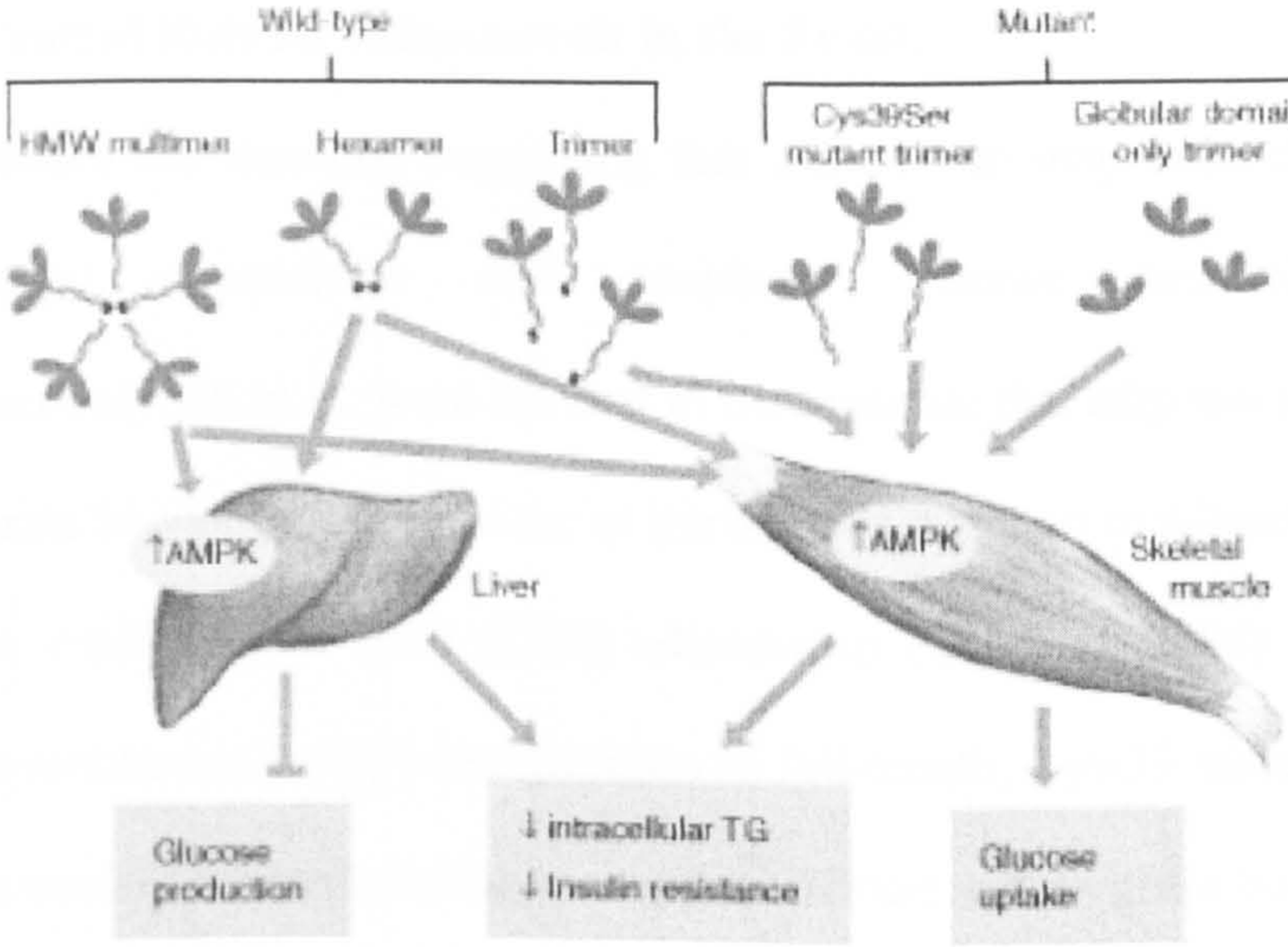

Figure 1.4.2.1 Illustration of target tissues of full-length adiponectin and the two trimeric forms of adiponectin; the Cys-39 mutant and the globular head domain. Full-length adiponectin can exert both effect both hepatic and peripheral skeletal muscle metabolism, whereas the trimeric forms of adiponectin affect only peripheral skeletal muscle tissue. HMW, high-molecular-weight; AMPK, AMP-activated protein kinase; TG, triglyceride.

In 2003, two putative adiponectin receptors were identified (Yamauchi *et al.* 2003); these novel receptors, termed ADIPOR1 and ADIPOR2, contain seven

transmembrane domains and were noted to be functionally distinct from G protein-coupled receptors (Yamauchi *et al.* 2002). ADIPOR1 is predominantly expressed in skeletal muscle and exhibits high affinity for globular adiponectin and low affinity for full-length adiponectin; whereas, ADIPOR2 is abundantly expressed in the liver and has intermediate affinity for full-length adiponectin (Yamauchi *et al.* 2002). A novel extracellular receptor, T-cadherin, has been observed to bind the more prevalent larger multimeric forms of adiponectin (Hug *et al.* 2004). Unlike ADIPORs, T-cadherin is expressed widely in endothelial and smooth muscle cells (Hug *et al.* 2004). The precise role in T-cadherin is unknown; however, it has been suggested to function as a co-receptor in modulating responses to adiponectin (Hug *et al.* 2004).

1.4.2.2 A Central Role for Adiponectin in the Brain.

Recent evidence has emerged suggesting that adiponectin may function centrally to affect energy expenditure and peripheral glucose metabolism. Using radioimmunoassay, Qi *et al.* were the first to demonstrate that adiponectin could cross the blood-brain barrier (BBB) in mice; as intravenous injection of adiponectin resulted in a rise in cerebrospinal fluid (CSF) adiponectin (Qi *et al.* 2004). Interestingly, intracerebroventricular (i.c.v) administration of full-length, Cys-39 mutant or globular adiponectin was shown to stimulate energy expenditure and decrease body weight (Qi *et al.* 2004); suggested to be attributable to enhanced lipid oxidation by peripheral action on skeletal muscle. Additonally, ADIPOR1 and ADIPOR2 have been shown to be expressed in the central nervous system (CNS) (Ahima 2005), further suggesting a central role for adiponectin. Limited studies to date have addressed the potential role of adiponectin in the brain and, in some cases have provided negative data regarding its importance in the CNS (Pan *et al.* 2006; Spranger *et al.* 2006). Further studies are

therefore required to elucidate the precise mechanistic action of adiponectin and its various oligomeric forms in the CNS.

1.4.3 Tumour Necrosis Factor-α.

TNF-α is a 26 kDa transmembrane protein that is cleaved into its 17 kDa soluble bioactive form. TNF-α was initially identified as an endotoxin-induced factor, secreted by macrophages and causing necrosis of tumours (Cerami *et al.* 1985). The first association between obesity, increased TNF-α expression and insulin action was reported by Hotamisligil and colleagues. The group identified TNF-α expression in white adipose tissue of obese rodents and humans (Hotamisligil *et al.* 1993; Hotamisligil *et al.* 1995), which further correlated with increasing adiposity and insulin resistance (Hotamisligil *et al.* 1993; Fernandez-Real *et al.* 2003; Hotamisligil 2003). Additional rodent studies revealed that *ob/ob* mice null for TNF-α or TNF receptor have improved insulin sensitivity and are more resistant to the development of T2DM, when compared to TNF-sufficient *ob/ob* mice (Uysal *et al.* 1997). Furthermore, immunoabsorption of TNF-α in rodent models of obesity was shown to increase insulin sensitivity (Hotamisligil *et al.* 1996). Furthermore, elevated levels of TNF-α in states of obesity and insulin resistance have been suggested to directly interfere with insulin signal transduction, through phosphorylation of critical serine residues in IRS-1 (Hotamisligil *et al.* 1996). Serine phosphorylation of IRS-1 results in early degradation (Trayhurn *et al.* 2001), thus causing desensitisation of insulin signalling (Hotamisligil *et al.* 1994). Subsequent studies revealed that adipocytes expressed both TNF receptors in soluble and membrane bound forms (Ruan *et al.* 2003). The production of TNF-α is mediated through activation of specific transcription factors, such as NF-κB and c-Jun N-terminal kinase (JNK). Such

activation of NF-κB causes profound changes in the gene expression profile of adipose tissue (Ruan *et al.* 2002). Many of these genes are thought to negatively impact on insulin signalling and lipid metabolism. It is now recognised that TNF-α is a multifunctional cytokine, influencing apoptosis, insulin signalling and many inflammatory processes (Hotamisligil *et al.* 1994).

1.4.4 Interleukin-6.

IL-6 is cytokine commonly associated with the immune system. IL-6 is functionally involved in the release of acute phase reactants from the liver, during the innate immune response. IL-6 has further been associated with complications such as melanoma, cachexia and T2DM. IL-6 is produced by a number of cells, including activated macrophages, lymphocytes, skeletal muscle cells and WAT (Febbraio *et al.* 2002). However, its major source may be from WAT, since approximately 30% of systemic IL-6 in humans is contributed by mature adipocytes (Mohamed-Ali *et al.* 1997); suggesting involvement of IL-6 with adipocyte metabolism and obesity-related insulin resistance. An increase in fat mass positively correlates with elevated circulating levels of IL-6 (Kern *et al.* 2001; Skurk *et al.* 2002), with a reduction in IL-6 further associated with weight loss (Bastard *et al.* 2000). IL-6 plasma concentrations are also upregulated in murine and human insulin resistance (Fried *et al.* 1998; Pradhan *et al.* 2001), with baseline IL-6 levels independently predicting future risk of developing T2DM (Kern *et al.* 2001; Vozarova *et al.* 2001). Alternatively, administration of recombinant IL-6 in rodents and humans has shown to induce hepatic gluconeogenesis, which in turn, leads to hyperglycaemia and compensatory hyperinsulinemia (Stith and Luo 1994; Tsigos *et al.* 1997). In contrast, several studies have demonstrated that insulin enhances IL-6 mRNA and protein expression in 3T3-

L1 adipocytes and human adipocytes (Vicennati *et al.* 2002; Fasshauer and Paschke 2003). Furthermore, studies have also revealed that elevated levels of IL-6 impair insulin signalling via distinct mechanisms. *In vitro* analysis using cultured hepatocytes and 3T3-L1 adipocytes demonstrated that IL-6 decreased activation of IRS-1 (Rotter *et al.* 2003); such an affect was mediated through the 'suppressor of cytokine signalling (SOCS) family of proteins (Senn *et al.* 2003). SOCS inhibit insulin signalling directly through the insulin receptor (Mooney *et al.* 2001). This data therefore suggests that IL-6 is pro-insulin resistance. Taken together, obesity and T2DM are associated with an increased state of inflammation; in which IL-6, along with TNF-α, play integral roles as mediators (Pickup *et al.* 1997; Engeli *et al.* 2003; Esposito *et al.* 2003).

1.4.5 Leptin.

Leptin was discovered in 1994 by positional cloning of the gene responsible for obesity in the *ob/ob* mouse (Zhang *et al.* 1994). This gene encoded a 146 amino acid protein, 16 kDa in size and, secreted predominantly by adipocytes (Madej *et al.* 1995). Soon after, the leptin receptor (OB-Rb) was cloned (Tartaglia *et al.* 1995) and defects were found in the gene in the genetically obese *db/db* or *ob/ob* mice (**Figure 1.4.5**) (Chen *et al.* 1996; Chua *et al.* 1996). As deficiency or mutations in the protein were associated with obesity in rodents, it was not surprising that leptin treatment to *ob/ob* mice resulted in a weight loss and improved diabetic status (Pelleymounter *et al.* 1995). Initial reports indicated that leptin serves as a key mediator in a negative feedback loop from adipose tissue to the brain, supressing appetite in response to excess adipose tissue mass (Considine *et al.* 1996; Van Gaal *et al.* 1999). However, initial rodent data regarding the biology of leptin did not readily translate to humans.

Few leptin mutations were present in humans and conversely, plasma leptin levels were severely elevated in obese subjects (Considine *et al.* 1996).

Figure 1.4.5 The genetically obese, leptin deficient (*ob/ob*) mouse.

Leptin administration is known to cause weight loss in excess of the effects of reduced food-intake (Levin *et al.* 1996). As such, leptin was proposed to regulate energy expenditure through mechanisms via the CNS; as central administration of leptin to rhesus monkeys demonstrated increases in norepinephrine and a significant reduction in food-intake (Tang-Christensen *et al.* 1999). Mutations resulting in complete leptin loss in humans (Montague *et al.* 1997; Strobel *et al.* 1998), in addition to defects in the receptor, resulted in marked hyperphagia and severe obesity (Strobel *et al.* 1998). However, with acute treatment of leptin to such leptin-deficient subjects, hyperphagia was reduced, followed by a severe reduction in fat-mass (Farooqi *et al.* 1999). It was further reported that obese individuals are severely resistant to leptin action. Such leptin resistance may occur during transport of leptin into the CNS (Caro *et al.* 1996) or impairment of signalling downstream of the leptin receptor (Bjorbaek *et al.* 1999; El-Haschimi *et al.* 2000). It would appear from such leptin resistance, that the action of leptin is profound when systemic levels fall below normal physiological levels. Therefore, it is thus proposed that reduced leptin levels serve as a signal for negative energy balance when energy reserves are low (Havel 2004).

Once in circulation, leptin is transported across the blood-brain barrier, where it then exerts its effects by interacting with receptors located in the hypothalamus (Flier 2006). At this site, leptin interacts with the melanocortin-4 receptor, melanocyte-stimulating hormone and neuropeptide Y (NPY), a powerful stimulator of appetite (Halaas *et al.* 1995; Zierath *et al.* 1998). In contrast, leptin is also capable of increasing expression of anorexigenic proteins, proopiomelanocortin (POMC) and cocaine amphetamine-related transcript (CART), causing a decrease in appetite (Schwartz *et al.* 2000; Spiegelman *et al.* 2001).

1.5 Obesity, Insulin Resistance & Inflammation: Adipokines Link Metabolic & Inflammatory Pathways: The Emerging Paradigm.

Adipose tissue has received much attention over recent years not only for its endocrine characteristics, but equally for its immunological activity. Mounting evidence has focused on the notion that chronic low-grade inflammation and activation of the innate immune system are closely involved in the obesity-associated insulin resistance and the progression of T2DM (Dandona *et al.* 2004; Pickup 2004) Such chronic inflammatory responses in obesity are characterised by an abnormal level of cytokine production and increased activation of inflammatory signalling pathways. Various adipokines can themselves regulate several inflammatiory processes or alternatively, serve as markers of inflammation (Tilg *et al.* 2006). Such pro-inflammatory adipokines, referred to as 'adipocytokines' include TNF-α, IL-6, PAI-1, C-reactive protein (CRP), resistin and leptin. Dysregulation of systemic levels of adipocytokines may therefore contribute to chronic inflammation, resulting in endocrine abnormalities observed in obesity and T2DM. Consequently, these factors are considered to have dual regulatory metabolic and immunological properties. More

specifically, adipocytokines may have key roles in mediating inflammation, in particular, within innate immunity. It is therefore considered that adipose tissue represents an initiation site for an innate immune response; however, its molecular basis, precise mechanistic action in the pathogenesis of T2DM, is yet to be fully elucidated.

1.5.1 Innate Immunity.

The innate immune system is the body's rapid first line of defence against environmental threats, such as microbial pathogens and physical or chemical injury (Medzhitov and Janeway 2000; Takeda and Akira 2005). Such pathogens initiate a 'hard wired' non-specific, however rapid, reaction to infection. Unlike the adaptive immune response, instead of utilising T and B-lymphocytes, innate immunity uses Toll-like receptors (TLRs) to recognise specific microbial components, including bacteria, fungi, protazoa and viruses (Kaisho *et al.* 2000).

The TLRs were first characterised in *Drosophila* in 1996 (Lemaitre *et al.* 1996). One year later, a mammalian homolog of the Toll receptor, termed TLR-4, was shown to induce the expression of genes involved in inflammatory responses (Medzhitov *et al.* 1997). Since the initial discovery of TLR-4, 11 family members have been identified in mammalian systems (Takeda *et al.* 2005). TLR-4 is an essential receptor for lipopolysaccharide (LPS), from Gram-negative bacteria; whereas TLR-2 recognises fungen-derived components, such as zymosan, from Gram-positive bacteria (Takeda *et al.* 2002). Upon binding of microbial components, TLRs dimerise and, along with their co-receptor, CD14, both initiate downstream signalling events. This firstly involves the recruitment of a common adaptor protein, MyD88, which binds to the cytoplasmic domain of the Toll-like receptor (TLR) (Schnare *et al.* 2001). Such

receptor binding recruits downstream IL-1 receptor-associated kinase (IRAK) (O'Neill *et al.* 1998) to the receptor complex. IRAK is then autophosphorylated and recruits TNF receptor-associated factor-6 (TRAF-6); this leads to the activation of the 'inhibitor of NF-κB kinase' (IKK) complex, which consists of the catalytic subunits IKK-α and IKK-β and, the regulatory subunit, NEMO/IKK-γ. Activation of the IKK complex sequentially phosphorylates 'inhibitor of NF-κB' (IκB), therefore causing its degradation; simultaneously, such activation releases the sequestered transcription factor, NF-κB, thus allowing it to translocate from the cytoplasm into the nucleus to initate transcription of inflammatory molecules (**Figure 1.5.1**), such as TNF-α, IL-1 and IL-6 (Zhang *et al.* 2000; Barton *et al.* 2003; Goldstein 2004).

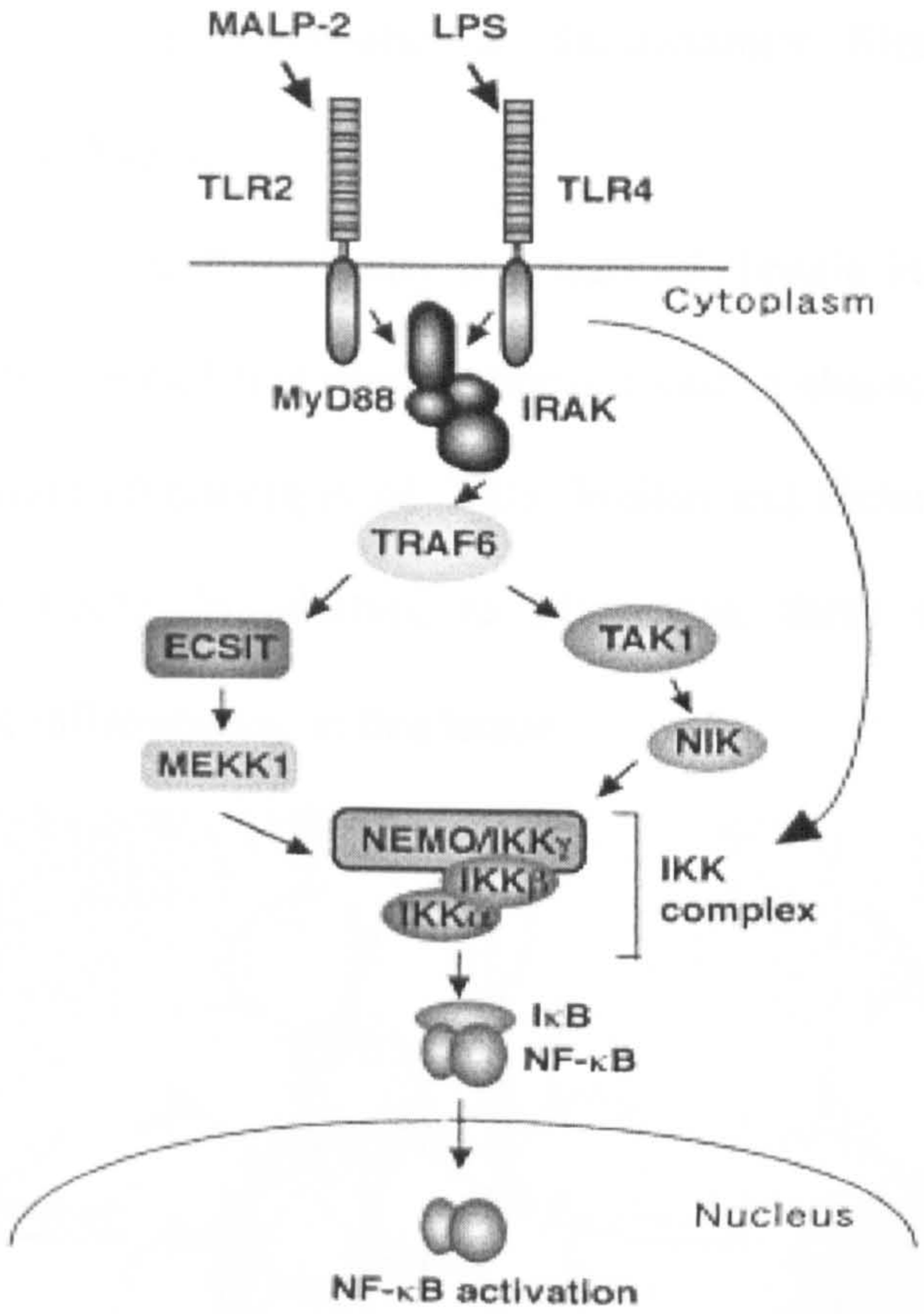

Figure 1.5.1 Key components of the innate immune signalling pathway. Upon activation, myeloid differentiation primary response gene-88 (MyD88) associates with the cytoplasmic domain of toll-like receptor (TLR), thus recuiting IL-1 receptor-associated kinase (IRAK) to the receptor. IRAK then activates TNF receptor associated factor-6 (TRAF-6), causing activation of the inhibitor of NF-κB kinase (IKK) complex (including inhibitor of NF-κB kinase-γ (NEMO/IKKγ), inhibitor of NF-κB kinase-β (IKKβ) and inhibitor of NF-κB kinase-α (IKKα)); this activated complex phosphorylates inhibitor of NF-κB (IκB), resulting in nuclear translocation of nuclear factor-κB (NF-κB), which induces the expression of inflammatory cytokines. Figure adapted from (Takeda *et al.* 2005). LPS, lipopolysaccharide; MALP-2, macrophage-activating lipopeptide-2; ECSIT, evolutionarily conserved signalling intermediate in toll pathways; MEKK1, mitogen-activated kinase kinase-1; TAK1, transforming-growth-factor-β-activated kinase-1; NIK, NF-κB-inducing kinase.

In vitro analysis has demonstrated a fully active pathway of innate immunity within 3T3-L1 adipocytes (Lin *et al.* 2000), thus emphasising that an increase in adipose tissue mass may be responsible for the elevated systemic levels of inflammatory factors observed in obese, insulin resistant and T2DM subjects. Future studies will however, have to address whether such a scenario exists in human isolated adipocytes.

1.5.2 Crosstalk between Metabolic & Inflamatory Signalling Pathways: Adipocytes & Macrophages.

Whilst it is now recognised that obesity is a state of chronic inflammation, several studies have recently reported that obese adipose tissue is characterised by profound macrophage infiltration (Weisberg *et al.* 2003; Wellen and Hotamisligil 2003; Xu *et al.* 2003) (**Figure 1.5.2**). In addition to adipocytes, these macrophages are a significant source of inflammation in this tissue.

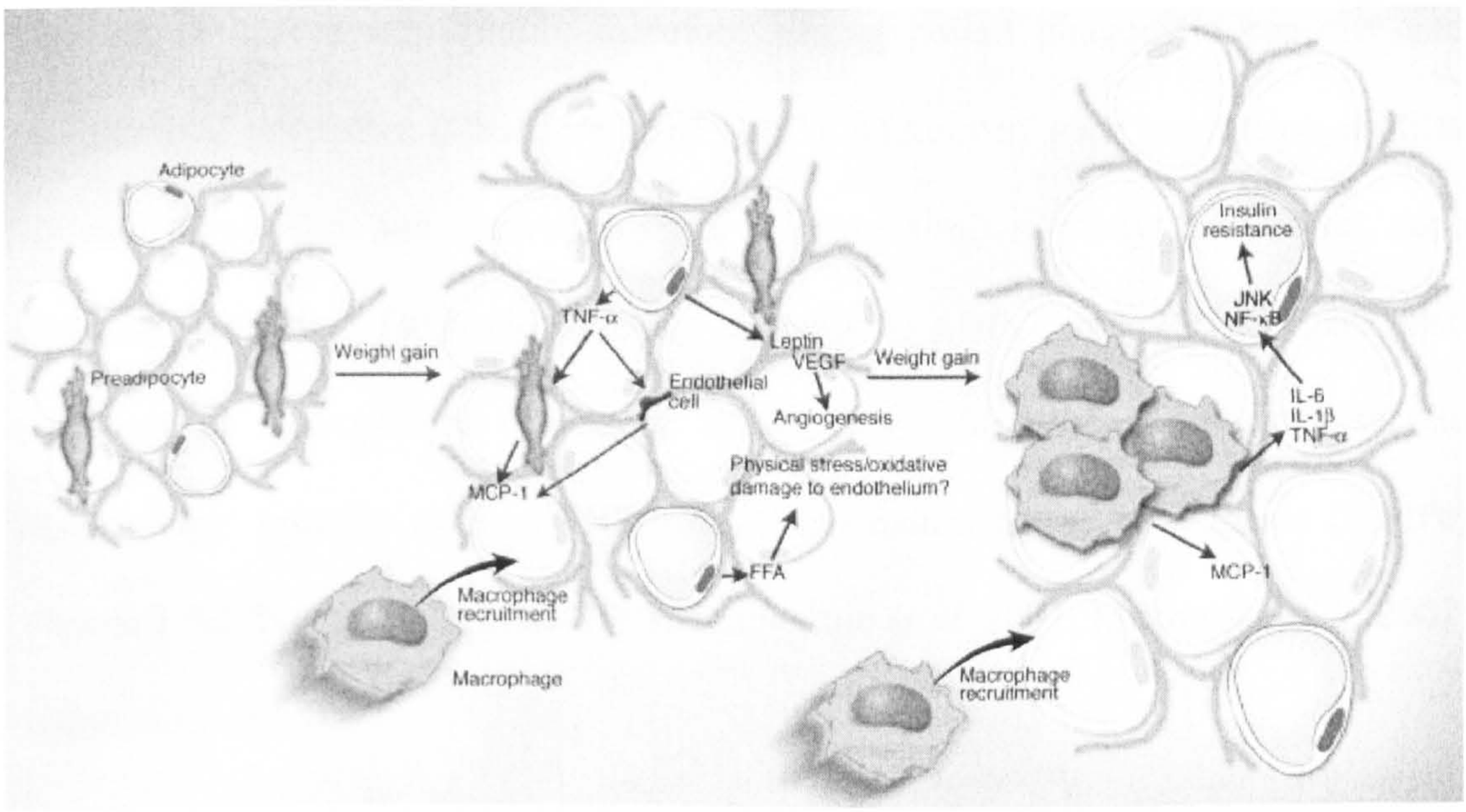

Figure 1.5.2.1 Illustration demonstrating progressive macrophage infiltration into expanding adipose tissue mass in the development of obesity. Adipose tissue becomes 'inflamed' in states of obesity; equally via infiltration of adipose tissue by macrophages and, as a consequence of adipocytes producing inflammatory cytokines. Figure obtained from (Wellen *et al.* 2003). MCP-1, monocyte chemotactic protein-1; VEGF, vascular endothelial growth factor; FFA, free fatty acid; JNK, c-Jun N-terminal kinase.

An interesting and novel concept is that the inflammatory response observed in obesity is 'triggered' by and resides predominantly in adipose tissue (Wellen *et al.* 2003); suggesting that adipocytes may propagate recruitment of systemic macrophages. Alterations in adipocyte size may thus cause modifications in the paracrine function of adipocytes. For example in obesity, the adipocyte begins to

secrete low levels of TNF-α, this then stimulates pre-adipocytes to produce monocyte chemotactic protein-1 (MCP-1) (Xu *et al.* 2003); such a stimulus then attracts macrophages into the adipose tissue. It is further acknowledged that adipocytes and macrophages possess similar functional and molecular roles in inflammatory pathways (Hotamisligil *et al.* 1993), suggesting an overlap in biology and a close interrelationship between adipocyte and macrophage lineages. For instance, pre-adipocytes have the capability to *trans*-differentiate into 'macrophage-like' cells in response to appropriate stimuli, therefore having potent phagocytic capacity and antimicrobial properties (Charriere *et al.* 2003). Moreover, gene expression profiles between adipocytes and macrophages are highly similar; many 'adipocyte' gene products, including fatty-acid binding proteins (FABPs) and PPAR-γ are also expressed in macrophages (Tontonoz *et al.* 1998). Similarly, adipocytes secrete 'macrophage' proteins such as TNF-α, IL-6 and matrix metalloproteinases (MMPs) **(Figure 1.5.2.2)** (Tontonoz *et al.* 1998; Bouloumie *et al.* 2001; Makowski *et al.* 2001; Hotamisligil 2003).

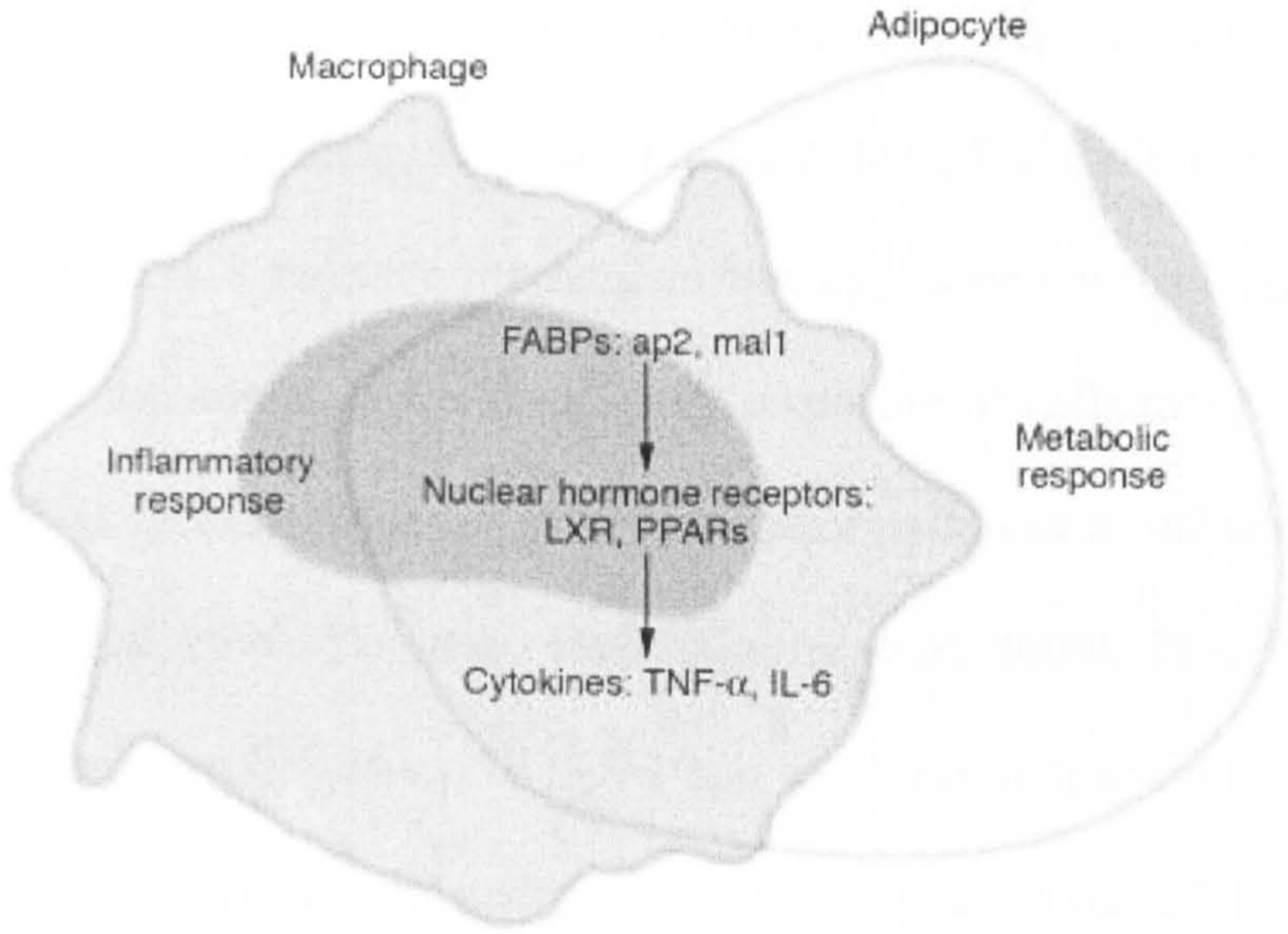

Figure 1.5.2.2 Diagram highlighting the integration of metabolic and immune factors in adipocytes and macrophages. Both cell-types share common features, such as expression of cytokines, FABPs and nuclear hormone receptors. Figure obtained from (Wellen *et al.* 2005). FABPs, fatty acid binding-proteins; ap2, adipocyte fatty acid-binding protein; mal1, adipocyte fatty acid-binding protein-5; LXR, Liver X receptor; PPARs, peroxisome proliferator activated receptors.

1.5.3 Inflammatory Pathways to Insulin Resistance: Overlap of Innate Immunity & Insulin Signalling: IKK-β & JNK as Central Mediators.

It is established that obesity is closely integrated with a state of inflammation; how exactly adipocytokines mediate insulin resistance is however, unclear. It has been suggested that activation of certain inflammation pathways impairs insulin signalling (Schling *et al.* 2002; Hotamisligil 2005). This paradigm emphasises crosstalk between innate immunity and the insulin signalling pathway; certainly identifying underlying mechanisms of these signalling pathways could distinguish central mediators of this convergence and presumably lead to the identification of key therapeutic targets.

1.5.3.1 The Insulin Signalling Cascade: JNK as an Inhibitor of Insulin Signalling.

Insulin affects cells through binding to its receptor on the surface of insulin-responsive cells. Such binding stimulates receptor autophosphorylation and

subsequent tyrosine phosphorylation of insulin receptor substrate (IRS) molecules to initiate downstream signalling, as detailed in **Chapter 1.3.1**. Inhibition of downstream insulin signalling is a primary mechanism through which inflammatory signalling leads to insulin resistance. More specifically, exposure of cells to TNF-α or elevated levels of NEFAs stimulates the inhibitory phosphorylation of serine residues of IRS-1 (Hotamisligil *et al.* 1996; Yin *et al.* 1998; Aguirre *et al.* 2000). This phosphorylation reduces tyrosine phosphorylation of IRS-1 and its ability to associate with the insulin receptor, thereby inhibiting downstream signalling (Paz *et al.* 1997; Aguirre *et al.* 2002). Several serine/threonine kinases are activated by inflammatory stimuli that inhibit insulin signalling, these include JNK and IKK (Zick 2003); of these candidates, JNK in particular, has recently emerged as a central metabolic regulator, playing a key role in the development of insulin resistance in obesity (Hirosumi *et al.* 2002).

The 3 members of the JNK group of serine/threonine kinases, JNK-1, -2 and, -3 belong to the 'mitogen-activated protein kinase' family of stress-activated protein kinases. Such kinases regulate cell development and function through their ability to mediate transcription by phosphorylating 'activator protein-1' proteins, including c-Jun and JunB (Derijard *et al.* 1994); of which are both implicated in the regulation of inflammatory genes (Davis 2000). In response to certain extracellular stimuli, such as elevated levels of NEFAs or TNF-α, or more recently established intracellular stimuli, such as endoplasmic reticulum (ER) stress, JNK is potently activated (Bennett *et al.* 2003). Such stimulation causes JNK to associate with and phosphorylate IRS-1 on Ser307 (Aguirre *et al.* 2000), thus impairing insulin action.

In obesity, JNK-1 is the predominant isoform in liver, skeletal muscle and adipocytes and, has been demonstrated to be upregulated in murine models of obesity (Hirosumi *et al.* 2002). Alternatively, loss of JNK-1 decreases adiposity, enhances insulin signalling and prevents the development of insulin resistance and diabetes in both genetic and diet-induced mouse models of obesity (Hirosumi *et al.* 2002). Additionally, synthetic JNK inhibitor and/or inhibitory peptide treatment has been reported to improve insulin sensitivity in mouse models of obesity and insulin resistance (Bennett *et al.* 2003). JNK inhibition in T2DM has therefore, the potential to serve as a viable therapeutic target for this disease and other chronic inflammatory diseases (Kaneto *et al.* 2004). Future studies examining JNK inhibitors in human adipose tissue would be of interest.

1.5.3.2 IKK-β as a Central Mediator of Inflammation & Insulin Resistance.

In addition to JNK, IKK-β has recently emerged as a central mediator of inflammatory and metabolic signalling systems. IKK-β can significant influence insulin signalling through two mechanisms. Firstly, IKK-β directly phosphorylates IRS-1 on serine residues (Yin *et al.* 1998; Gao *et al.* 2002), impairing insulin signalling. Secondly, IKK-β phosphorylates IκB, releasing NF-κB, (as detailed in **Chapter 1.5.1**) to initiate the production of inflammatory mediators (Shoelson *et al.* 2003). **Figure 1.5.3.2** below demonstrates the impact that JNK and IKK-β have on metabolic and inflammatory signalling systems.

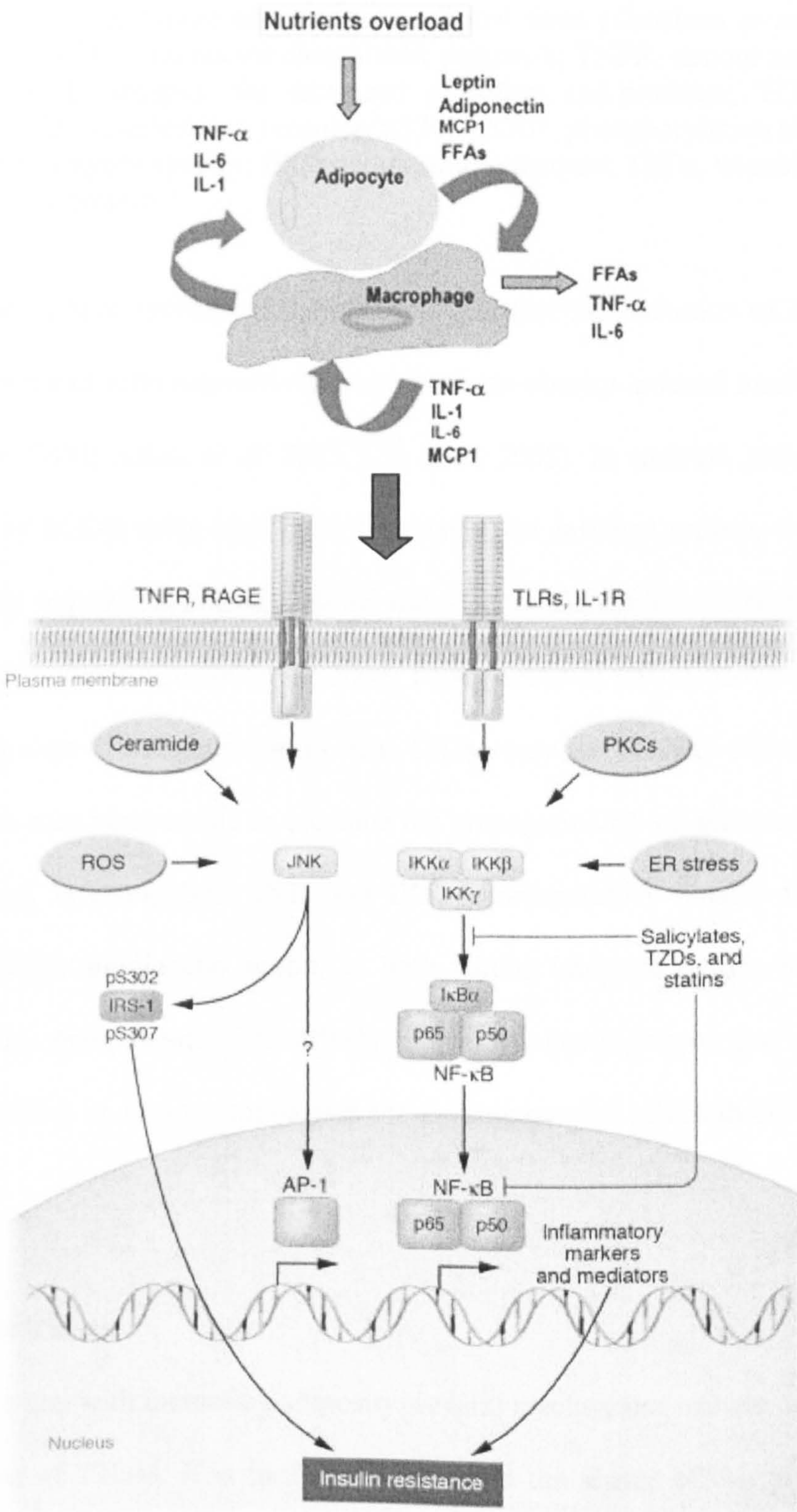

Figure 1.5.3.2 Hypothesised integration between metabolic and inflammatory pathways in obesity. Extracellular stimuli activating both nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK) signalling, include TNF-α, free fatty acids (FFAs) and microbial products. Intracellular stimuli include ER stress and protein kinase C (PKC) isoforms. Obesity-induced IKK-β activation causes NF-κB translocation and increased expression of inflammation molecules that cause insulin resistance. Obesity-induced JNK activation promotes serine phosphorylation of insulin receptor substrate-1 (IRS-1); this negatively regulates normal signalling through the insulin

receptor/IRS-1 axis. Figure obtained and adapted from (Shoelson *et al.* 2006) and (Chen 2006). MCP-1, monocyte chemotactic protein-1; TNFR, tumour necrosis factor receptor; RAGE, receptor for advanced glycation end-products; TLRs, toll-like receptors; IL-1R, interleukin-1 receptor; pS302/pS307, phosphorylation sites 302/307; ROS, reactive oxygen species; ER, endoplasmic reticulum; TZDs, thiozolidinediones; AP-1, activator protein-1.

Recent studies have revealed that tissue and cell-specific activation of IKK-β in the liver and myeloid cells respectively, contributes to obesity-induced insulin resistance (Rohl *et al.* 2004; Arkan *et al.* 2005; Cai *et al.* 2005). In contrast, pharmacological inhibition of IKK-β using high-dose salicylates, for instance aspirin, was shown to significantly improve insulin signalling and glucose metabolism in both obese mice and diabetic humans (Hundal *et al.* 2002; Perseghin *et al.* 2003). However, targeting single mediators of inflammation to treat T2DM may not be fully effective, as other components may compensate to continue the propagation of inflammatory responses. Nevertheless, targeting both JNK and IKK-β inflammatory kinases together may generate robust antidiabetic action, as both factors integrate signals from multiple inflammatory components. Collectively, recent studies highlight that activation of JNK and IKK-β in obesity emphasises the striking overlap of metabolic and immune pathways.

1.6 Aims of Thesis.

It is evident that with increasing adiposity, several mechanisms operate to enhance the pathogenisis of T2DM. It is further apparent that the source of this problem arises from adipose tissue itself. From studies to date, it appears that the equilibrium of adipose-derived factors is substantially dysregulated with obesity. As a consequence, adipocytokines such as resistin, TNF-α and IL-6 assemble to exert pathogenic effects, collectively contributing to the development of insulin resistance and T2DM.

Neveretherless, unraveling the mechanisms leading from obesity to inflammation will have significant implications for the design of novel therapies to reduce the morbidity and mortality of obesity.

This thesis will therefore predominantly explore the adipocytokine resistin and further, albeit to a lesser extent, key components of the innate immune system in the context of human adult and childhood obesity. Furthermore, these studies will investigate the pro-inflammatory actions of resistin in adipose tissue in concordance with factors of the innate immune pathway. Finally, the adipokine adiponectin, having a somewhat 'ying-and-yang' relationship with resistin in terms of obesity and inflammation, will be investigated in the context of central action and energy homeostasis.

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Chapter 2

General Methods & Materials

General Methods & Materials.

This section details the methods and materials most frequently used throughout the thesis. Precise information outlining specific methods for each study will be covered within each appropriate chapter; any further information on specific reagents or techniques used is noted in the Appendices.

2.1 Adipose Tissue Collection & Processing.

Abdominal Subcutaneous (AbSc) adipose tissue was collected from subjects undergoing elective, cosmetic surgery (liposuction) or in accordance with local ethics committee guidelines. All subjects with a history of malignancies or under treatment or suffering from any endocrine abnormalities were excluded from the study. Fresh primary adipose tissue was utilised the following ways; either frozen and stored (**Chapter 2.1.1**) or processed to isolate the mature adipocytes (**Chapter 2.1.2**).

2.1.1 Freezing Adipose Tissue.

Approximately 40 ml of adipose tissue obtained from liposuction was aliquoted into sterile 50 ml centrifuge tubes then flash frozen in liquid nitrogen. To ensure minimal protein damage or degradation, frozen AbSc adipose tissue samples were placed directly from liquid nitrogen into a -80°C freezer for storage until further use.

2.1.2 Isolation of Abdominal Subcutaneous Adipocytes: Collagenase Digestion.

Adipose tissue (25 ml) obtained from liposuction was poured directly into sterile 50 ml centrifuge tubes. For collagenase digestion, adipose tissue was suspended in 20 ml of warm collagenase solution then incubated at 37°C in a continuous shaking (100 cycles/min) water bath for 30-45 min, with additional shaking at 10 min intervals until

the consistency of the adipose tissue was a smooth homogeneous liquid. Following collagenase digestion, adipose tissue was filtered through a sterile fine cotton mesh (Medistore, UK) into another sterile 50 ml centrifuge tube. Centrifugation at 360 x g for 5 min separated the collagenase digested adipose tissue into three independent layers: the lower stromal-vascular fraction containing a pellet of pre-adipocyte cells, the central adipocyte layer and the upper supernatent fraction containing lysed adipocyte cells. The supernatent fraction was discarded and the adipocyte layer was gently poured into fresh sterile 50 ml centrifuge tubes, leaving the stromal-vascular fraction (containing various cell-types, such as pre-adipocytes, leukocytes and monocytes and macrophages). The adipocytes were washed twice by adding 30 ml of warm (37°C) culture medium (DMEM/F-12 Hams' phenol red free medium containing penicillin (100 units/ml) and streptomycin (100 μg/ml) (Sigma, UK)) to remove any traces of collagenase and blood. Previous assessment for potential blood/macrophage contamination of adipocyte fractions using immunohistochemistry, revealed no macrophage or monocyte contamination, as previously detailed (McTernan *et al.* 2002). The adipocytes were dispersed by gently rocking them through the medium before centrifugation at 190 x g for 30 s. The lower supernatant layer was then carefully removed using a 10 ml pipette and discarded. Approximately 1 ml of compact adipocyte cells (containing ~500,000 adipocytes, as determined by haemocytometer analysis) were added to individual sterile 25 cm^3 flasks containing 5 ml of culture medium (DMEM/F12 Hams' phenol red free medium containing 1% transferrin (Sigma, Dorset, UK), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Sigma, UK)). Cells were then incubated for either to 14 hr, 24 hr or a maximum of 48 hr with or without drug, or cytokine treatment (as described in Chapter 3 and Chapter 4).

2.1.3 Purification & Processing of AbSc Adipose Tissue Explants by Erythrocyte Lysis Buffer Digestion.

The purification and subsequent processing of whole adipose tissue explants yields non-collagenase treated adipose tissue, comprising of an intact extracellular matrix and thus containing several cell-types (as opposed to isolating one cell-type, for example adipocytes, as detailed in **Chapter 2.1.2**). Such cell-types in explanted adipose tissue include pre-adipocytes, adipocytes, monocytes, macrophages and leukocytes. For comparative studies between isolated adipocytes and whole adipose tissue explants, the following procedure was used: as previously described, freshly collected adipose tissue obtained from liposuction was poured (25 ml) directly into sterile 50 ml centrifuge tubes. For erythrocyte lysis buffer digestion, 20 ml of the lysis buffer (**Appendix I.2.1**) was added to the adipose tissue. The mixture was left to stand at RT for 15 min then centrifuged at 360 x g for 5 min. Centrifugation separated the digested adipose tissue into two independent layers: the lower blood liquid-phase fraction containing lysed erythrocytes and the upper adipose tissue layer. The lower blood fraction was discarded using a 10 ml pipette. The remaining adipose tissue layer was then washed twice with 30 ml of warm (37°C) culture medium (DMEM/F-12 Hams' phenol red free medium containing penicillin (100 units/ml) and streptomycin (100 μg/ml) (Sigma, UK)) to remove any traces of lysis buffer and blood. This process was repeated until a clean tissue layer remained, as assessed by the supernatant layer. The remaining tissue was then ready for culture, as described in Chapter 4.

2.1.4 Adipocyte Cell Viability Assessed by Trypan Blue Staining.

Viability of adipocytes was assessed using a dye-exclusion method. An aliquot of compact adipocytes was re-suspended in trypan blue dye (Sigma, Dorset, UK) containing PBS and distilled water (dH_2O). The sample mixture was then vortexed and incubated for 5 min at room temperature (RT). Adipocyte cell viability was assessed by analysing an aliquot of the homogenous mixture using a haemocytometer. Both viable and non-viable cells were analysed under a light microscope and counted using a cell counter. Viable adipocytes did not take up the dye, whereas non-viable cells stained blue as they take up the dye.

2.2 Extraction of Protein from AbSc Adipocytes.

The following methods for the extraction of protein from mature adipocyte cells are used throughout the thesis. Any specific alteration of the treatment of adipocytes in the studies covered is outlined in the appropriate chapters.

2.2.1 Extraction of Protein from AbSc Adipocytes using Sodium Dodecyl Sulphate (SDS).

Adipocytes were cultured in an incubator (37°C, 95% O_2, 5% CO_2 mix) for either 14 hr, 24 hr or a maximum of 48 hr with culture medium (DMEM/F12 Hams' phenol red free medium containing 1% transferrin, penicillin (100 units/ml) and streptomycin (100 μg/ml)) with or without the drug or cytokine treatment of interest. Following incubation, adipocytes suspended in culture medium (DMEM/F12 Hams' phenol-red free medium containing 1% transferrin, penicillin (100 units/ml) and streptomycin (100 μg/ml)) were removed from each flask and aliquoted into sterile 15 ml microfuge tubes before centrifugation at 190 x g for 30 s. The lower conditioned

medium layer was then carefully removed and pipetted into 1.5 ml eppendorfs and stored at -80°C until further analysis. From the remaining adipocyte fraction, 500 μl was aliquoted into 1.5 ml eppendorfs and boiling 4% SDS (BioRad, Hercules, California, USA) solution was added (400 μl) into each tube, heated at 95°C for 4 hr before storage at -80°C.

2.2.2 Extraction of Protein from AbSc Adipocytes using Radio-Immunoprecipitation Assay (RIPA) Buffer.

As previously described, adipocytes were cultured in an incubator (37°C, 95% O_2, 5% CO_2 mix) for either 14 hr, 24 hr or a maximum of 48 hr in culture medium (DMEM/F12 Hams' phenol-red free medium containing 1% transferrin, penicillin (100 units/ml) and streptomycin (100 μg/ml)) along with the treatment of interest. Adipocytes suspended in culture medium (DMEM/F12 Hams' phenol red free medium containing 1% transferrin, penicillin (100 units/ml) and streptomycin (100 μg/ml)) were removed from each flask and placed in 15 ml microfuge tubes before centrifugation at 190 x *g* for 30 s. Conditioned medium was carefully pipetted into labelled eppendorfs and stored at -80°C until analysis. From the remaining adipocyte fraction, 500 μl was aliquoted into a 1.5 ml centrifuge tube then 200 μl of RIPA buffer (**Table 2.2.2**) was added into each tube with gentle shaking to mix the contents. Prior to clarification, the sample mixture was then flash frozen in liquid nitrogen to prevent denaturing of protein aggregates. Following thawing, samples were spun at 16,060 x *g* at 4°C for 30 min. Infranatant containing proteins isolated from the adipocyte cells was removed from under the lipid layer, using a syringe and fine bore hyperdermic needle, then stored at -80°C until further use.

Reagent	Quantity	Final Concentration
Phosphate Buffered Saline (PBS) (pH 7.6)	9 ml	120 mM
NonIdet P40	0.1 ml	1% (v/v)
Sodium Deoxycholate	0.05 mg	20% (w/v)
10% SDS	0.1 ml	0.1% (v/v)
100 ug/ml ALLN (protease inhibitor)	1000 μg	100 ug/ml
Complete Mini Protease Inhibitor Cocktail*	1 Tablet	1X (v/v)

Table 2.2.2 Contents of RIPA buffer mix. *Roche diagnostics, Germany.

2.2.3 Extraction of Protein from Whole AbSc Adipose Tissue using RIPA.

Protein samples from whole AbSc adipose tissue for use in Western blot analyses were extracted using RIPA buffer (**Table 2.2.2**). Whole adipose tissue was collected from -80°C storage, then, without allowing the sample to defrost, ~100 mg of adipose tissue was cleaved off using a sterile scalpel and forceps then transferred into a 5 ml bijou tube. RIPA buffer (600 μl) was added to the tissue. The sample mixture was then homogenised using a rotor-stator homogeniser (PowerGen125, Fisher Scientific, UK) at 1100-4200 x g for 3-5 s. The homogenised mixture was then transferred to 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen. Once thawed, the samples were centrifuged at 60,060 x *g* for 30 min at 4°C. Using a fine bore needle and syringe, liquid from below the solid layer was extracted and aliquoted into fresh 0.5 ml microcentrifuge tubes and stored at -80 °C until further use.

2.3 Quantification of Protein.

For the determination of protein, the BioRad DC (detergent compatible) protein assay kit was used, containing Reagent S (Surfactant solution), Reagent A (an alkaline Copper Tartrate solution) and Solution B (dilute Folin Reagent) (BioRad, Hercules, California, USA). The BioRad DC protein assay allowed the quantification of protein extracted from adipocytes and adipose tissue, without alteration from SDS detergent, a component used in both protein extraction methods previously detailed.

To determine protein content of extracted proteins, aliquots of frozen sample were thawed and vortexed to ensure a homogenous sample distribution. A solution containing 20 μl Reagent S and 980 μl Reagent A was pre-mixed in a glass test-tube. To each polypropylene cuvette (Starstedt, Germany), 125 μl of the pre-mixed solution was added. Extracted protein from adipose tissue (3 μl) or adipocytes (5 μl) was then added to the pre-mixed solution. Solution B (1 ml) was added to each cuvette containing the sample mixture then incubated for 15 min at RT. Microfilm was placed over each cuvette and inverted to achieve uniform colour dispersion.

A standard curve of absorbance against protein concentration was generated using known dilutions of Bovine Serum Albumin (BSA) (First Link LTD, UK) (2 μg/μl) in the range of 0-100 μg/μl, using the method described above. The absorbance of protein samples and standard curve were then determined using a spectrophotometer (6505 UV/VIS, Jenway, UK) at a wavelength of 655 nm. Protein concentrations were thus deduced (μg/μl) from the standard curve.

2.4 Western blot Analyses of Isolated Adipocytes & Whole Adipose Tissue.

Western blotting is an immunoassay-based technique that allows the sizing, identification and quantification of specific proteins. In brief, proteins of interest undergo electrophoresis through a gel. Proteins are separated according to their molecular weight (M_r). Following electrophoresis, proteins are electrophoretically transferred onto a polyvinylidene-fluoride (PVDF)™ or nitrocellulose membrane. Proteins of interest are then detected by probing with antibodies raised against them. Proteins are then visualised using a chemiluminescent detection system. This method is detailed below in full.

2.4.1 Preparation of Protein Samples for Electrophoresis.

Quantified protein samples were thawed and vortexed. Equal volumes of protein (20-60 μg; relating to Chapter 3 and Chapter 4) were then transferred into 1.5 ml eppendorfs containing loading buffer, at a minimum ratio of 1:2 (sample: loading buffer) (**Table 2.4.1**). Varying quantities dH_2O were added to the sample mixture to standardise final volumes. A rainbow molecular weight marker (Amersham Biosciences, UK) (14,300-220,000 Da) was diluted 1:5 with loading buffer and loaded alongside each set of samples (i.e. minimum 1 lane per gel), providing a visual protein size record with which protein samples could be compared (**Appendix III.3.2**). The sample mixtures were then heated at 95°C for 5 min to denaturise and linearise the proteins.

REAGENT	QUANTITY	FINAL CONCENTRATION
Tris HCl (pH 6.8) (BioRad, Hercules, CA, USA)	625 μl	125 mM
SDS (10%) (Sigma, UK)	500 μl	4%
Glycerol (Sigma, UK)	1000 μl	20% (w/v)
DTT (Sigma, UK)	200 μl	$6.5x10^{-3}$ mM (w/v)
Bromophenol Blue	125 μl	$2.5x10^{-3}$ mM (w/v)
dH_2O	250 μl	N/A

Table 2.4.1 Quantities of reagents in loading buffer for Western blot analysis.

2.4.2 Electrophoretic Protein Separation.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their size (4% stacking gel, pH 6.8; 8-15% resolving gel, pH 8.8 (percentage of resolving gel was chosen according to the size of the protein of interest). The reagents and their quantities for each gel used throughout the thesis are shown in **Table 2.4.2.**

RESOLVING GEL REAGENT	QUANTITY FOR 8% GEL	QUANTITY FOR 12% GEL	QUANTITY FOR 15% GEL
Protogel*	5.3 ml	8 ml	9.9 ml
Protogel Resolving Buffer*	5.2 ml	5.2 ml	5.2 ml
dH_2O	9.3 ml	6.6 ml	4.5 ml
Ammonium Persulphate†	200 µl	200 µl	200 µl
TEMED¶	20 µl	20 µl	20 µl

STACKING GEL REAGENT	QUANTITY FOR 4% GEL
Protogel*	1.3 ml
Protogel Stacking Buffer*	2.5 ml
dH_2O	6.1 ml
Ammonium Persulphate†	50 µl
TEMED¶	10 µl

Table 2.4.2 Components of resolving gel (8%, 12% and 15%) and stacking gel (4%) used for Western blot analysis.
* See Appendix 1 for full details.
† 0.1% (w/v) (BioRad, Hercules, California, USA)
¶ (BioRad, Hercules, California, USA)

The gel casting unit was constructed according to the manufacturers' instructions. Glass plates were slotted into the clamp assembly and water-tightness tested to ensure no leaks. The resolving gel was prepared as detailed above, vortexed and poured to ~15 mm of the total height of the glass plates. To enhance polymerisation and reduce meniscus formation, ~3 mm of 70% isopropanol was carefully layered over the resolving gel. The gel was then left to set for ~30 min at RT. Once set, the surface of the resolving gel was gently washed with dH_2O and the stacking gel prepared. This gel solution was poured onto the surface of the resolving gel and wells created by

inserting a comb (1.5 mm thickness, 10 teeth of 5 mm wide each) at an angle to minimise introducing air bubbles into the gel. Any loss of gel was immediately replaced. Once the stacking gel was set, the comb was removed vertically and wells were flushed vigorously with a syringe containing electrode buffer. Gels encased within the glass plates were transferred to a tank containing 1L of 1X electrode buffer (**Appendix I**) and secured into place creating two separate reservoirs, allowing ionic movement only through the gels. Wells were flooded and any leakages between the two reservoirs were stopped. Samples were loaded using specific loading tips (Fisher, UK) and air bubbles expelled. Samples were resolved by electrophoresis.

2.4.3 Electrophoretic Transfer (Blotting).

Immobilon-P^{TM} PVDF membranes (0.45 μm) (Millipore, Bedford, Massachusetts, USA) were briefly immersed in 100% methanol (Fisher Scientific, UK), washed in dH_2O for 1 min then soaked in transfer buffer (**Appendix I**) until use. For smaller sized proteins, Westran PVDF membranes (0.2 μm) (Geneflow Ltd., UK) were briefly immersed in 100% methanol and soaked in Transfer Buffer for 5 min. After ~1 hr, gels containing the resolved proteins were removed from the glass plate housing. Stacking gels were discarded and the resolving gels were soaked in transfer buffer for 10 min. Fibre pads and filter paper sets (2 sets per gel) were soaked in transfer buffer for 10 min. Two pieces of filter paper were laid over a saturated fibre pad and gel layered on to the filter paper (orientated to be identical to loading sequence) expelling all air bubbles. One piece of permeabilised Immobilon-P^{TM} membrane was then layered over the gel, ensuring that no bubbles interrupted the contact between the gel and membrane. This was sandwiched between further layers of saturated filter paper and fibre pads. These stacks were transferred to transfer casings and placed into a tank

containing 1L of transfer buffer. Proteins were transferred electrophoretically at a constant voltage of 100V for 1hr.

2.4.4 Primary Antibody Application.

Following the transfer of proteins, membranes were removed from between the filter paper and fibre pads layers and trimmed to mark orientation and membrane number. Membranes were incubated with 10% non-fat milk solution (Marvel Milk Powder, Premier Brands, Merseyside, UK) diluted in 0.5% PBS (Tween 20 (0.1% (v/v), Sigma, Dorset UK) and placed on an orbital shaker for either 1 hr at RT or overnight for ~14 hr at 4°C (conditions vary depending on the protein of interest). This procedure prevents the primary antibody binding directly to the PVDF membrane and further blocks non-specific binding of proteins to the membrane. Following blocking, membranes were rinsed 3 times in PBS and once in 0.1% PBS-T. Primary antibody was then prepared in sufficient quantity to cover the surface of the membrane at a concentration of 0.1% PBS and 0.1% PBS-T and incubated in a 50 ml centrifuge tube for 1 hr at RT or 4°C overnight on an orbital shaker. After ~14 hr at 4°C, membranes were washed 3 times with PBS and once in PBS-T. Membranes were returned to the shaker for three 10 min washes in excess 0.1 % PBS-T.

2.4.5 Secondary Antibody Application.

Secondary antibody was made up in 0.1% PBS/PBS-T. Membranes were incubated at RT for 1 hr on an orbital shaker (80-100 cycles/min). Following incubation with secondary antibody, membranes were then rinsed three times in 0.1% PBS/PBS-T with three further 10 min washes in excess 0.1% PBS-T.

2.4.6 Immunodetection of Antibody Labelled Proteins.

The secondary antibody allows the detection of specific proteins by serving as a catalyst for luminol, a substrate that luminesses, which part of an ECL plus Western blotting detection system (Amersham Pharmacia Biotech., Little Chalfont, Buckinghamshire, UK). The reaction is based on the oxidation of the cyclic diacylhydrazide luminol. Secondary antibody conjugated with horseradish-peroxidase (HRP) binds to the specific primary antibody that is attached to the protein of interest. Combined HRP and peroxide catalyses the oxidation of the lumigen PS-3 acridan substrate generating thousands of acridinium ester intermediates per minute (**Figure 2.4.6**). These intermediates react with peroxide under slightly alkaline conditions to produce a sustained, high intensity chemiluminescence. The light exuding from the reaction can be visualised by application of photographic X-ray film or real-time digital photography.

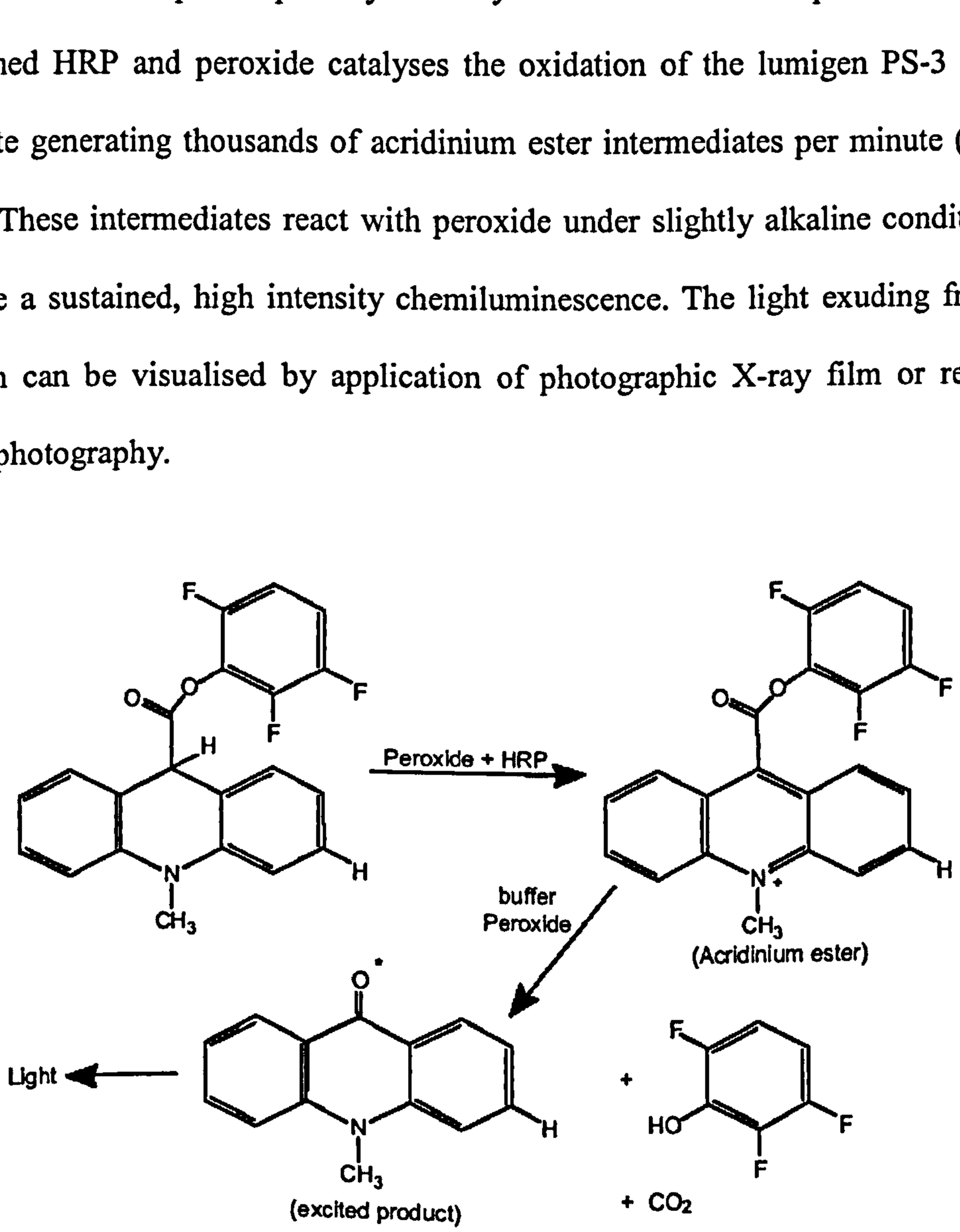


Figure 2.4.6 Chemiluminescent reaction of Lumigen PS-3 with horseradish-peroxidase (HRP) (Modified from Isacsson and Watermark 1974).

Following secondary antibody incubation and subsequent washes in PBS (120 mM, pH 7.6), membranes were ready for the immunodetection of proteins. Membranes were firstly placed protein side-up on saran wrap. ECL plus Solution A (Tris Buffer) was mixed with Solution B (Acridin Solution in Dioxane and Ethanol) at a 1/40 ratio to a volume required to cover each membrane (4 ml/membrane). This mixture was incubated on the membrane for 5 min at RT. Following incubation, ECL plus was discarded and the corner of the membrane blotted on tissue paper to remove excess ECL mixture. Membranes were then sandwiched between two sheets of clear plastic and excess ECL was removed with tissue paper and then placed into a film cassette. The filters were then exposed to photographic X-ray film (Kodak, UK) for varying times depending on the antibody used (1-60 min).

2.4.7 Quantification of Western blot Protein Bands.

Autoradiographs were quantified by gel blot analysis using UVP Gel Blot Analysis System (UVP, UK) and appropriate statistical analysis as detailed in individual results chapters (Chemigenius, Syngene, Cambridge, UK).

2.5 Extraction & Quantification of RNA.

2.5.1 RNA Isolation & Purification from Adipose Tissue for Quantitative PCR.

A column-based method (RNeasy Lipid Tissue Mini Kit for total mammalian mRNA isolation (Qiagen, West Sussex, UK)) was implemented to extract mRNA from the adipose tissue in accordance with manufacturer's instructions. This process included a deoxyribonuclease (DN*ase*) digestion step with 5μl DN*ase* enzyme diluted in 5 μl reaction buffer (DN*ase* Kit, Sigma, UK) **(Appendix I.3.1)** to remove any

contaminating genomic DNA. The tubes containing these reactants were incubated at standard RT for 15 min before the reaction was quenched through the addition of 5 μl of stop solution (**Appendix I.3.1**) and immediate incubation at 70°C for 10 min. RNA was quantified with a spectrophotometer using a ratio of readings at a wavelength of 260 and 280 nm. Once quantified the isolated RNA samples were stored at -70°C.

2.5.2 Reverse Transcription of Isolated mRNA from Adipose Tissue.

An aliquot containing 1 μg of mRNA from each sample was pipetted into a sterile microcentrifuge tube along with 0.5 μl of random hexamers mixed in equal quantities. To standardise, nuclease free water was then added to each aliquot to make up a final volume of 10 μl. Each reactant mixture was then incubated at 70°C for 10 min. Reverse transcriptase (RTn) master mix was then prepared (**Table 2.5.2**) in sufficient quantities to allow for the required number of RTn reactions. After a thorough vortex, 10 μl of master mix were added to each incubated sample of mRNA. Each sample was then vortexed, centrifuged briefly and heated at 37°C. After 1 hr, the reaction was quenched by a consecutive incubation at 95°C for 5 min to denature the enzymes. The resultant cDNA product was stored at -20°C for further use. The theory behind reverse transcription is outlined in **Appendix II.2.3.**

Contents of Reverse Transcription Master Mix	Concentration in Final 20 µL Volume	Quantity Added (µL)
Reverse Transcription 10X Reaction Buffer (Mg^{2++} free)	1x (v/v)	2.0
Mg^{2++} (25 mM)	5 mM	4.0
dNTPS (10 mM)	1 mM	2.0
AMV (20 U/µl)	0.5 u/µl (v/v)	0.5
RNasin (40 U/µl)	1 u/µl (v/v)	0.5
Nuclease free H_2O	5%	1.0

Table 2.5.2 The contents of the reverse transcription master mix. The final volume of cDNA sample is 20 µl where 1 µl equals 50 ng of single stranded cDNA.

2.5.3 Quantitative Real-Time PCR.

The real-time PCR reaction was performed in 25 µl volumes on 96 well plates, in a reaction buffer (Taqman universal PCR master mix), containing 3 mM Mn(Oac)2, 200 pM dNTPs, 1.25 units ampliTaq gold polymerase, 1.25 units ampErase UNG, 100-200 nmol Taqman probe, 900 nmol primers and 25-125 ng cDNA. All reactions were multiplexed with the housekeeping gene for the 18S ribosomal subunit, provided as a pre-optimised control probe (PE Biosystems, UK) enabling data to be expressed in relation to an internal reference to allow for differences in RT-PCR efficiency. Data were obtained as the cycle number at which logarithmic PCR plots cross a calculated threshold line or the Ct value. In accordance of the manufacturer's guidelines ΔCt values were determined (ΔCt=Ct of the target gene minus Ct of the housekeeping gene). Measurements were carried out in triplicate and the target gene probes were

labelled with the fluorescent label FAM, and the housekeeping gene with the fluorescent label VIC. Reactions are as described in **Table 2.5.3**.

Thermal Cycler	Times and Temperatures			
	First Steps		1 of 44 cycles	
			Melt	Anneal/ Extend
ABI Prism 7700 Sequence Detector	Hold	Hold	Cycle	
	2 min 50°C	10 min 95°C	15 s 95°C	1 min 62°C

Table 2.5.3 RT-PCR reaction conditions as pre-set on the ABI 7700.

2.5.4 Data Handling & Statistical Analysis.

For RNA analysis to exclude potential bias due to averaging data, which had been transformed through the equation $2^{-\Delta\Delta Ct}$, all statistics were performed at the ΔCt stage. For both mRNA and protein findings, statistical analysis was undertaken using an unpaired Students' t-test. The threshold for significance was $p<0.05$. Data in the text and figures are presented as mean±SD.

2.6 Separation of Serum from Blood.

In preparation of serum samples, whole blood was drawn directly into a BD-vacutainer® (BD) serum tube containing silica clot activator. Each sample was left at RT for 30 min to allow the blood to clot. Once clotted, the samples were centrifuged at 698 x g for 15 min at RT. Supernatant was carefully drawn off; flash frozen then stored in labelled 1.5 ml centrifuge tubes at -80°C.

2.7 Collection & Processing of Cerebrospinal Fluid (CSF).

Lumbar puncture was performed as part of a diagnostic evaluation. The sampling of CSF was performed according to standardised procedures with the examined subject in a lateral recumbent position and lumbar puncture at the L3-L4 or L5-L6 interspace with a standard needle. Prior to spinal anaesthesia injection, a clear volume of CSF was extracted. CSF samples were passed through a 0.2 microl syringe filter (Whatman, Florham Park, NJ, USA), aliquoted, flash frozen and stored at -80°C until analysis.

2.8 Enzyme Linked Immunosorbent Assay (ELISA) & Radioimmunoassay (RIA).

All ELISA and RIA based assays applied in this thesis were performed using ready prepared commercially available kits. The sources of these kits are outlined in the relevant chapters. In all cases, both ELISA and RIA analyses were carried out in accordance of the manufacturers' instructions of the individual kits. All the corresponding CV, intra- and interassay values are given for each individual assay in the relevant chapters. Appropriate statistical analysis for ELISA and RIA was carried out using a statistical software package (SPSS for Windows, Version 14.0, Woking, UK).

2.8.1 Principles of ELISA & RIA Analysis.

ELISA is an immunological technique for the detection and quantification of specific proteins. An antibody, specific to the protein of interest, is coated onto individual wells of a microtiter plate. Standardised samples (for example, serum samples), in which the protein of interest is contained, are then pipetted into the wells. During the first incubation, antigens of the protein of interest bind to the immobilised antibody,

on the base of the well. The wells are then washed and an antibody specific for the protein of interest is added. During the second incubation, this antibody serves as a detection antibody by binding to the immobilised protein captured during the first incubation. After removal of excess antibody, a HRP-labelled antibody is added, which binds to the detection antibody to complete the four-member sandwich. Following a third incubation and subsequent washing, a substrate solution is added, which is activated by the bound enzyme to produce colour. The colour intensity is directly proportional to the concentration of the protein of interest present in the original sample. The intensity of the colour is then read on a plate-reader (Tecan, UK), alongside a standard curve that is set up concurrently with the samples of interest.

2.9 Principles of Bioplex Luminex Assays.

The luminex multiplex system is a method of measuring several target proteins of interest simultaneously from small amounts of conditioned media, serum or plasma. The technology utilises 5.6 μm microspheres that are internally dyed with red and infra-red molecules. The molecular reactions then take place on the surface of microsphere sets that have been colour-coded using a blend of different fluorescent intensities of the two dyes. By using this method, multiple distinct microsphere sets can be created. The microspheres serve as molecular carriers that capture a sample; they are then tagged with a fluorescently-labelled reporter tag that binds to the captured sample on the microsphere. The microspheres are then injected into the instrument using microfluidics to align them in single file, where they pass through two lasers. One laser illuminates the colours inside the microsphere to identify which bead is being read and the second laser excites the colour on the bead surface. The

advanced optics capture the colour signals, digital signal processing translates the signals into real-time and quantitates data for each reaction.

2.9.1 Laboratory Methodology for Luminex Assay.

Multiple serum analytes were assayed using LincoPlex kits (Linco Research, Missouri, USA) details of individual kits are described in the relevant chapters.

2.9.2 Panel Preparation.

The human adipokine standard cocktail was reconstituted with 250 µl deionised and prepared into seven standards by serial dilution. The human adipokine controls were reconstituted with 250 μl dH_2O and allowed to settle for at least 5 min. Wash buffer (Linco Research, Missouri, USA) (10X) was brought to RT and mixed to bring all salts into solution. This was then diluted with 270 ml of dH_2O to form a 1X solution. Bottles containing the antibody beads were sonicated for 30 s and vortexed for 1 min. Each antibody (150 μl) was added to the mixing bottle and brought to final volume of 3 ml with bead diluent. The filter plate was blocked by pipetting 200 μl of assay buffer into each well of the microtiter plate. This was sealed and mixed on a plate shaker for 10 min at RT. Assay buffer was removed by vacuum being careful not to invert plates and the bottom of the plates dried by using paper towels. Assay buffer (25 μl) was added to the 0 Standard (Background) wells. Each Standard or Control (25 μl) was added to the appropriate wells. Assay buffer (25 μl) was added to all wells. The sample of interest (25 μl) was then added the appropriate designated wells. A solution containing the mixed micospheres/beads (25 μl) was then added to each well. The plate was sealed and covered with aluminium foil to reduce light

interference in on the reaction, then incubated with agitation on a plate shaker overnight (16-18 hr) at 4°C.

2.9.3 Antibody Incubations.

Following overnight incubation, the plate and reagents were allowed to warm to RT before continuing with the assay. The fluid was gently aspirated by vacuum. The plate was then washed 3 times with 200 μl per well of wash buffer, and wash buffer removed using vacuum filtration between each wash. The bottom of the plate was then dried by using paper towels. Detection antibody cocktail (50 μl) was pipetted into each well having allowed the detection antibody to warm to RT prior to addition. This was then sealed, covered with aluminium foil, and incubated with agitation on a plate shaker for 30 min at RT.

2.9.4 Detection & Analysis.

Streptavidin-Phycoerythrin (SAPE) (50 μl) was then added to each well having allowed the SAPE to warm to RT prior to addition. This was sealed, covered with aluminium foil, and incubated with agitation on a plate shaker for 30 min at RT. All contents were then gently removed by vacuum. The plate was washed 3 times with 200 μl per well wash buffer, with the wash buffer removed by vacuum filtration between each wash. Excess buffer was removed from the bottom of plate with tissue. Sheath fluid (100 μl) was then added to all wells. The plate was then covered with aluminium foil and the beads were re-suspended on a plate shaker for 5 min. The plate was then read on the Luminex^{100} multiplex reader (BioRad, UK). The median data was then evaluated using the appropriate software using 5-parameter data reduction.

2.10 Principles of Fast Protein Liquid Chromatography (FPLC): Separation of Protein Complexes by Size-fractionation.

Gel-filtration (also termed size-exclusion chromatography) is a liquid-chromatography technique that separates proteins according to their size (sub-fractionation) as they pass through a high performance gel-filtration medium, pre-packed in a glass column (in this case, the Superdex 200 10/300 GL column was utilised). Ultimately, allowing analysis of the molecular-weight distribution within a protein of interest, further allowing the separation of monomers from their corresponding aggregates.

To perform a separation, gel filtration medium is packed into a column to form a 'packed bed'. The packed bed is a porous matrix consisting of stable and inert spherical particles. The medium is equilibrated with a choice of column running buffer (**Appendix I.4**) that fills the pores of the matrix and the space in between the particles. The liquid inside the pores is called the 'stationary phase' and this is in equilibrium with the liquid outside the particles, the 'mobile phase'.

The sample of interest is then applied to the column. The buffer (mobile phase) and sample move through the column. Molecules diffuse in and out of the pores of the matrix (partitioning of the sample between the mobile and stationary phase). Smaller molecules move further into the matrix and so stay longer on the column. As buffer passes continuously through the column, molecules that are larger than the pores of the matrix are unable to diffuse into the pores and pass through the column. Smaller molecules diffuse into the pores and are delayed in their passage down the column. Large molecules leave the column first followed by smaller molecules in order of their size. The entire separation process takes place as one total column volume.

2.10.1 Equilibration of the GL Column (Sephadex 200 10/300).

To equilibrate the GL Column prior to use, sterile Column running buffer was firstly de-gased for 20 min using a vacuum pump to remove air-bubbles. Following de-gasing, 15 ml of the column running buffer (25 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$; pH 8.0) **(Appendix I.4)** was flushed through the column at a constant flow-rate of 0.25 mS/ml and pressure of 100 psi for 1 hr at RT. Following equilibration, the column was ready for sample injection as described below.

2.10.2 Preparation & Injection of Serum Prior to Size-fractionation.

Prior to use, serum samples were gently thawed over ice. Once thawed, 30 μl of sample was spun at 16,060 x g for 10 min. Column running buffer (20 μl) (25 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$; pH 8.0) was added to the sample. The sample mixture was then injected into a GL column (Superdex 200 10/300, Amersham Biosciences Corp., New Jersey, USA). Samples were run down the column at a flow-rate of 0.70 mS/ml, the pressure of 100 psi and conductivity set to ~20 m/s. In total, 25 (200 μl) gradient-fractions were collected sequentially from the sample. Serum fractions were then analysed by quantitative Western blot analysis **(Chapter 6)**.

2.10.3 Preparation & Injection of CSF Prior to Size-fractionation.

Adiponectin knockout mouse serum (20 μl) (kindly donated by A. Nawrocki, Scherer laboratory, New York, USA) was added to 200 μl of CSF sample to mimic *in vivo* conditions and serve as a protein carrier. The sample mixture was then spun at 16,060 x g for 10 min before injected into the Superdex 200 10/300 GL column undiluted. In total, 25 (200 μl) gradient-fractions were sequentially retrieved from the sample. The CSF fractions (200 μl) were further vacuum concentrated for 1 hr (SpeedVac Plus, USA). Fractions were subsequently analysed by Western blot analysis **(Chapter 6)**.

2.10.4 Visualisation of Sub-fractionated Protein & Subsequent Statistical Analysis.

Following Western blot procedure, fluorescently-labelled protein bands were then visualised using an Infrared Imager (LI-COR Biosciences, Nebraska, USA) using imaging software (Odyssey Infrared Imaging System; Version 1.2). A typical protein Western blot following sub-fractionation and scanned using the described Infrared Imager and software system is shown in **Figure 2.10.4**.

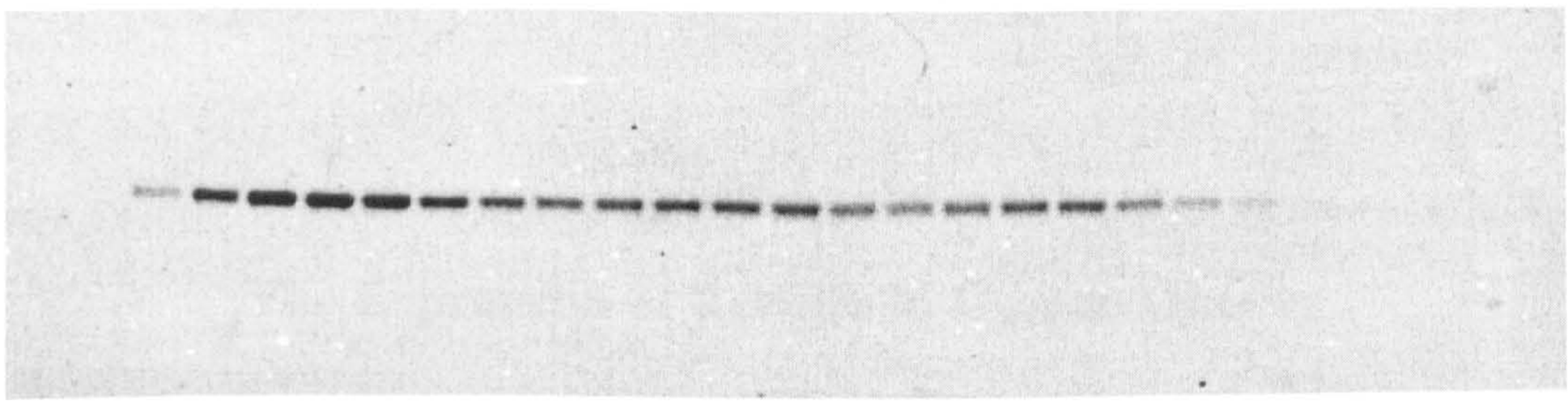


Figure 2.10.4 An example of a Western blot following sub-fractionation protein. The Infrared Imager allowed visualisation of proteins, probed with a fluorescent-dye conjugated antibody system (as detailed in **Chapter 6**).

CHAPTER 3

The Expression of Resistin in Human Obesity

& the Innate Immune Signalling Pathway in Obesity & T2DM

3.1 Introduction.

Resistin, the recently discovered 14 kDa adipokine, is a member of the RELM family of cysteine-rich proteins (Steppan *et al.* 2001). Initially, rodent studies implicated resistin as a factor linking obesity and diabetes (Steppan *et al.* 2001). Subsequent analysis demonstrated that resistin administration or transgenic over-expression severely induces hepatic insulin resistance by increasing glucose production (Rajala *et al.* 2003; Rangwala *et al.* 2004). Alternatively, resistin anti-sense oligonucleotide treatment reverses hepatic insulin resistance and normalises glucose homeostasis (Muse *et al.* 2004); similarly, resistin knockout mice exhibit lower blood glucose levels due to reduced hepatic glucose production (Banerjee *et al.* 2004). Similar metabolic defects occur in peripheral skeletal muscle tissues, as transgenic over-expression of resistin and adenovirus-mediated hyper-resistinemia in rats was shown to impair insulin-stimulated glucose utilisation (Pravenec *et al.* 2003; Satoh *et al.* 2004). Resistin therefore serves primarily to exacerbate hepatic insulin sensitisation *in vivo* (Rajala *et al.* 2003; Banerjee *et al.* 2004; Muse *et al.* 2004; Rangwala *et al.* 2004), with secondary peripheral effects on lipid oxidation in skeletal muscle and adipose tissues (Pravenec *et al.* 2003; Satoh *et al.* 2004).

With regards to obesity, systemic levels of resistin are increased in various diet-induced and genetic models of obesity (Steppan *et al.* 2001; Lee *et al.* 2004), which are further concordant with increasing levels of insulin, glucose and lipids (Rajala *et al.* 2004). Additionally, high-fat fed mice have induced adipocyte differentiation, as denoted by fatty acid binding-protein-2 (FABP-2) expression and a positive correlation with resistin expression (Asensio *et al.* 2004). Human adipose tissue studies have demonstrated increased resistin expression in obese adipose tissue

(Degawa-Yamauchi *et al.* 2003), particularly central abdominal depots (McTernan *et al.* 2002; McTernan *et al.* 2002). Furthermore, obese subjects have higher circulating resistin levels in comparison to lean subjects (Degawa-Yamauchi *et al.* 2003; Schaffler *et al.* 2004; Vendrell *et al.* 2004), which positively correlate with BMI (Azuma *et al.* 2003; Degawa-Yamauchi *et al.* 2003; Yannakoulia *et al.* 2003; Vozarova de Courten *et al.* 2004) and body-fat content (Zhang *et al.* 2002). In contrast, circulating resistin levels are significantly reduced following moderate weight loss (Valsamakis *et al.* 2004) and post gastric bypass (Vendrell *et al.* 2004).

In addition to its role in obesity, the concept of resistin as a pro-inflammatory adipokine in humans is emerging (Bokarewa *et al.* 2005; Silswal *et al.* 2005). Circulating levels of resistin are associated with TNF-α receptor-2 (TNF-R2) and are predictive of coronary atherosclerosis (Reilly *et al.* 2005). Furthermore, endotoxemia has been shown to increase serum resistin levels, concurrently with soluble TNF-R2 levels in T2DM patients (Lehrke *et al.* 2004). The precise role of resistin inflammation is, however, yet to be established.

It is recognised that obesity is associated with a pro-inflammatory milieu, leading to chronic activation of the innate immune system (Dandona *et al.* 2004); this eventually causes progressive impairment of glucose tolerance. Innate immunity provides the first line of defence against invading pathogens and noxious stimuli (Goldstein 2004). The innate immune signal transduction pathway is initiated when certain microbial components or endogenous lipoproteins are recognised by a group of pattern-recognition receptors, the TLRs (Takeda *et al.* 2005). TLR activation causes its association with the cytoplasmic adapter molecule MyD88 through its Toll-interleukin

1 receptor-resistance (TIR) domain (Hoebe *et al.* 2004). MyD88 recruitment thus triggers a downstream signalling cascade, which includes activation of the common signalling adapter molecule, TRAF-6. Consequently, sequential activation of downstream kinases and the primary transcriptional regulator, NF-κB, thus initiates transcription of inflammatory factors (Zhang *et al.* 2001).

Reduced atherosclerosis in MyD88-null mice has been proposed to link elevated serum cholesterol levels to activation of innate immune signalling pathways (Bjorkbacka *et al.* 2004). Moreover, NF-κB is activated in rodent liver in two common models of obesity, high-fat diet and genetic hyperphagia (*ob/ob* mice and *fa/fa* rats) (Cai *et al.* 2005); such hepatocelluar activation of NF-κB in mice further caused profound hepatic insulin resistance and moderate systemic insulin resistance (Cai *et al.* 2005). Recent rodent studies thus highlight associations between increased lipid levels and insulin resistance with components of the innate immune pathway. The expression of these key intermediates of the innate immune pathway in obese and type 2 diabetic human adipose tissue are, however, yet to be determined.

It has been acknowledged that with increasing adiposity, there is profound macrophage infiltration into expanding adipose tissue (Weisberg *et al.* 2003; Xu *et al.* 2003); macrophages may thus represent an alternative site of an innate immune response within adipose tissue. It has further been proposed that the initial innate immune response originates from pre-adipocytes and adipocytes (Wellen *et al.* 2003); secreting low levels of TNF-α and monocyte chemotactic protein-1 (MCP-1), respectively. Such factors 'initiate' an innate immune inflammatory response, thereby recruiting circulating macrophages into the expanding adipose tissue; consequently

macrophages serve to 'amplify' such an inflammatory response in obese adipose tissue. Alternatively, it has been proposed that macrophage recruitment may arise from phenotypic alteration of pre-adipocytes (Charriere *et al.* 2003), possessing potent phagocytotic capacity (Wellen *et al.* 2003). Furthermore, many genes that are critical to adipocytes, such as PPAR-γ and FABP-2, are also expressed in macrophages (Tontonoz *et al.* 1998; Makowski *et al.* 2001). Similarly, adipocytes have been shown to express inflammatory genes, such as TNF-α (Xu *et al.* 2003). It has further been proposed that increased adipose tissue mass is responsible for the elevated circulating levels of resistin in obesity. In rodents, resistin is expressed primarily in adipocytes (Steppan *et al.* 2001). In humans however, although the macrophages that have infiltrated adipose tissue in states of obesity serve as the primary source of circulating resistin, it has not been fully substantiated as to whether or not human resistin is expressed and secreted from adipocytes, particularly within states of obesity.

The focus of this study was therefore to (1) establish the association between increasing adiposity and the expression of resistin and CD45, a known marker for macrophages, in human isolated Abdominal Subcutaneous (AbSc) adipose tissue (AT) (2) investigate whether resistin is expressed in human isolated AbSc adipocytes at a protein level, in relation to obesity; (3) examine the innate immune pathway in human AbSc adipose tissue, in particular the expression of key intracellular components of the pathway, in relation to increasing adiposity and type 2 diabetic status, (4) validation of a commercially available resistin ELISA. Finally, determine whether systemic levels of resistin are influenced by the antigenic stimuli, LPS and zymosan, within human isolated AbSc adipocytes.

3.2 Subjects, Methods & Materials.

3.2.1 Subjects.

For non-diabetic studies, whole AbSc AT was obtained from a human non-diabetic Caucasian population (BMI: 26.5±5.9 kg/m^2; age: 36-49 yrs; n=35; 4 smokers, 31 non-smokers; all female subjects) undergoing elective liposuction surgery. Those patients receiving endocrine therapy (steroids, hormone replacement therapy or thyroxine), anti-inflammatory therapy (aspirin, cyclooxygenase-2 inhibitors), statins, TZDs or any antihypertensive therapy were excluded from the study. For diabetic studies, whole AbSc AT was obtained from Caucasian type 2 diabetic subjects (BMI: 28.2±1.6 kg/m^2; age: 61±4 yrs; n=6; male subjects, n=5; female subjects, n=1; all non-smokers) and BMI-matched Caucasian non-diabetic subjects (BMI: 29.2±1.9 kg/m^2; age: 46±2 yrs; n=6; male subjects, n=2; female subjects, n=4; all non-smokers) by liposuction elective surgery. Studies were performed with the approval of the local ethics committee with informed consent being obtained from all subjects prior to enrolment.

3.2.2 Isolation & Treatment of Mature AbSc Adipocytes.

AbSc AT was digested in collagenase (2 mg/ml; Worthington Biochemical, USA) to isolate the mature adipocytes, as previously outlined in **Chapter 2.1.2**. Aliquots of mature adipocyte cells (1 ml; ~500,000 adipocytes) were then cultured in 5 ml of phenol red-free DMEM:F-12 medium, containing 15 mM glucose, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1% transferrin, with or without antigenic stimuli treatment, as highlighted below.

For antigenic stimuli studies, adipocytes were maintained in the culture medium for 14 h, treated with either a bacterial endotoxin, LPS (100 ng/ml; Sigma-Aldrich Company Ltd., Poole, UK) or a fungal antigen, zymosan (30 μg/ml; Sigma-Aldrich Company Ltd., Poole, UK) or untreated to serve as matched paired controls. Dose and time-responses for LPS and zymosan were previously established (LPS: 1-100 ng/ml; 14, 24 and 48 h; zymosan: 1-100 μg/ml; 14, 24 and 48 h). Adipocytes maintained in untreated media were used as controls. A trypan blue dye (Sigma-Aldrich) exclusion method was used to assess the viability of the adipocytes, as previously documented in **Chapter 2.1.4**. Following treatment, the adipocytes and conditioned media were separated by centrifugation (360 x g for 2 min). Adipocyte conditioned media were removed and stored at -80°C. Adipocyte cells were then re-suspended in either 4% SDS or RIPA buffer solution for extraction of protein, (as previously detailed in **Chapter 2.2.2**) then stored at -80°C.

3.2.3 Protein Determination & Western blot Analysis.

Homogenised human AbSc AT and isolated adipocytes were re-suspended in 4% SDS or RIPA buffer solution, as previously detailed in **Chapter 2.2.** Protein concentrations were determined using the Bio-Rad DC (Detergent Compatible) protein assay kit (Bradford 1976). Western blot analysis was performed using the method previously described in **Chapter 2.5**. In brief, 20-60 μg of protein was loaded onto a 8-15% polyacrylamide gel (Geneflow Ltd., Fradley, UK). A human resistin polyclonal antibody (1:3000, Linco Research, Missouri, USA) was utilised to assess resistin expression. rhResistin (1 μg/ml; Phoenix Pharmaceuticals, Belmont, CA, USA) was further used to confirm the specificity of the primary antibody. Resistin was developed using an anti-guinea-pig HRP secondary antibody (Biogenesis Ltd., Poole, UK). A

polyclonal MyD88 antibody (1:250, TCS Cellworks, UK) and a polyclonal TRAF-6 antibody (1:500, TCS Cellworks, UK) were used to assess MyD88 and TRAF-6 protein expression respectively. Both MyD88 and TRAF-6 were developed using an anti-rabbit HRP secondary antibody (The Binding Site, Birmingham, UK). Protein expression of NF-κB (1:250, TCS Cellworks, UK) was assessed using mouse monoclonal antibodies. Equal protein loading was confirmed by examining α-tubulin (1:5000) (The Binding Site, Birmingham, UK) protein expression. No statistical difference was observed in α-tubulin expression for all samples analysed. For reducing conditions, samples were mixed in a 1:2 ratio with sample buffer containing 20% β-mercaptoethanol. A chemiluminescent detection system ECL/ECL^{+} (Amersham Pharmacia Biotech, Little Chalfont, UK) enabled visualisation of bands, whilst intensity was determined using densitometry (Genesnap, Syngene, UK).

3.2.4 RNA Extraction & Quantitative RT-PCR.

RNA was extracted from whole AT using the RNeasy Lipid Tissue Mini Kit (Qiagen, UK). RNA extraction was followed by a deoxyribonuclease (DN*ase*) digestion step to remove any contaminating genomic DNA. 1 μg of RNA was reverse transcribed using RevertAid H Minus M-MuLV reverse transcriptase (Helena Biosciences Europe, Sunderland, UK) and random hexamers in 20 μl reaction volumes, according to the manufacturers' instructions. Messenger RNA levels were determined using an ABI 7500 Real-time PCR Sequence Detection system. The reactions were performed in 25 μl volumes in reaction buffer containing TaqMan Universal PCR Master Mix, 150 nmol TaqMan probe, 900 nmol primers and 50 ng cDNA (for CD45 expression, a known marker for macrophages) or 115 ng cDNA (for resistin expression). Previously determined quantitative primer and probe sequences for the resistin and CD45 genes

were utilised (McTernan *et al.* 2003). All reactions were multiplexed with the housekeeping gene 18S, provided as a pre-optimised control probe (Applera, Cheshire, UK), enabling data to be expressed as delta cycle threshold (ΔCt) values (ΔCt=Ct of 18S subtracted from Ct of gene of interest) in order to correct for differences in the efficiency of reverse transcription. Measurements were carried out on at least three occasions for each sample.

3.2.5 Assessment of Resistin Secretion from Treated AbSc Adipocytes.

Conditioned media from cultured control untreated AbSc adipocytes and LPS or zymosan treated adipocytes, were assayed using the previously validated human resistin ELISA (Phoenix Europe GmbH, Germany) (Intra-assay CV, <3%; Inter-assay CV, <10%). Minimum Detectable Concentration (sensitivity of assay) was 0.016 ng/ml.

3.2.6 Statistical Analyses.

Protein expression data between control and treatments were compared using an unpaired Student's t-test. Due to the normal distribution of resistin, an unpaired t test was used to assess protein expression data. Results are presented as mean±SD. Analyses were carried out using the SPSS (SPSS Inc. 14.0, Woking, UK) statistical software package. Correlation analyses were calculated using a Pearsons' Correlation Coefficient test. The threshold for significance was $p<0.05$.

3.3 Results.

3.3.1 Resistin & CD45 mRNA Expression in Human AbSc Adipose Tissue.

Resistin mRNA expression in AbSc AT (AT obtained form all Caucasian subjects) was evaluated with increasing adiposity (BMI range, 19.2-37.04 kg/m^2; n=24; all female subjects). Results demonstrated that resistin expression positively correlates with increasing BMI in AT (ΔCT range, 25.0-30.7; r^2=0.461; p<0.001) (**Figure 3.3.1**).

Further analysis of CD45 expression with increasing adiposity showed a similar but weaker correlation (ΔCT range, 20.0-23.6; r^2=0.226; p<0.02) (**Figure 3.3.1**).

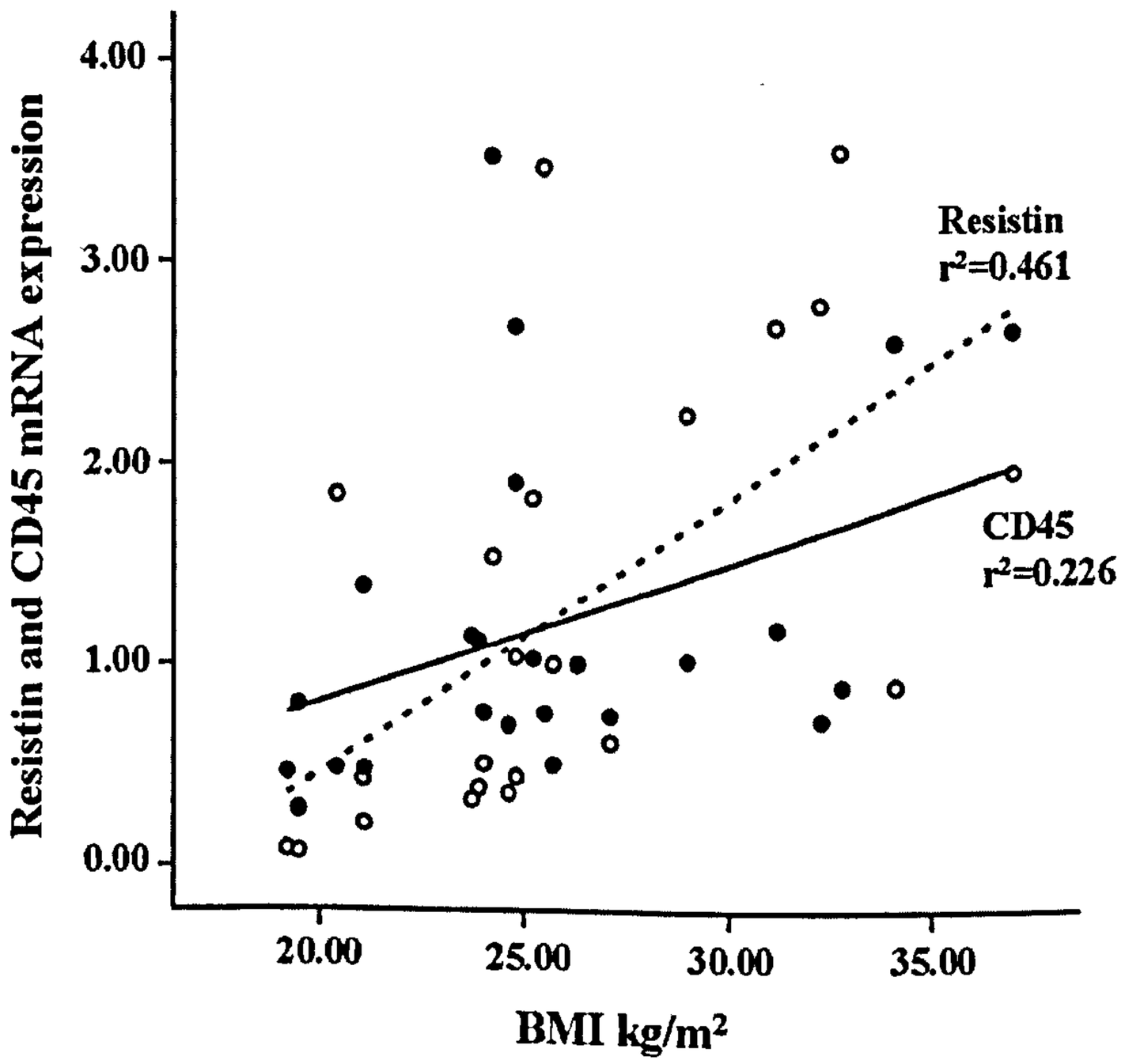


Figure 3.3.1 Relative resistin mRNA expression levels (ringed data points) and CD45 mRNA (solid data points) expression levels in AbSc AT positively correlates with increasing adiposity. The median ΔCt, in the range of values, was assigned an arbitrary value of 1; the other expression levels were standardised to this. (Resistin: r^2=0.461; p<0.001; CD45: r^2=0.226; p<0.02).

3.3.2 Resistin Protein Expression in Lean & Obese Adipose Tissue & Isolated Adipocytes.

Resistin mRNA data was confirmed with assessment of resistin protein expression in AbSc AT in lean (BMI: 21.2±1.4 kg/m^2, n=8) and overweight (BMI: 33.9±4.6 kg/m^2, n=8) subjects. Resistin protein expression in Abd Sc AT was 1.5-fold higher in obese AT (BMI: 33.9±4.6 kg/m^2, n=8) when compared with lean AT (BMI: 21.2±1.4 kg/m^2, n=8) ($p<0.001$) (**Figure 3.3.2.1**).

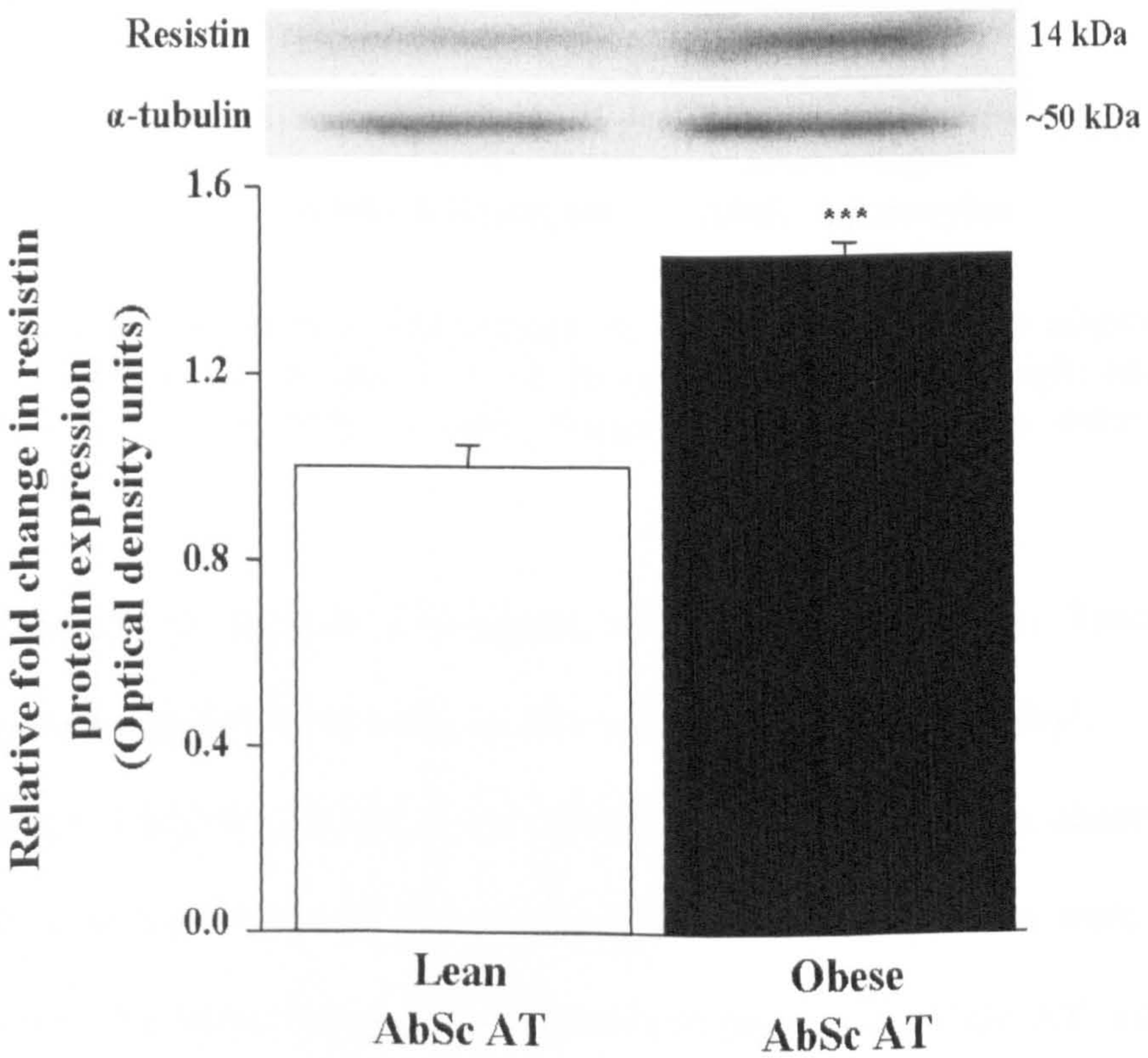


Figure 3.3.2.1 The relative fold change in resistin expression in AT of lean subjects (BMI: 21.2±1.2 kg/m^2; n=8) when compared to obese subjects (BMI: 33.9±4.6 kg/m^2; n=8) ***, $p<0.001$.

Furthermore, when examining isolated AbSc adipocytes, a 2.2-fold higher level of resistin protein expression was observed in overweight subjects (BMI: 28.3±2.7 kg/m^2, n=4) in comparison to lean subjects (BMI: 23.2±1.6 kg/m^2, n=4; $p<0.001$) (**Figure 3.3.2.2**).

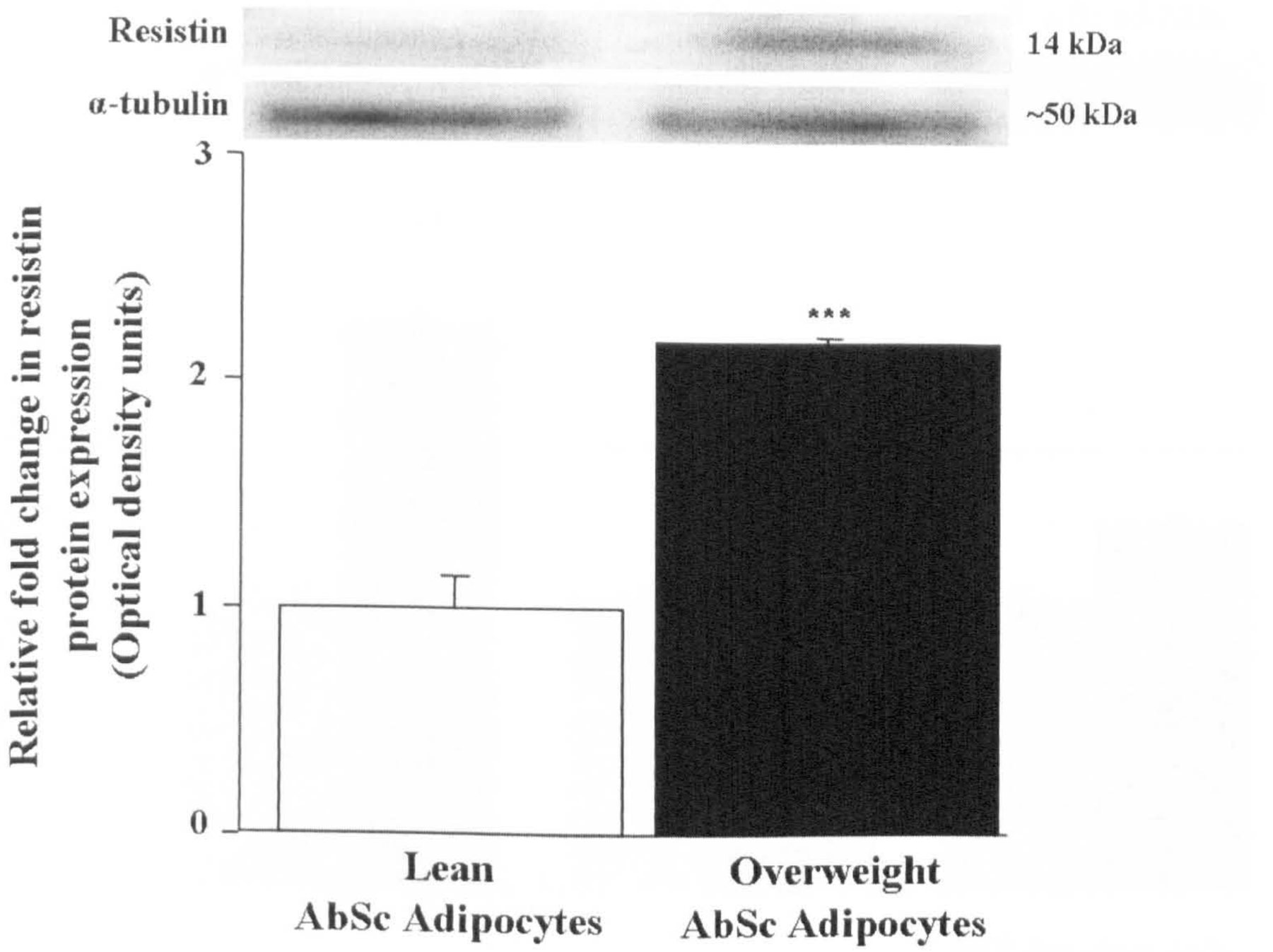


Figure 3.3.2.2 The relative fold change in resistin expression in adipocytes of lean subjects (BMI: 23.2±1.6 kg/m^2; n=4), in comparison to overweight subjects (BMI: 28.3±2.7 kg/m^2; n=4) ***, $p<0.001$. Equal protein loading was determined by α-tubulin.

3.3.3 Analysis of Protein Expression of Components of the Innate Immune Pathway in Lean & Obese Adipose Tissue: The 'Effect of Obesity'.

Assessment of MyD88, TRAF-6 and NF-κB protein expression in obese AbSc AT in comparison to lean AbSc AT demonstrated that these components were all increased within AbSc AT taken from obese subjects compared with AbSc AT taken from lean subjects (MyD88: 1.94-fold increase in obese AbSc AT, $p<0.001$, n=6; TRAF-6: 1.13-fold increase in obese AbSc AT, $p<0.05$, n=6; NF-κB: 1.23-fold increase in obese AbSc AT, $p<0.05$, n=6) (**Figure 3.3.3**).

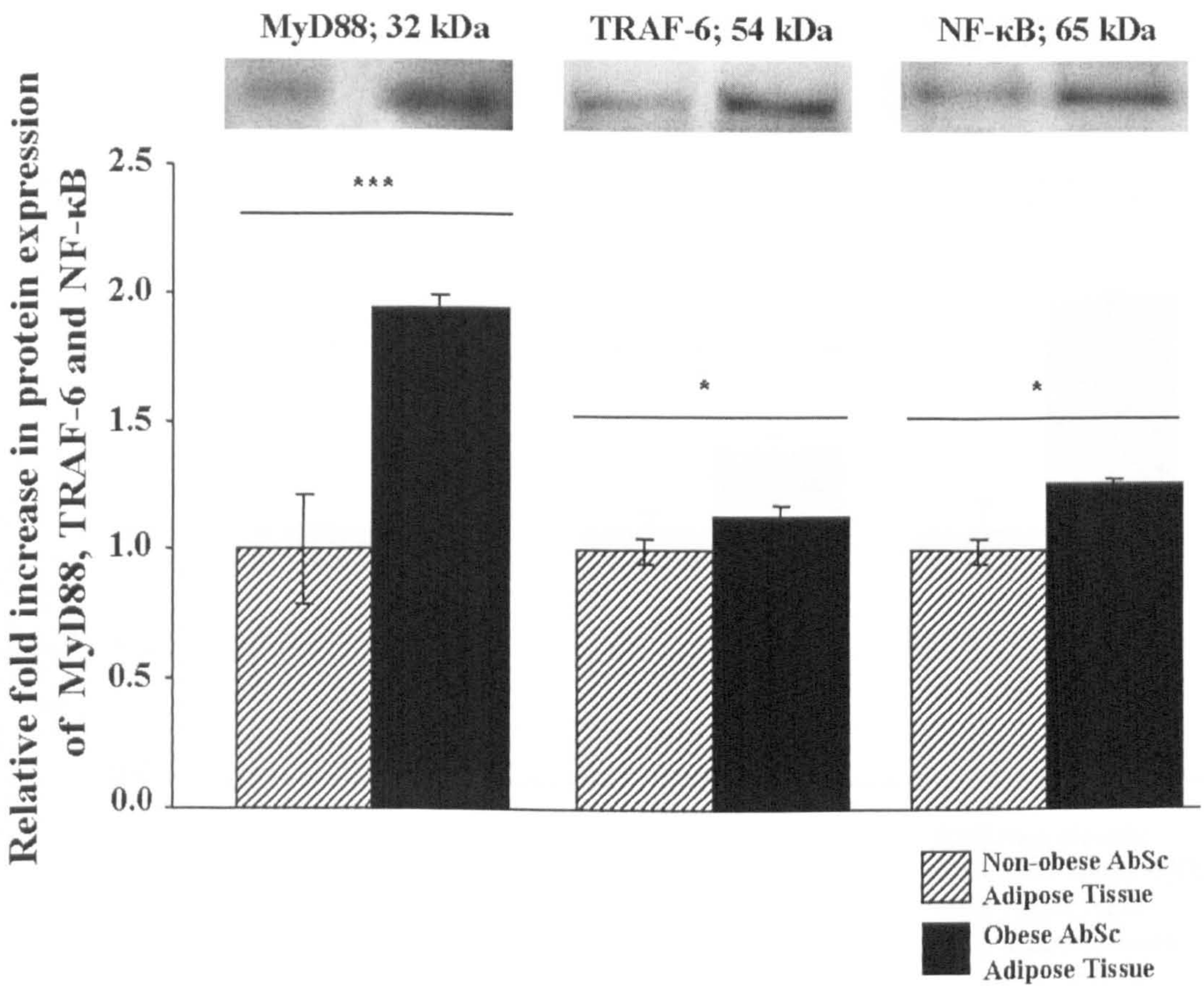


Figure 3.3.3 The relative fold change in MyD88, TRAF-6 and NF-κB protein expression in AbSc AT from obese subjects (BMI: 32.6± 1.1 kg/m^2; n=8) in comparison to AbSc AT from lean subjects (BMI: 23.0±1.1 kg/m^2; n=8) *, $p<0.05$; ***, $p<0.001$. MyD88, myeloid differentiation primary response gene-88; TRAF-6, TNF receptor-associated factor-6; NF-κB, nuclear factor-κB.

3.3.4 Assessment of Protein Expression of Factors of the Innate Immune Pathway in AbSc AT from T2DM Subjects & Non-diabetic Controls: The 'Effect of Diabetic Status'.

Western blot analysis of MyD88, TRAF-6 and NF-κB revealed that protein expression of these components were significantly increased within AbSc AT from T2DM subjects in comparison to non-diabetic AbSc AT (MyD88: 1.38-fold increase in T2DM AT, $p<0.01$, n=6; TRAF-6: 1.32-fold increase, $p<0.01$, n=6; NF-κB: 1.93-fold increase in T2DM AT, $p<0.001$, n=6) (**Figure 3.3.4**).

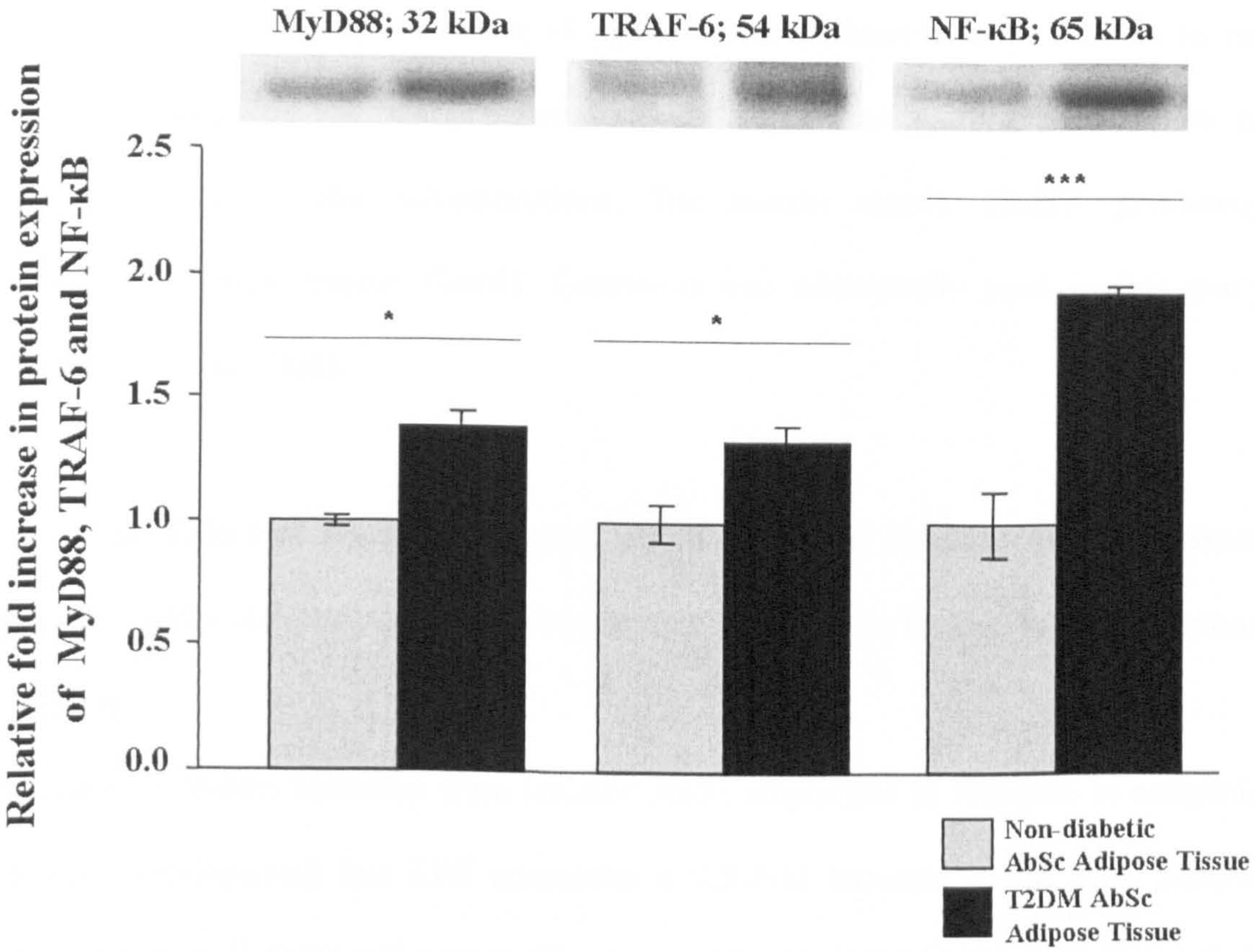


Figure 3.3.4 The relative fold change in MyD88, TRAF-6 and NF-κB protein expression in AbSc AT from T2DM subjects (BMI: 28.2±1.6 kg/m^2; n=6) in comparison to AbSc AT from non-diabetic subjects (BMI: 29.2±1.9 kg/m^2; n=6) *, $p<0.05$; ***, $p<0.001$. MyD88, myeloid differentiation primary response gene-88; TRAF-6, TNF receptor-associated factor-6; NF-κB, nuclear factor-κB.

3.3.5 Resistin Assay Validation: Recovery of Recombinant Resistin & Cross-reactivity with RELMs.

For this study, the R&D Systems human resistin ELISA (resistin range: 0-10 ng/ml) was validated for recovery of spiked recombinant resistin and potential cross-reactivity with other RELMs. Known concentrations of recombinant human resistin (rhResistin) (1, 5 and 10 ng/ml; R&D Systems, UK) were added to pooled serum (10.5 ng/ml). The recovery of spiked resistin was above 80% efficiency. To assess cross-reactivity with other RELMs, known concentrations of RELM-α/FIZZ 1 or RELM-β/FIZZ 2 partial-peptides (1, 2.5, and 5 ng/ml; Alpha Diagnostics, Eastleigh, UK) and rhResistin (5 ng/ml) were co-incubated with pooled serum (10.5 ng/ml), an aqueous solution or

serum matrix containing rhResistin (5 ng/ml). The addition of both RELMs to the different treatments did not interfere with the resistin assay, nor alter known or expected serum resistin concentrations. The human resistin ELISA previously validated (Phoenix Europe GmbH, Germany) was additionally used in this study (McTernan *et al.* 2003).

3.3.6 The Effect of Antigenic Stimuli on the Level of Resistin Secretion from Isolated AbSc Adipocytes: An Introduction to Resistin in the Innate Immune Pathway.

Analysis of resistin secretion from isolated AbSc adipocytes in response to antigenic stimuli, demonstrated that LPS stimulates a 2.2-fold increase in the level resistin secretion from isolated adipocytes (Control: 1.24±0.2 ng/ml; LPS: 2.75±0.4 ng/ml; $p<0.001$; n=8). Similarly, zymosan also shown to enhance a 2.5-fold increase in the level of resistin secretion from adipocytes when compared to control adipocytes (Control: 1.24±0.2 ng/ml; zymosan: 3.1±0.3 ng/ml; $p<0.001$; n=8) (**Figure 3.3.6**).

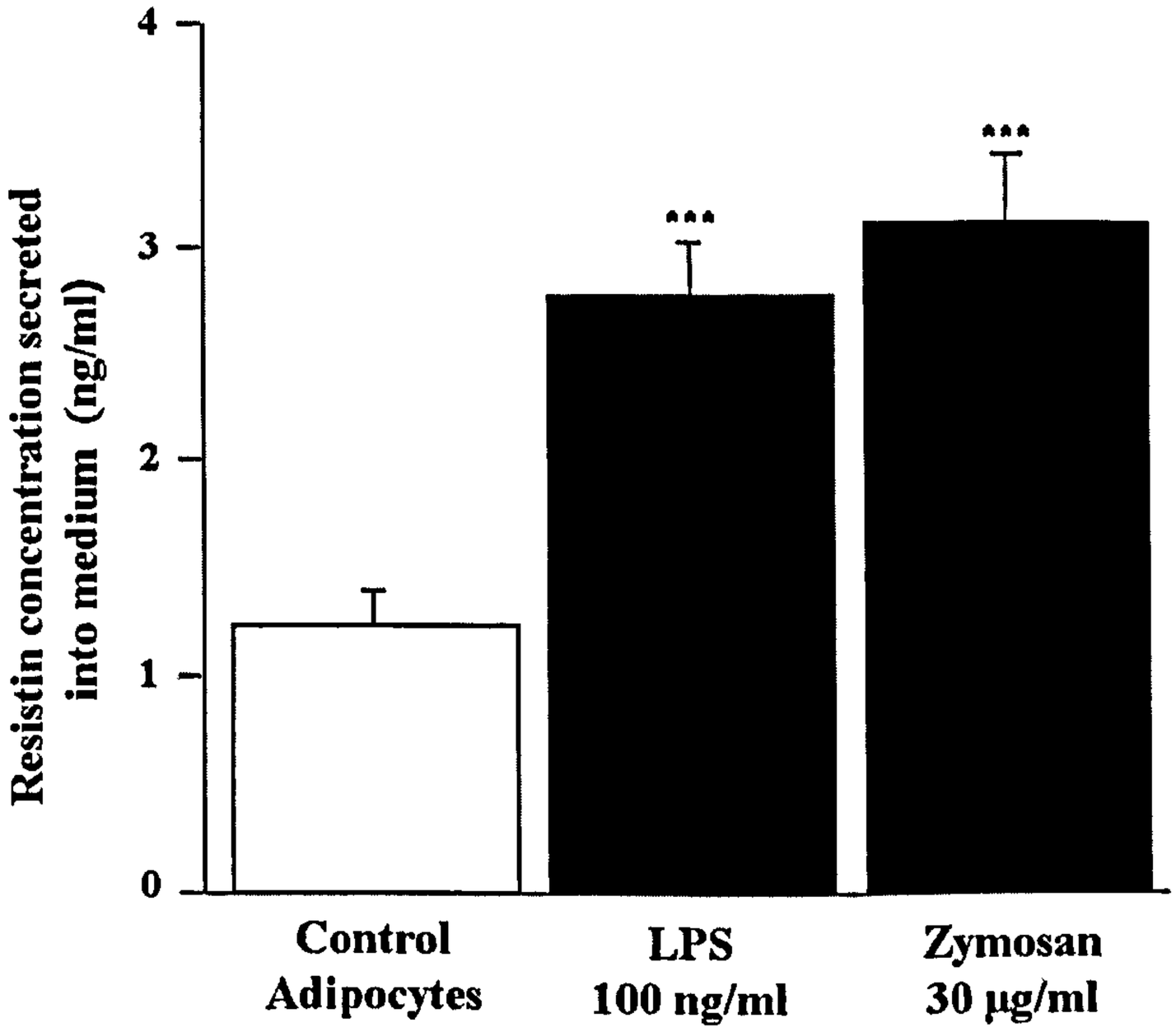


Figure 3.3.6 The level of resistin secretion from isolated adipocytes in response to the antigenic stimuli, lipopolysaccharide (LPS) or zymosan (n=8). Controls were untreated adipocytes. Values obtained with LPS or zymosan treatments were compared to these untreated controls. ***, $p<0.001$.

3.4 Discussion.

This study firstly demonstrated that resistin expression positively correlates with increasing adiposity in AbSc AT. Furthermore, an established marker of macrophages, CD45, demonstrated a comparable, albeit weaker correlation with increasing adiposity. Such observations suggest that other cells, in addition to macrophages, may contribute to elevated levels of resistin in states of obesity; for instance pre-adipocytes (McTernan *et al.* 2002) or adipocytes. To explore this, resistin protein expression was investigated in human AbSc AT and isolated AbSc adipocytes. It was established that the level of resistin protein expression was increased in AT obtained from obese subjects in comparison with AT from lean subjects. Similarly, an increased level of resistin was expressed in AbSc adipocytes isolated from obese AT in comparison with AbSc adipocytes isolated from lean AT. Further studies included the examination of the adipocyte as a potential site for an innate immune response.

Previous mRNA studies have demonstrated that resistin gene expression is most predominant in human macrophages, whilst virtually undetectable in isolated human adipocytes (Savage *et al.* 2001; Patel *et al.* 2003), thus suggesting a similar scenario at the protein level. Initial and current studies however, demonstrate resistin protein expression and secretion from human isolated adipocytes; this has been confirmed by recent observations (Pagano *et al.* 2005; Curat *et al.* 2006). Possible explanations in such diverse quantitative levels between resistin mRNA expression and circulating resistin levels include differences in post-transcriptional and post-translational modifications (Rajala *et al.* 2003; Asensio *et al.* 2004), consequently affecting the secretory rates of resistin. Alternatively, increased systemic levels of resistin may enhance transcript degradation rates via negative feedback mechanisms, or the

initiation and recruitment of inhibitors of transcription. The secreted form of resistin is believed to possess paracrine properties; this implies that the majority of regulation of resistin may occur at the protein level.

It is now acknowledged that increased infiltration of macrophages into adipose tissue occurs in states of obesity (Weisberg *et al.* 2003; Xu *et al.* 2003). Macrophages are thus considered to account for the majority of circulating levels of resistin in states of obesity (Kaser *et al.* 2003; Patel *et al.* 2003). In addition to this, adipocytes have been shown to secrete approximately a quarter of the total amount of resistin produced by macrophages (Curat *et al.* 2006), suggesting that both cell-types are responsible for the elevated levels of resistin in circulation. Adipocytes may, therefore, make a considerable contribution to the increased systemic levels of resistin in humans, which may become increasingly relevant in states of increased adipose tissue mass. More specifically, the adipocyte may therefore serve as an undervalued contributor to the circulating levels of resistin in obesity. Future studies will further have to address resistin levels in adipose tissue and isolated adipocytes in relation to ethnicity and gender. Yannakoulia *et al.* reported that systemic resistin levels are higher in females than males; whether this is true for adipose tissue protein expression levels is yet to be determined.

This study demonstrated that in addition to the adipokine resistin, protein expression of key intermediates of the innate immune signalling cascade, in particular, MyD88, TRAF-6 and NF-κB is significantly increased in obese AT in comparison to lean AT. Moreover, Western blot analysis further revealed that MyD88, TRAF-6 and NF-κB protein expression was also significantly increased in AbSc AT obtained from T2DM

subjects in comparison to BMI matched non-diabetic AbSc AT. Such increased expression levels of components of innate immune pathway implies that in states of obesity and T2DM, there is enhanced activation of innate immunity. Consequently, this may produce abnormal levels of inflammatory molecules, such as TNF-α and IL-6, thus contributing to an altered pro-inflammatory milieu and potentially exacerbating insulin resistance. Taken together, this study re-affirms interrelationships between excess adipose tissue mass, inflammation, insulin resistance and T2DM.

Whilst a role for resistin and components of the innate immune pathway in human obesity are highlighted, a paradigm of resistin as a pro-inflammatory factor is emerging. To further analyse resistin in relation to the innate immune pathway, we examined whether bacterial and fungal antigenic stimuli, previously known to stimulate an inflammatory cascade in fully intact 3T3-L1 adipocytes (Lin *et al.* 2000), could influence the level of resistin secretion from isolated human AbSc adipocytes. Following validation of a commercially available ELISA, it was established that the bacterial antigen LPS, in addition to the fungal antigen zymosan, increased the level of resistin secretion from isolated adipocytes. Such observations coincide with recent studies, demonstrating that endotoxemia increases circulating resistin levels in healthy subjects (Lehrke *et al.* 2004), highlighting antigenic stimuli can increase resistin levels *in vivo*. Collectively, these studies suggest that resistin may directly contribute to an altered pro-inflammatory cytokine status and, therefore promote inflammation.

In conclusion, this study suggests that adipocytes may be a contributory source of resistin in human obesity. Furthermore, resistin responds to LPS and zymosan treatment, suggesting an involvement of resistin in the innate immune pathway in human adipose tissue. Further studies thus examining resistin function in concordance

with factors of the innate immune signalling pathway would prove beneficial in defining a role for resistin in inflammation.

CHAPTER 4

The *In vitro* Pro-inflammatory Actions of rhResistin on Components of the Innate Immune Pathway & Insulin Signalling Cascade

4.1 Introduction.

The association between central obesity, insulin resistance and T2DM has been established; however, the underlying mechanisms of this association remain unclear. Besides its metabolic functions, increased adipose tissue mass is now recognised to have immunological characteristics, primarily through the secretion of adipokines, such as leptin, TNF-α and IL-6 (Rajala *et al.* 2003). Such inflammatory mediators are increased in the insulin resistant states of obesity and T2DM, which suggests that inflammatory mechanisms may underly these conditions. Within this context, adipose tissue is now considered to integrate metabolic and immune functions. Such a duality of function may represent a conserved evolutionary mechanism, as suggested by observations examining the 'fat body' in *Drosophila* fruitfly; in which a single cell-type with developmental heritage serves as a primary integrator for both pathogen and nutrient-sensing pathways (Tzou *et al.* 2002).

The adipokine resistin was originally described as a potential molecular link between obesity and insulin resistance in rodents; this has however, remained somewhat controversial in humans, due to contrasting data (Steppan *et al.* 2004). In rodents, resistin is expressed primarily in adipocytes and employs a more metabolic role, by impairing glucose tolerance and inducing liver-specific antagonism of insulin sensitivity *in vivo* (Rangwala *et al.* 2004). In humans however, a more 'pro-inflammatory' function for resistin has been defined (Bokarewa *et al.* 2005; Silswal *et al.* 2005). Although resistin gene expression is largely confined to macrophages (Savage *et al.* 2001; Patel *et al.* 2003), recent studies have reported resistin protein expression and secretion from human isolated adipocytes (McTernan *et al.* 2002; McTernan *et al.* 2003; Pagano *et al.* 2005; Curat *et al.* 2006).

Human serum profiles have highlighted increased circulating levels of resistin in obese, insulin resistant and T2DM states, which further correlate with CRP (McTernan *et al.* 2003), a marker of inflammation and a recently established predictor of cardiovascular disease (CVD) (Ridker *et al.* 2002). Such a correlation between CRP and serum resistin has also been identified by subsequent studies on pre-diabetic, T2DM subjects (Stejskal *et al.* 2003; Shetty *et al.* 2004) and individuals with acute rheumatoid arthritis (Bokarewa *et al.* 2005), further identifying resistin as a potential pro-inflammatory marker. Although the majority of studies report associations between resistin and inflammatory conditions, the precise mechanistic action of resistin in inflammation, particularly in concordance with components of the innate immune pathway, is currently unclear.

Innate immunity serves as the first line of defence against pathogens, contributes to the eradication of such pathogens and further forms the basis of adaptive immunity. Furthermore, the innate immune system is a potential candidate for the production of elevated circulating levels of cytokines in obesity and T2DM. Previous studies have demonstrated the presence of a fully intact pathway of innate immunity in the adipocyte (Lin *et al.* 2000), activated when specific pattern-recognition receptors, the Toll-like receptors (TLRs), recognise and bind a broad spectrum of microbial components. For instance, TLR-4 binds the bacterial antigen LPS, through its co-receptor, CD14; alternatively, TLR-2 binds the fungal antigen, Zymosan. Interestingly, activation of TLR-4 by LPS has been shown to induce TLR-2 expression in 3T3-L1 adipocytes (Lin *et al.* 2000), highlighting potential crosstalk between TLR activation systems. The activation of TLRs initiates a cascade of downstream intracellular signalling events, which in turn cause phosphorylation, hence degradation of IκBα (Doyle *et al.* 2006).

Consequently, this allows the sequestered transcription factor, NF-κB, to translocate into the nucleus, initiating the production of immunomodulatory factors, such as IL-6, IL-1 and TNF-α. Several serine/threonine kinases are activated during the innate immune response that influence insulin signalling (Hirosumi *et al.* 2002). For example, IKK-β mediates activation of NF-κB; whereas JNK, a central metabolic regulator, contributes to the development of insulin resistance in obesity (Hirosumi *et al.* 2002; Tuncman *et al.* 2006). The activation of JNK and IKK-β within innate immunity, particularly in obesity and T2DM, highlights crosstalk between metabolic and immune pathways.

An integration of metabolic and immune systems may thus reflect the mode of resistin action within adipocytes and immune cells, exerting metabolic and immune functions in both cell-types. It has recently been reported that resistin impairs insulin signalling, via the upregulation of 'suppressor of cytokine signalling-3' (Steppan *et al.* 2005) and further inhibits glucose transport in 3T3-L1 adipocytes (Fu *et al.* 2006). Additionally, resistin promotes glucose-dependent lipogenesis and lipid accumulation in human macrophages (Rae *et al.* 2006). On the other hand, the pro-inflammatory functions of resistin in human macrophages (Silswal *et al.* 2005) and 3T3-L1 adipocytes (Fu *et al.* 2006) have been documented. Resistin may thus function in adipocytes to influence both metabolic and pro-inflammatory changes, suggesting that the effects of resistin are to some extent linked. Such a duality in function for resistin may be a consequence of the crosslink initially proposed between metabolic and inflammatory pathways in adipocytes and immune cells (Weisberg *et al.* 2003; Xu *et al.* 2003). Where resistin may influence key factors in the sequential stages from one signal transduction pathway, this may consequently alter components from another.

The aims of this study were therefore to examine the effect of recombinant human resistin (rhResistin) on the expression of components of the innate immune pathway, progressively through TLRs and intracellular factors mediating NF-κB. Additionally, determine whether rhResistin may influence the expression of JNK, a key inhibitor of the insulin signalling cascade in isolated human AbSc adipocytes. Furthermore, evaluate the combined effects of rhResistin, insulin and RSG on the pro-inflammatory response. Finally, determine the effects of NF-κB inhibitor or JNK inhibitor on the level of resistin secretion from isolated AbSc adipocytes.

4.2 Subjects, Methods & Materials.

4.2.1 Subjects.

AbSc adipose tissue (AT) was obtained from a human Caucasian non-diabetic population (BMI: 26.5±5.9 kg/m^2: age 36-49 yrs; n=35; all female subjects; 4 smokers, 31 non-smokers) undergoing elective liposuction surgery. Patients receiving endocrine therapy (steroids, hormone replacement therapy or thyroxine), anti-inflammatory therapy (aspirin, cyclooxygenase-2 inhibitors), statins, TZDs or any antihypertensive therapy were excluded. Studies were performed with the approval of the local ethics committee with informed consent being obtained from all subjects prior to enrolment.

4.2.2 Isolation of Mature Adipocytes.

AbSc AT was digested in collagenase (2 mg/ml; Worthington Biochemical, USA) to isolate the mature adipocytes, as previously detailed in **Chapter 2.1.2**. Following isolation of the adipocytes, cells were re-suspended in either 4% SDS or RIPA Buffer solution (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris) for extraction of protein. Adipocytes were additionally maintained in phenol red-free DMEM:F-12 medium containing 15 mM glucose, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1% transferrin with the various treatment regimens described in the following sub-section.

4.2.3 Treatment of Cultured Adipocytes.

For pro-inflammatory cytokine secretion studies, isolated AbSc adipocytes were treated with rhResistin (30 ng/ml, 48 h; Phoenix Pharmaceuticals, Belmont, CA, USA) (endotoxin concentration below 0.1 ng/μg, at final concentrations of 10-50 ng/ml). To assess whether resistin has a 'direct' influence on the level of cytokine secretion,

resistin treated adipocytes were further cultured with human resistin polyclonal antibody (10 μg/ml, 48 h; Linco Research, Inc., Missouri, USA). Dosage of resistin antibody was based on data previously documented (Bokarewa *et al.* 2005). AbSc adipocytes were also treated with insulin alone (10 nM; Sigma-Aldrich Company Ltd., Poole, UK) or in combination with rosiglitazone (RSG) (10^{-8} M; GlaxoSmithKline, Harlow, UK). rhResistin, insulin and RSG concentrations and time-points were chosen based on secretion data previously described (McTernan *et al.* 2003). Adipocytes were further treated with rhTNF-α (10, 50, 100 ng/ml; Biosource Europe, S. A., Belgium) or rhIL-6 (10, 50, 100 ng/ml; Sigma-Aldrich). Additional assessment of resistin secretion included the treatment of isolated AbSc adipocytes with NF-κB inhibitor (SN50, CalBiochem, Nottingham, UK) (50 μg/ml; 24 h). A dose and time-course study was performed to assess changes in the level of resistin secretion at 14, 24, and 48 h with control and NF-κB inhibitor-treated adipocytes (10, 25, 50 and 100 μg/ml). AbSc adipocytes were also treated with JNK inhibitor (SP600125, A. G. Scientific, Inc., San Diego, USA) (10 μM; 24 h). JNK inhibitor conditions were based on data previously described (Baan *et al.* 2006). For protein expression analysis, adipocytes were treated with increasing concentrations of rhResistin, using the previously established time-point (10, 30, 50 ng/ml; 48 h). For all treatment regimens, adipocytes maintained in untreated media were used as controls. A trypan blue dye (Sigma-Aldrich) exclusion method was used to assess viability of the adipocytes; as described in **Chapter 2.1.4.** Following treatment, adipocyte cells and conditioned media were separated by centrifugation (360g for 2 min). Adipocyte conditioned media was removed, aliquoted and stored at -80°C, whilst adipocyte protein was extracted (as previously detailed in **Chapter 2.2.2**), then stored at -80°C.

4.2.4 Processing AbSc Adipose Tissue to Obtain Explants & Treatment of the Explanted Adipose Tissue.

To assess differences between isolated adipocytes and matched whole explant adipose tissue, liposuction material was processed using erythrocyte Lysis Buffer, as detailed previously in **Chapter 2.1.3**. The resulting adipose tissue explant was treated with either NF-κB inhibitor (SN50, CalBiochem, Nottingham, UK) (50 μg/ml; 24 h) or JNK inhibitor (SP600125, A. G. Scientific, Inc., San Diego, USA) (10 μM; 24 h). AbSc AT explants were maintained in untreated media to serve as controls. Following treatment, AbSc AT explants and conditioned media were separated by centrifugation (360g for 2 min). AbSc explants and conditioned media were removed, aliquoted and stored at -80°C.

4.2.5 Protein Determination & Western blot Analysis.

AbSc adipocytes were re-suspended in 4% SDS or RIPA buffer, as previously described **(Chapter 2.2)**. Protein concentrations were determined using the Bio-Rad DC (Detergent Compatible) protein assay kit (Bradford, 1976). Western blot analysis was performed using the procedure highlighted in **Chapter 2.5**. In brief, 20-60 μg of protein was loaded onto a 8-15% polyacrylamide-gel (Geneflow Ltd., Fradley, UK). Human TLR-2 monoclonal and TLR-4 polyclonal antibodies were utilised (1:500 and 1:1000, respectively; Insight Biotechnology Ltd., Wembley, UK). TLR-2 and TLR-4 were developed using an anti-sheep/goat conjugated HRP secondary antibody (The Binding Site, Birmingham, UK). Polyclonal anti-JNK1 & 2 SAPK phosphospecific and MyD88 antibodies (1:1,750; Biosource UK, Belgium and 1:250; TCS Cellworks, UK respectively) were utilised. JNK and MyD88 were both developed using an anti-rabbit HRP secondary antibody (The Binding Site, Birmingham, UK). Protein expression of

NF-κB, (1:250; TCS Cellworks, UK), IKK-β (1:500; TCS Cellworks, UK) and IKK-α (1:500; Abcam, UK) in Abd Sc adipocytes was assessed using mouse monoclonal antibodies. Equal protein loading was confirmed by examining α-tubulin (1:5000; The Binding Site, Birmingham, UK) protein expression. No statistical difference was observed in α-tubulin expression for all samples analysed. For reducing conditions, samples were mixed in a 1:2 ratio with sample buffer containing 20% β-mercaptoethanol. A chemiluminescent detection system ECL/ECL$^+$ (Amersham Pharmacia Biotech, Little Chalfont, UK) enabled visualisation of bands, whilst intensity was determined using densitometry (Genesnap, Syngene, UK).

4.2.6 Assessment of Resistin, TNF-α & IL-6 Secretion from Isolated AbSc Adipocytes.

The human resistin ELISA previously validated (Phoenix Europe GmbH, Germany) was used in this study (McTernan *et al.* 2003). Conditioned media from rhTNF-α or rhIL-6 treated adipocytes was assessed using the human resistin ELISA from R&D Systems, UK (intra-assay CV 4.7%, inter-assay CV 8.4%); validation of this resistin ELISA is described in **Chapter 3.3.6**. Conditioned media from AbSc adipocytes treated with rhResistin, insulin, or insulin in combination with RSG, was assayed for IL-6 and TNF-α (QuantiGlo ELISA, R&D Systems, Abingdon, UK) (IL-6, intra-assay CV 3.1%, inter-assay CV 2.7%; TNF-α, intra-assay CV 6.7%, inter-assay CV 11.0%). For NF-κB and JNK inhibitor studies, resistin concentrations were measured using Bioplex assays (Linco Research, Missouri, USA) with a sensitivity of 6.7 pg/ml (intra-assay CV 1.4-7.9%, inter-assay CV <21%).

4.2.7 Statistical Analysis.

Protein expression data between control and treatments were compared using an unpaired Students' t-test. Data is presented as mean±SD. Analyses were carried out using the SPSS (SPSS Inc. 14.0, Woking, UK) software package. The threshold for significance was $p<0.05$. Correlation analyses were calculated using a Pearson's Correlation Coefficient test.

4.3 Results.

4.3.1 The Pro-inflammatory Effect of Resistin on TLR-2 & TLR-4 Protein Expression in Isolated AbSc Adipocytes.

For protein expression studies, rhResistin was shown to stimulate TLR-2 (90 kDa) expression in AbSc adipocytes when compared to matched untreated control adipocytes (Control: 1.00±0.11; TLR-2: 1.28±0.10; $p<0.001$, n=6) (**Figure 4.3.1**). However, no significant alteration in TLR-4 (90 kDa) protein expression in response to rhResistin was observed (Control: 1.00±0.05; TLR-4: 0.98±0.07; p=N.S, n=6) (**Figure 4.3.1**); this was expected, due to the known constitutive expression of TLR-4 in other tissues.

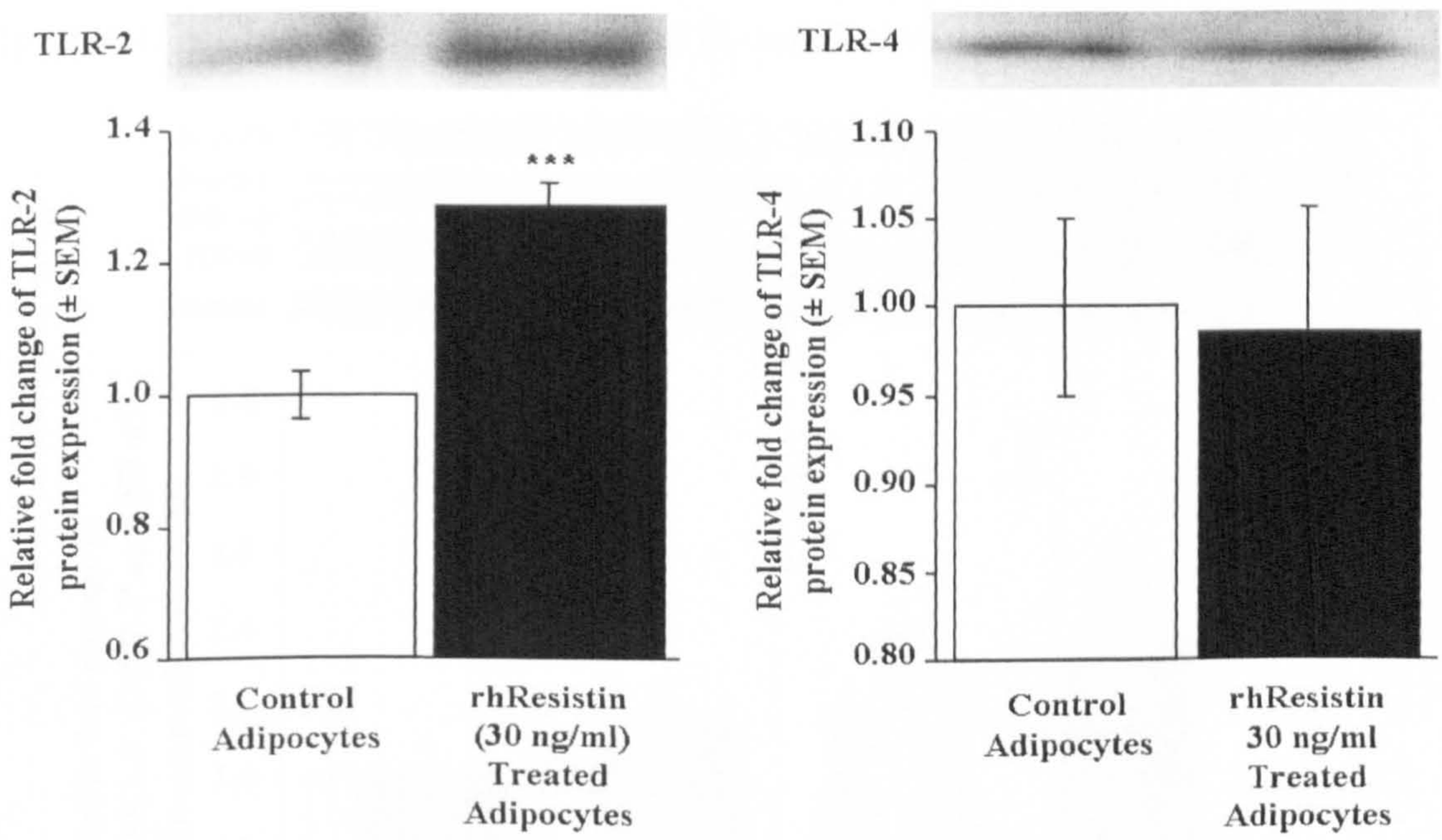


Figure 4.3.1 Relative fold-change of TLR-2 (n=6; ***, $p<0.001$) and TLR-4 (n=6; p=N.S) protein expression in AbSc adipocytes treated with rhResistin. Expression for each component is shown as relative fold difference compared to their matched control untreated adipocytes. Equal protein loading was determined by α-tubulin.

4.3.2 The Effect of Resistin on Key Components of the NF-κB Pathway & Insulin Signalling Cascade in Isolated AbSc Adipocytes.

When examining the effect of rhResistin on components of the NF-κB pathway in Abd Sc adipocytes, rhResistin was shown to stimulate MyD88 expression in the isolated Sc adipocytes (Control: 1.00±0.13; MyD88 50 ng: 1.80±0.04; ↑$p<0.01$, n=6) (**Figure 4.3.2**). Similarly, NF-κB (Control: 1.00±0.04; NF-κB 50 ng: 1.37±0.02; ↑$p<0.05$, n=4) expression was increased in response to rhResistin dose (**Figure 4.3.2**). Additionally, rhResistin was shown to further upregulate factors of the insulin signalling cascade; as both phosphospecific JNK1 (Control: 1.00±0.03; JNK1-P 50 ng: 1.29±0.05; ↑$p<0.05$, n=6) and phosphospecific JNK2 (Control: 1.00±0.08; JNK2-P 50 ng: 1.53±0.03; ↑$p<0.001$, n=6) expression was increased in response to rhResistin (**Figure 4.3.2**).

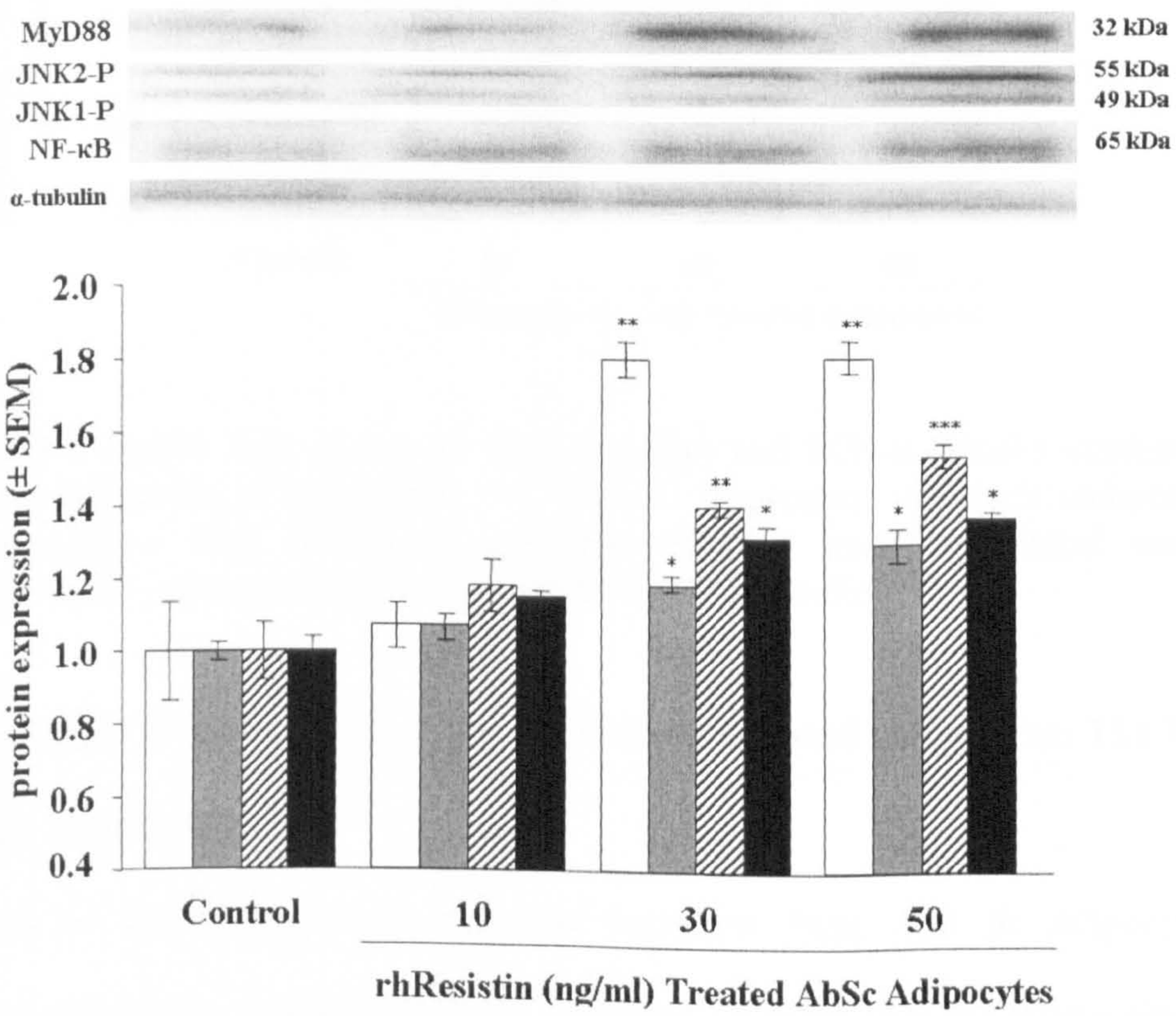


Figure 4.3.2 The relative fold change of MyD88 (white), JNK1-P (grey), JNK2-P (stripes) and NF-κB (black) expression in response to rhResistin in isolated AbSc adipocytes. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Expression for each component is shown as relative fold difference compared to their matched control untreated adipocytes only. Equal protein loading was determined by α-tubulin.

4.3.3 The Effect of Resistin on the IKK Complex.

When examining the expression of the catalytic subunits of the IKK complex, both IKK-β and IKK-α were upregulated in response to increasing rhResistin concentrations (Control: 1.00±0.04; IKK-β 50 ng: 1.17±0.03 ↑$p<0.01$, n=4) (Control: 1.00±0.06; IKK-α 50 ng: 1.50±0.02 ↑$p<0.01$, n=4) (**Figure 4.3.3**).

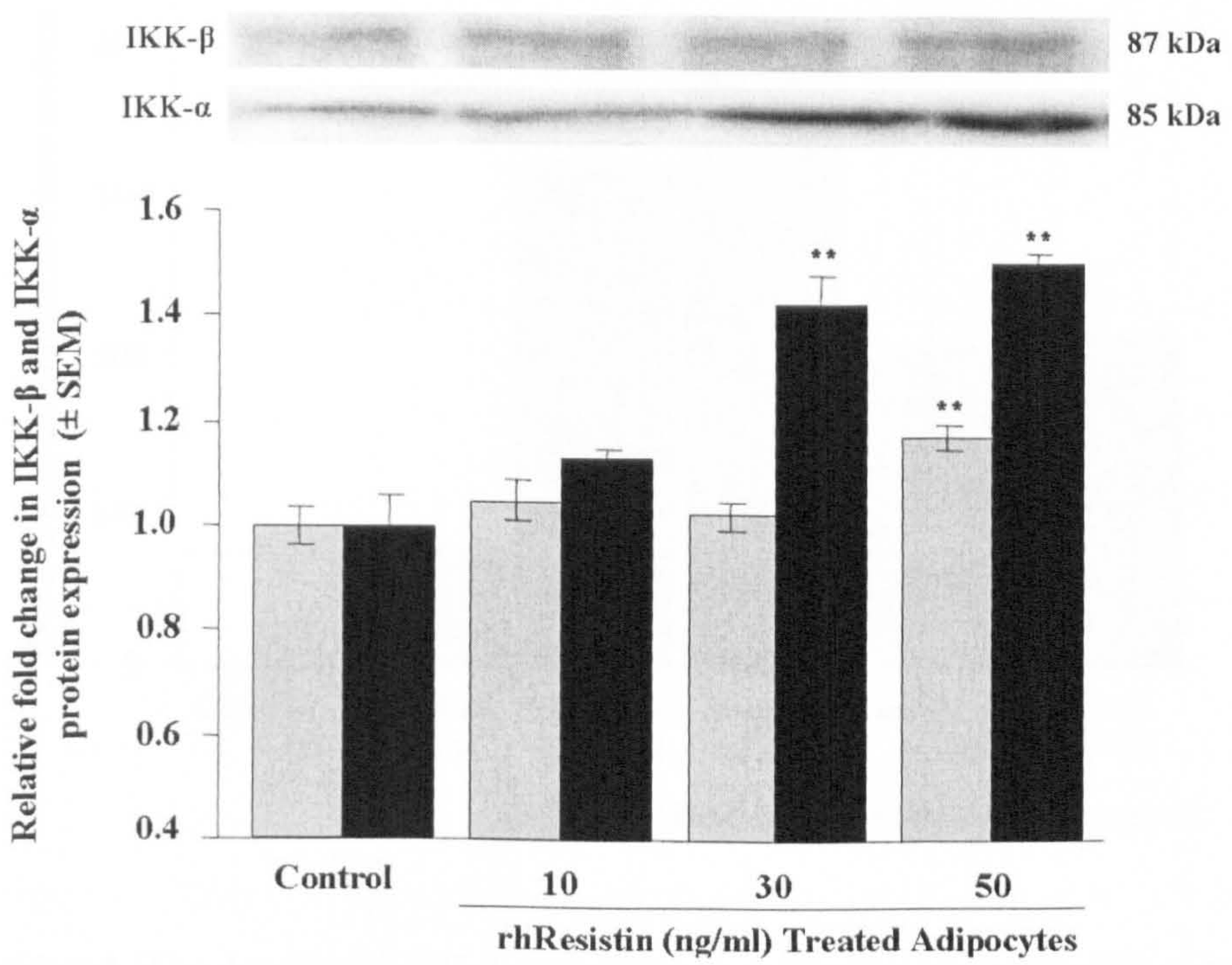


Figure 4.3.3 Relative fold change of IKK-β (grey) and IKK-α (black) expression in response to rhResistin in adipocytes. **, $p<0.01$. Expression for each component is shown as relative fold difference compared to their matched control untreated adipocytes. Equal protein loading was determined by α-tubulin.

4.3.4 Regulation of TNF-α & IL-6 Secretion from Isolated Adipocytes: The Effects of Resistin, Insulin & RSG.

Assessment of pro-inflammatory cytokine secretion from Abd Sc adipocytes in response to rhResistin, insulin and RSG treatment revealed that rhResistin alone (30 ng/ml), and in combination with insulin (10 nM), significantly increased the level of TNF-α secretion from AbSc adipocytes (Control: 74±10 pg/ml; rhResistin: 435±36.5 pg/ml; $p<0.001$). Additionally, RSG (10^{-8} M) was shown to significantly reduce this

resistin-stimulated increase in TNF-α secretion from Abd Sc adipocytes ($p<0.001$); following this reduction, TNF-α secretion levels still however, remained higher than the control ($p<0.01$) **(Figure 4.3.4.1)**.

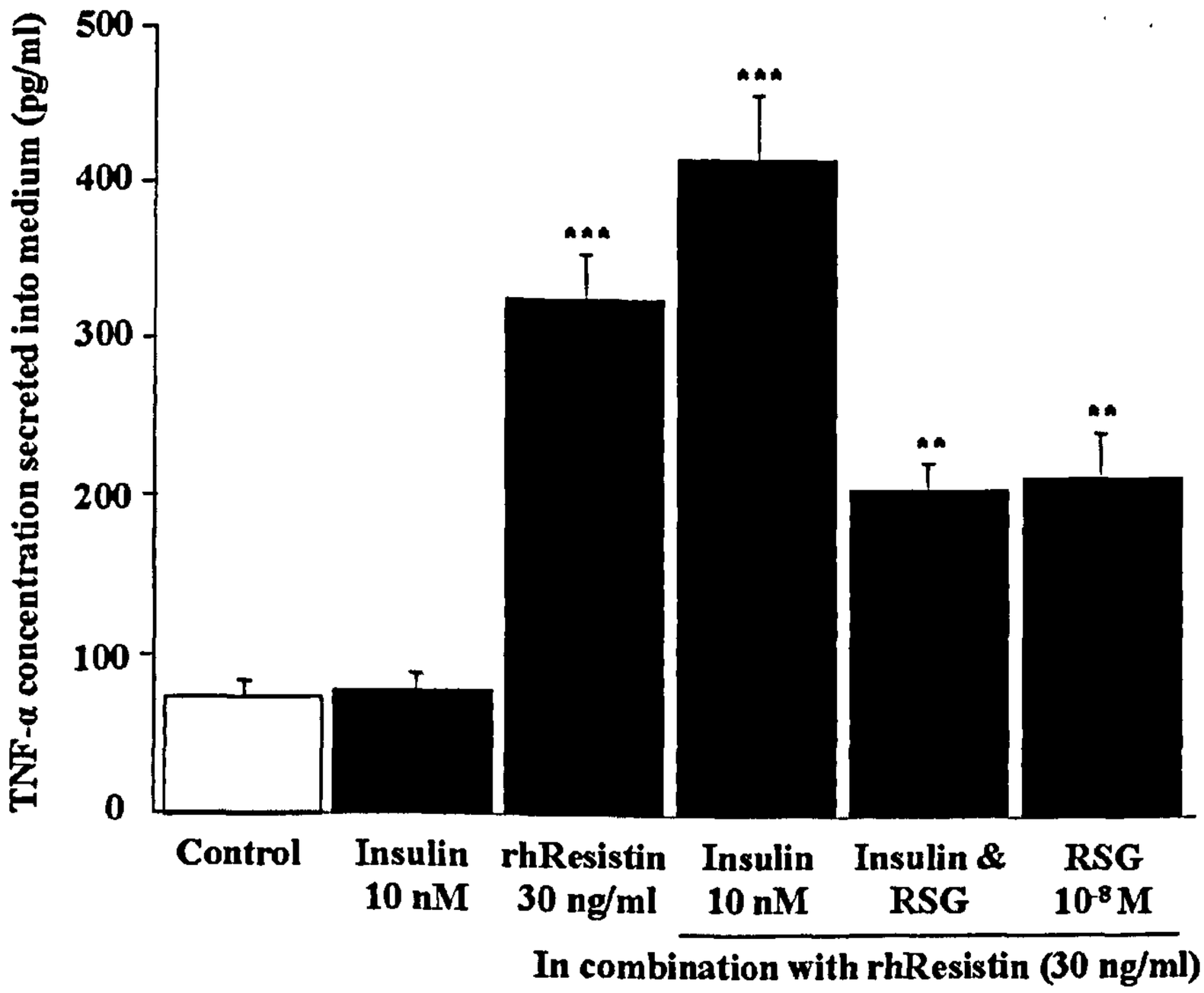


Figure 4.3.4.1 The level of TNF-α secretion in response to rhResistin alone, insulin alone or in combination with RSG, or RSG alone (n=8). Control samples were adipocytes maintained in medium in the absence of treatment. Values obtained with control samples were compared with those of resistin alone (***, $p<0.001$), with insulin (***, $p<0.001$) and RSG (**, $p<0.01$). A comparison of resistin in combination with insulin *versus* resistin, insulin and RSG was also performed. **, $p<0.01$.

Similar to the effect on TNF-α, rhResistin (30 ng/ml) and insulin (10 nM) significantly increased the level of IL-6 secretion from isolated AbSc adipocytes (Control: 1962±130 pg/ml; rhResistin: 2906.4±297.0 pg/ml; $p<0.01$). Furthermore, RSG was shown to reduce this resistin-induced increase in IL-6 secretion from the adipocyte cells (**Figure 4.3.4.2**).

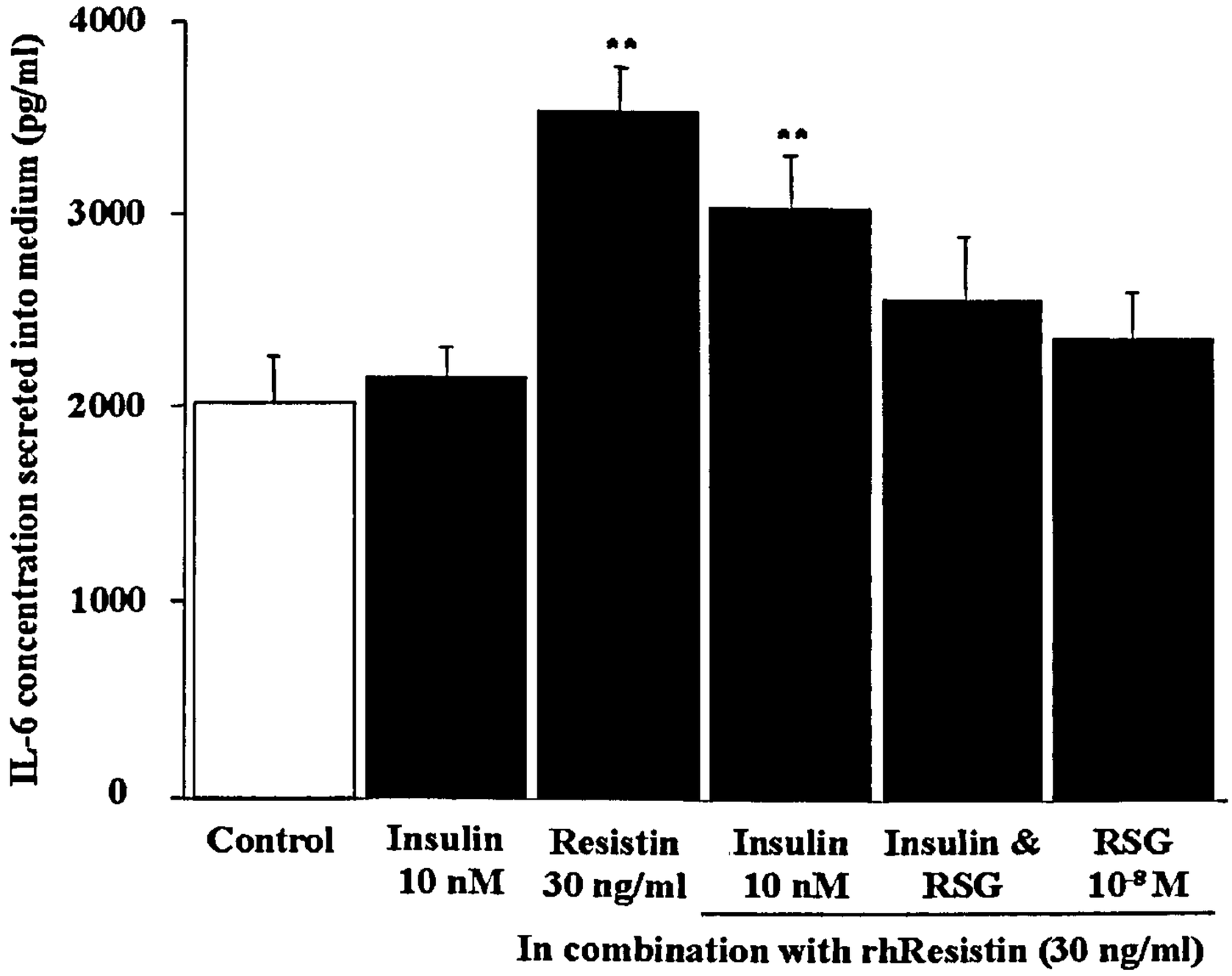


Figure 4.3.4.2 The level of IL-6 secretion in response to rhResistin alone, in combination with insulin and RSG, or RSG alone (n=8). Values obtained with controls were compared with those of resistin alone and in the presence of insulin. **, $p<0.01$.

4.3.5 A 'Direct' Effect of rhResistin on the Level of TNF-α & IL-6 secretion from Isolated AbSc Adipocytes.

To assess the specificity of resistin-induced effects, additional experiments were performed using monoclonal anti-resistin antibodies (Abs). The anti-resistin Abs were added to resistin-treated adipocytes prior to analysis of pro-inflammatory cytokine secretion from the isolated adipocytes. It was demonstrated that addition of anti-resistin Abs could significantly reduce the level of TNF-α secretion from isolated AbSc adipocytes (Control: 76.5±6.2 pg/ml; rhResistin (30 ng/ml): 89.2±4.6 pg/ml; Anti-resistin Abs (10 μg/ml): 71.5±5.9 pg/ml; $p<0.05$). A similar scenario was also demonstrated for the level of IL-6 secretion from AbSc adipocytes (Control: 362.0±22.6 pg/ml; rhResistin (30 ng/ml): 1115.5±40.6 pg/ml; Anti-resistin Abs (10 μg/ml): 351.5±55.9 pg/ml; $p<0.01$). This consequently reinforces the potential for

resistin exerting a significant impact on the level of cytokine secretion from human AbSc adipocytes.

4.3.6 The Effect of TNF-α & IL-6 on the Level of Resistin Secretion from Isolated Adipocytes: Feedback Mechanism Between Resistin, TNF-α & IL-6?

To establish whether a pro-inflammatory cytokine feedback mechanism exists within AbSc adipocytes, we further examined the level of resistin secretion from isolated adipocytes treated recombinant human TNF-α (rhTNF-α) and recombinant human IL-6 (rhIL-6). However, resistin secretion from adipocytes was unaffected by rhTNF-α, at any concentration up to 100 ng/ml (Control: 135±19 pg/ml; 10 ng/ml rhTNF-α: 129±15 pg/ml; 50 ng/ml rhTNF-α: 141±11 pg/ml; 100 ng/ml rhTNF-α: 116±11 pg/ml; n=12) **(Figure 4.3.6.1)**.

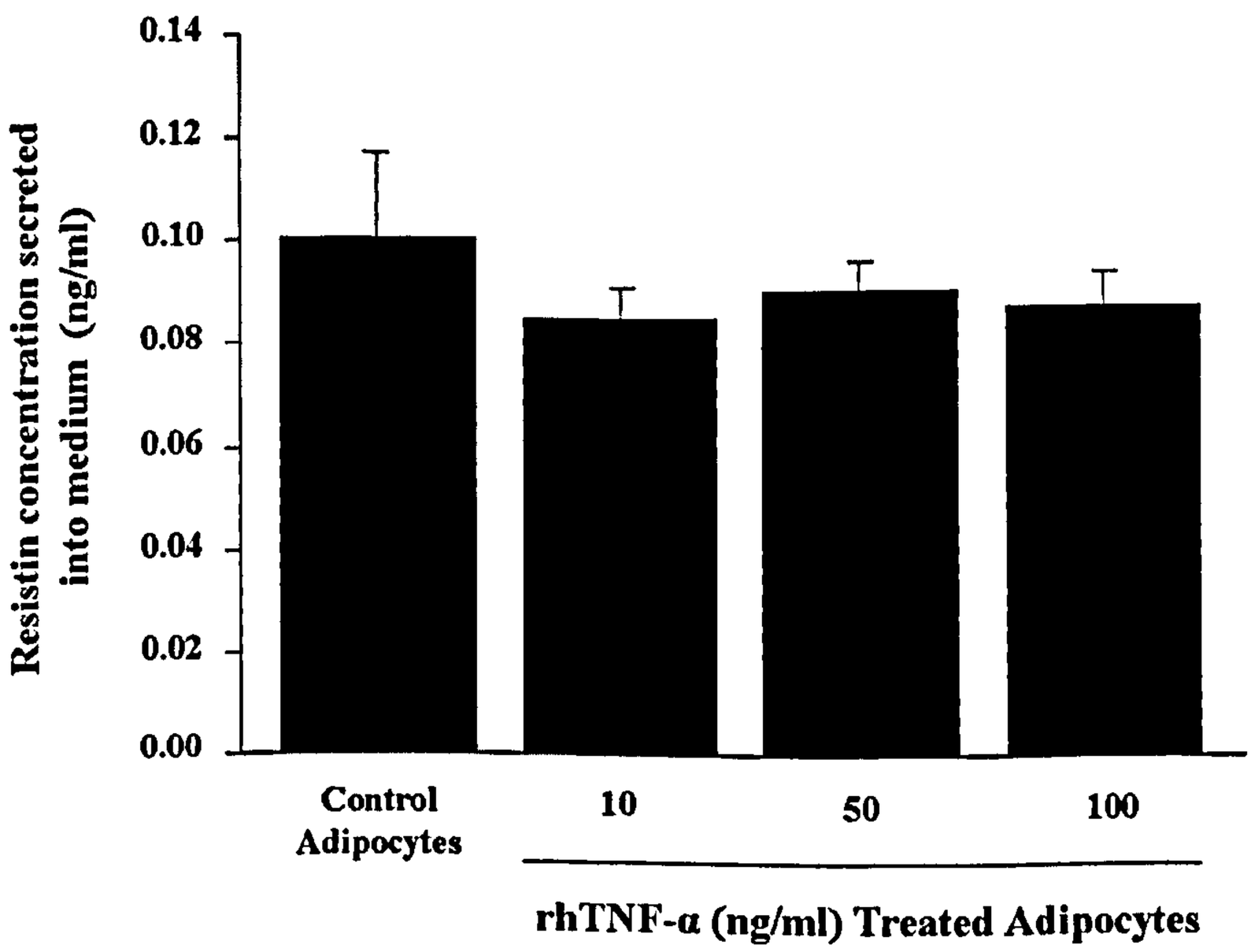


Figure 4.3.6.1 The level of resistin secretion from isolated AbSc adipocytes in response to increasing concentrations of rhTNF-α (n=12; p=N.S). Matched untreated AbSc adipocytes were used as controls.

Furthermore, rhIL-6 also had no significant effect on the level of resistin secretion from Abd Sc adipocytes (Control: 129±12 pg/ml; 10 ng/ml rhIL-6: 135±13 pg/ml; 50 ng/ml rhIL-6: 123±10 pg/ml; 100 ng/ml rhIL-6: 125±12 pg/ml; n=8) **(Figure 4.3.6.2)**.

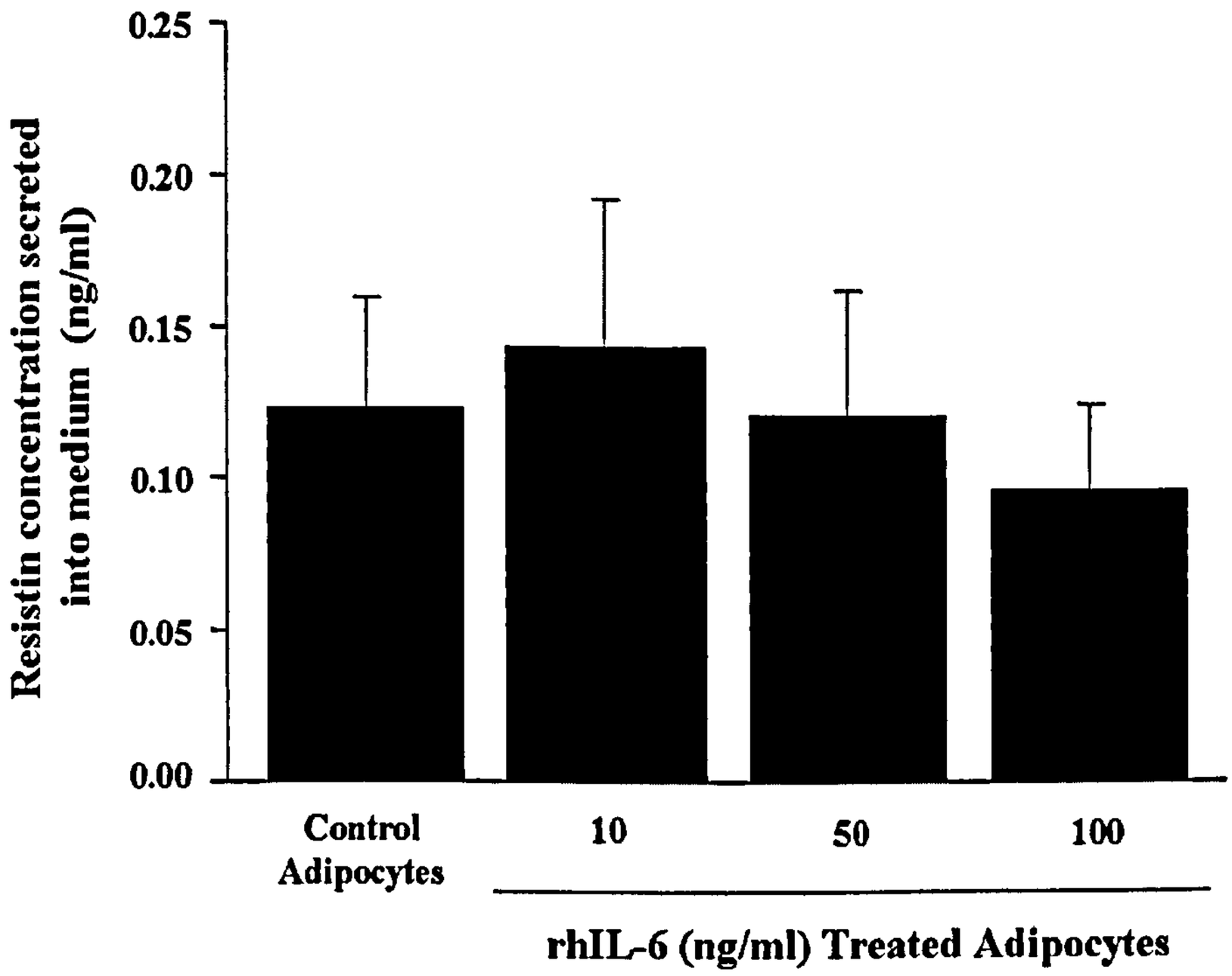


Figure 4.3.6.2 Resistin secretion from AbSc adipocytes in response to increasing concentrations of rhIL-6 (n=8; p=N.S). Control matched samples were adipocytes maintained in medium in the absence of treatment.

4.3.7 The Effects of NF-κB or JNK Inhibitors on the Level of Resistin Secretion from Isolated AbSc Adipocytes.

When examining the effects of JNK or NF-κB inhibitor on resistin secretion from isolated adipocytes, the level of resistin secretion was significantly reduced with NF-κB inhibitor treatment (Control: 83.1±20.5 pg/ml; NF-κB inhibitor: 61.6±16.6 pg/ml; n=7, $p<0.05$) **(Figure 4.3.7)**. Similarly, with the addition of JNK inhibitor, the level of resistin secretion from AbSc adipocytes was shown to significantly decrease (control: 101.5±19.2 pg/ml; JNK inhibitor: 77.0±11.4 pg/ml; n=8, $p<0.05$) **(Figure 4.3.7)**.

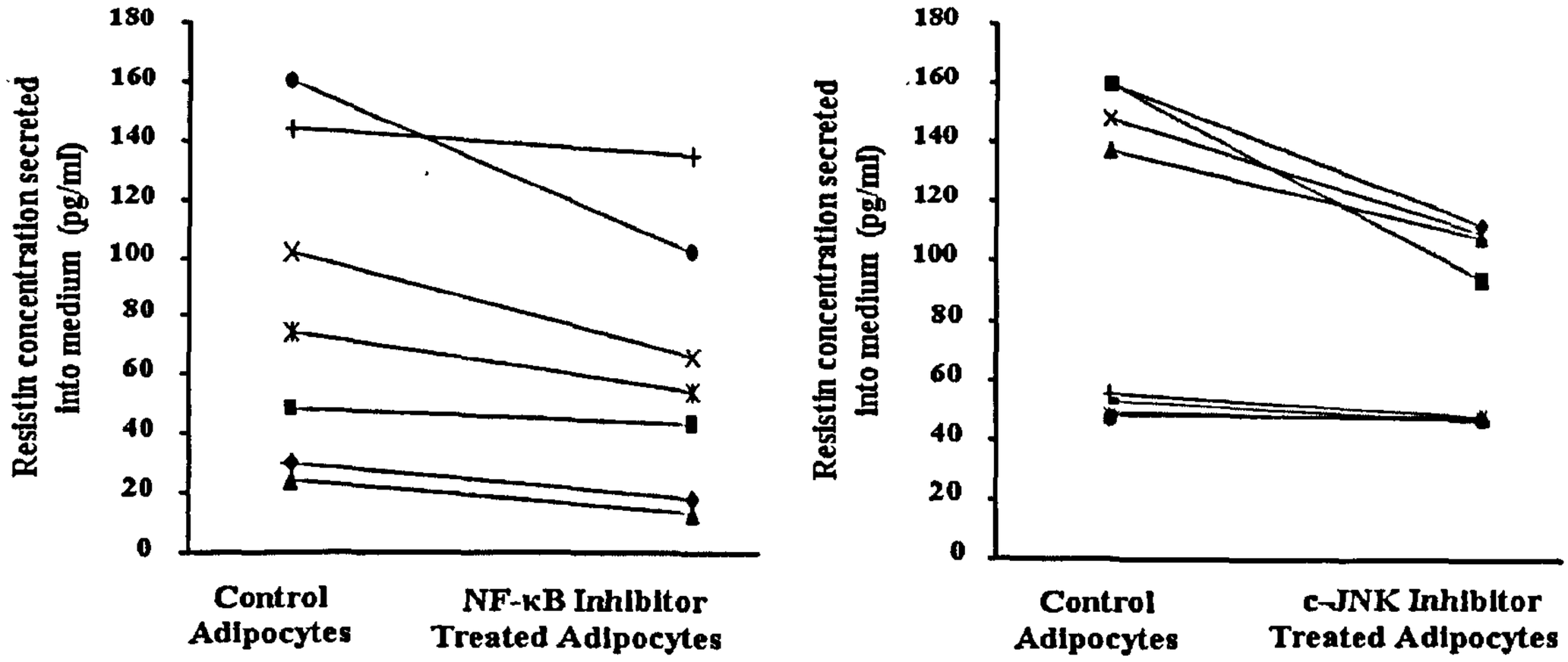


Figure 4.3.7 The level of resistin secretion from isolated AbSc adipocytes in response to NF-κB inhibitor (n=7; $p<0.05$) (left). Resistin secretion from AbSc adipocytes in response to JNK inhibitor (n=8; $p<0.05$) (right). In both figures, inhibitor treated AbSc adipocytes were compared to matched control untreated adipocytes from the same patient.

4.3.8 The Effects of NF-κB or JNK Inhibitors on the Level of Resistin Secretion from AbSc Adipose Tissue Explants.

The level of resistin secretion from AbSc adipose tissue explants was significantly reduced with NF-κB inhibitor treatment (Control: 1225.9±195.4 pg/ml; NF-κB inhibitor: 913.0±147.4 pg/ml; n=8, $p<0.05$) (**Figure 4.3.8**). However, no significant difference in the level of resistin secretion was observed for JNK inhibitor treated AbSc adipose tissue explants (Control: 125.9±63.9 pg/ml; JNK inhibitor: 205.7±59.8 pg/ml; n=8, p=N.S) (**Figure 4.3.8**).

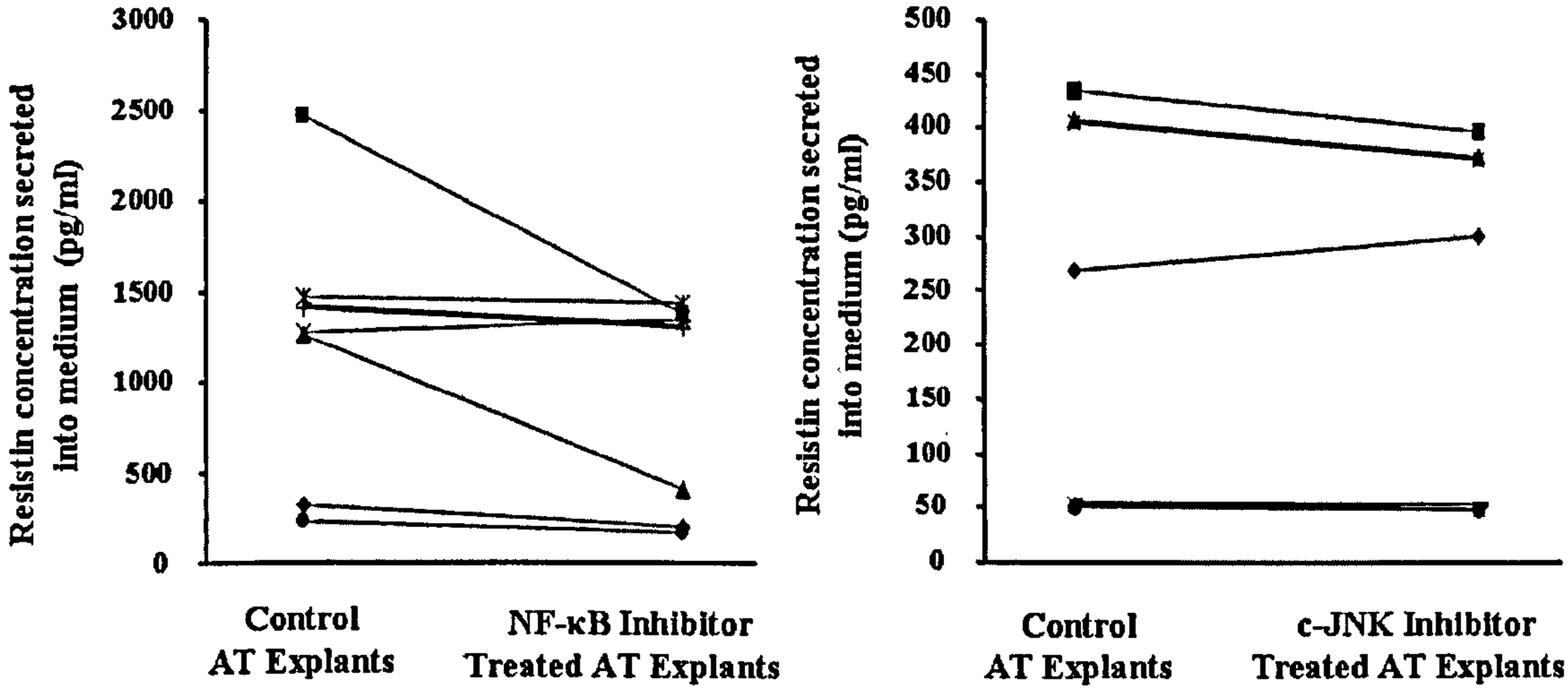


Figure 4.3.8 The level of resistin secretion from AbSc adipose tissue (AT) explants in response to NF-κB inhibitor (n=8; $p<0.05$) (left). Resistin secretion from AbSc AT explants in response to JNK inhibitor (n=8, p=N.S) (right). In both cases, inhibitor treated AbSc adipose tissue explants were compared to matched control untreated AbSc adipose tissue explants from the same patient.

4.4 Discussion.

This study demonstrated the pro-inflammatory actions of resistin in isolated human AbSc adipocytes. It was firstly determined that rhResistin stimulated protein expression of a key receptor in the innate immune pathway, TLR-2, in addition to two central metabolic and inflammatory kinases, JNK and IKK-β respectively. It was further established that rhResistin positively influenced the secretion of pro-inflammatory cytokines, such as TNF-α and IL-6, from human AbSc adipocytes. Furthermore, induction of cytokine secretion by rhResistin is attenuated by the insulin sensitiser, RSG. Collectively, these findings implicate resistin in the stimulation of pro-inflammatory cytokine release from human AbSc adipocytes; additionally, this further highlights human adipose tissue as an important site for the progression of low-grade inflammation.

Whilst a more pro-inflammatory role for resistin is emerging in humans, the metabolic actions of resistin remain uncertain. Rodent studies implicate the liver as the major physiological target of resistin action; as administration of exogenous resistin was shown to impair glucose tolerance and cause hepatic insulin resistance (Rajala *et al.* 2003). Similarly, adenovirus mediated hyper-resistinemia was shown to abrogate hepatic and peripheral insulin action (Sato *et al.* 2005). Conversely, resistin null mice exhibit low fasted blood glucose levels, due to reduced hepatic glucose production (Banerjee *et al.* 2004). Moreover, *in vitro* adipocyte studies highlight that resistin impairs insulin-stimulated glucose uptake (Steppan *et al.* 2005) and the insulin signalling cascade itself (Graveleau *et al.* 2005). It was additionally reported that human resistin has properties similar to its murine counterpart; whereby mouse and human resistin impair glucose transport (Graveleau *et al.* 2005).

To address the potential mechanism of resistin action in human AbSc adipocytes, studies examined whether rhResistin could influence the protein expression of key components of the innate immune pathway and insulin signalling cascade. It was observed that resistin upregulated the expression of TLR-2, MyD88 and NF-κB in AbSc adipocytes. When examining the key intermediate activating NF-κB, the IKK complex, protein expression of the catalytic subunits IKK-β and IKK-α increased in response to rhResistin treatment. Interestingly, rhResistin also upregulated phosphospecific JNK1 and JNK2 protein expression, suggesting NF-κB activation may overlap into a JNK-mediated pathway. Such a role for JNK as a mediator of NF-κB activation has been identified in macrophages and alveolar epithelial cells (Li *et al.* 2003; Roeder *et al.* 2004), consistent with crosstalk between metabolic and inflammatory pathways. Alternatively, elevated TNF-α and IL-6 levels induced by resistin may activate JNK and NF-κB systems, rather than via a direct effect by resistin.

To further examine the importance of the NF-κB and JNK signalling on resistin action in human adipocytes, cells were treated with NF-κB or JNK inhibitors. It was demonstrated that by inhibiting either NF-κB or JNK, the level of resistin secretion from isolated AbSc adipocytes was significantly reduced, thus suggesting a potential overlap of resistin function in both inflammatory and insulin signalling pathways. Furthermore, whilst NF-κB inhibition appeared to reduce the level of resistin secretion from AbSc adipose tissue explants, no effect was observed on resistin secretion with JNK inhibitor treatment. Consequently, although an overlap between NF-κB and JNK systems in resistin action is implicated for isolated AbSc adipocytes, resistin may have more prominent effects via the NF-κB pathway in whole AbSc adipose tissue explants. The significance of the NF-κB pathway for resistin-induced inflammatory effects

within human peripheral-blood mononuclear cells has previously been highlighted (Bokarewa *et al.* 2005).

In vitro studies further demonstrated that resistin increases the level of TNF-α and IL-6 secretion from human isolated adipocytes. This is consistent with recent reports, whereby human resistin increased TNF-α and IL-12 secretion from human macrophages (Silswal *et al.* 2005) and further, stimulated the secretion of pro-inflammatory cytokines TNF-α, IL-6 and monocyte chemoattractant protein-1 from 3T3-L1 adipocytes (Fu *et al.* 2006). This study also determined that the addition of anti-resistin Abs to rhResistin-treated adipocytes significantly reduces the level of TNF-α and IL-6 secretion from AbSc adipocytes. This therefore suggests that resistin may directly contribute to an altered cytokine status and thus promote inflammation. It is widely acknowledged that systemic levels of TNF-α and IL-6 are elevated in states of obesity and T2DM (Hotamisligil *et al.* 1995). Therefore, future analysis examining cytokine secretion levels in response to rhResistin in chronically obese, pre-and post TZD-treated patients, may further elucidate the mechanistic action of resistin in obesity-related inflammation.

The current literature documents the metabolic effects of resistin in the human macrophages (Rae *et al* 2006) and 3T3-L1 adipocytes (Steppan *et al* 2004, Fu Y *et al* 2006). Similarly, the pro-inflammatory effects of resistin are reported in human macrophages (Silswal *et al* 2005) and 3T3-L1 adipocytes (Fu Y *et al* 2006). Our study further demonstrated that resistin exerts pro-inflammatory effects in metabolically active cells i.e. human isolated adipocytes. Resistin may thus function in adipocytes to influence both metabolic and pro-inflammatory changes, suggesting that the effects of resistin are to some extent linked. Such a duality in function for resistin may be a

consequence of the crosslink initially proposed between metabolic and inflammatory pathways in adipocytes and immune cells (Weisberg *et al.* 2003; Xu *et al.* 2003). Collectively, these studies suggest a potential overlap between metabolic and immune functions for human resistin.

Finally, it is known that hyper-restinemia contributes to an inflammatory response. rhResistin was shown to alter the level of cytokine release from cultured adipocytes when compared to control. Insulin was utilised to observe the effects of RSG in this system; as such, this study demonstrated that the PPAR-γ agonist, RSG, attenuated this resistin-induced secretion of TNF-α and IL-6 from AbSc adipocytes. Although the mechanisms for this are as yet unclear, it is known that the resistin gene promoter contains a PPAR-γ binding site (Patel *et al.* 2003), through which RSG may coordinate the recruitment of transcriptional co-repressors (Aranda *et al.* 2001), thereby suppressing resistin expression at the genetic level. However, this did not appear to be the mechanism through which the observations in this study were mediated, as exogenous resistin was used to stimulate cytokine production. This suggests that RSG can act downstream of the resistin promoter to mitigate resistin-mediated TNF-α and IL-6 stimulation, potentially via NF-κB (Ye *et al.* 2004).

In conclusion, this study demonstrated that resistin can influence the secretion of pro-inflammatory cytokines from isolated human AbSc adipocytes. The intracellular mechanism for such positive mediation of resistin on TNF-α and IL-6 release appears to act via the NF-κB pathway and furthermore, potentially influences the insulin signalling cascade. Consequently, the elevated levels of pro-inflammatory factors,

enhanced by resistin, may thus contribute to the pro-inflammatory milieu proposed in obesity-related insulin resistance and T2DM.

CHAPTER 5

Resistin & Bacterial Endotoxin in Sub-clinical Inflammation Associated with Childhood & Adolescent Obesity

5.1 Introduction.

The prevalence of childhood obesity and its related co-morbidities are increasing at an alarming rate worldwide (Hedley *et al.* 2004), to such an extent that obesity is now a worldwide paediatric health risk factor (Freedman *et al.* 2004). Over the past decade, 20% of children are now classified as overweight in Europe (Lobstein *et al.* 2004). Obesity is an important early risk factor for much of adult morbidity and mortality (Dietz 1998). In parallel to this, the incidence of obesity-related T2DM is rising dramatically (Weiss *et al.* 2005). In particular, there is an increased number of young obese populations diagnosed with the metabolic syndrome (MS), which at present, affects a quarter of overweight children and adolescents (Invitti *et al.* 2003; Duncan *et al.* 2004) and worsens with the degree of adiposity. The MS is manifested by the coexistence of central obesity, hypertension, dyslipidemia, insulin resistance and glucose intolerance; all of which are risk factors for coronary heart disease and T2DM (Ninomiya *et al.* 2004). Many of these metabolic and cardiovascular complications of obesity may be present in as many as 30% of obese adolescents (Cook *et al.* 2003). Similarly, circulating levels of low-grade inflammatory markers are elevated with increasing childhood adiposity, such as TNF-α, CRP and PAI-1. Moreover, decreased systemic levels of adiponectin have also been associated with MS (Ryo *et al.* 2004; Dandona *et al.* 2005). As such, childhood obesity and its later progression to T2DM are therefore now associated with sub-clinical inflammation and increased pro-inflammatory cytokine production.

The adipokine resistin, a member of a cysteine-rich RELM family of protein (Steppan *et al.* 2001), has demonstrated a pertinent role in regulating fasting glucose level (Banerjee *et al.* 2004) and reducing insulin sensitivity *in vivo* (Pravenec *et al.* 2003; Satoh *et al.* 2004) and *in vitro* (Moon *et al.* 2003; Steppan *et al.* 2005). Recent studies

have further implicated human resistin as a key player in several inflammatory processes (Lehrke *et al.* 2004; Bokarewa *et al.* 2005; Silswal *et al.* 2005). As previously detailed, several studies have documented contradictory findings on resistin in relation to adult obesity. Supportive findings include increased resistin expression (McTernan *et al.* 2002; Degawa-Yamauchi *et al.* 2003; Pagano *et al.* 2005) and, elevated systemic concentrations (Azuma *et al.* 2003; Valsamakis *et al.* 2004) in adult obesity. However, others report no changes in resistin levels following weight loss (Lee *et al.* 2003; Vendrell *et al.* 2004). Whilst is it apparent that the incidence of childhood and adolescent obesity is increasing, new questions are emerging in the field of resistin; does resistin follow a similar pattern of expression in juvenile obesity and insulin resistance as in adult obesity? To date, limited studies have addressed the role of resistin as a potential marker of juvenile obesity (Gerber *et al.* 2005; Reinehr *et al.* 2006). Such studies however, did not support a relationship between resistin, insulin resistance index and weight status in children (Gerber *et al.* 2005; Reinehr *et al.* 2006). Further studies are therefore required to fully establish whether resistin is a critical participant in childhood and adolescent obesity.

Another adipokine, adiponectin, is a protein secreted exclusively by adipocytes (Scherer *et al.* 1995), which possesses anti-diabetic, anti-inflammatory and anti-atherogenic properties (Trujillo *et al.* 2005). Both *in vivo* and *in vitro* studies have revealed that adiponectin decreases the production of pro-inflammatory cytokines and enhances hepatic insulin action (Ouchi *et al.* 2003; Brakenhielm *et al.* 2004; Goldstein *et al.* 2004; Shetty *et al.* 2004; Kadowaki *et al.* 2005). Circulating adiponectin levels are decreased in obesity, T2DM and coronary artery disease and, are further negatively correlated with inflammatory markers and the MS (Cnop *et al.* 2003; Engeli *et al.* 2003; Schulze *et al.* 2005). Finally, hypoadiponectinemia is now considered a

biomarker of insulin resistance, associated with an increased risk of developing T2DM (Choi *et al.* 2004; Duncan *et al.* 2004; Langenfeld *et al.* 2004; Kadowaki *et al.* 2005).

Whilst it is acknowledged that sub-clinical inflammation may integrate obesity and its progression to T2DM, the underlying mechanisms of this association are unclear. Previous studies have suggested that the bacterial endotoxin, LPS, potentially derived from commensal bacteria in the human gastrointestinal (GI) tract, may contribute to sub-clinical inflammation. Human adipose tissue has been demonstrated to express Toll-like receptors (Creely *et al.* 2006), which initiate an innate immune inflammatory response in the presence of endotoxin, thus producing inflammatory cytokines, such as TNF-α, IL-6 and PAI-1. Elevated systemic levels of endotoxin may thus exacerbate inflammatory cascades and contribute to the abnormal circulating levels of adipocytokines in obesity and a pro-inflammatory or pro-thrombotic milieu (Dandona *et al.* 2005). In this respect, endotoxin may potentially serve as one of many factors modulating the progression of obesity-associated inflammation and T2DM. Several studies using cohorts of obese children have demonstrated elevated circulating levels of PAI-1 and CRP, with data further highlighting an association with central adiposity and an increased risk of developing insulin resistance, T2DM, thrombosis and fibrosis (Sudi *et al.* 2001; Invitti *et al.* 2004; Ford *et al.* 2005; Valle *et al.* 2005). Moreover, systemic levels of markers of vascular injury and atherogenesis, such as PAI-1 and soluble intercellular adhesion molecule type-1 (sICAM-1), are increased in childhood and adolescent obesity (Halle *et al.* 2004; O'Brien *et al.* 2006; Syrenicz *et al.* 2006).

The aims of this study were 1) Firstly, to examine the circulating levels of resistin in a cohort of children and adolescents with varying degrees of obesity, with particular emphasis on gender differences. 2) Secondly, compare resistin levels in the following

sub-groups: (i) obese only, (ii) insulin resistant only, (iii) high triglyceride and low HDL and, (iv) MS only. 3) Finally, examine bacterial endotoxin as a potential mediator of sub-clinical inflammation in childhood obesity, by investigating the relationship between endotoxin and various markers of inflammation.

5.2 Subjects, Methods & Materials.

5.2.1 Subjects.

A total of 114 Caucasian obese children and adolescents with varying degrees of obesity were analysed in this study (BMI: 35.0±5.2(SD) kg/m^2; age: 14±2 yrs; female subjects (n=74); male subjects (n=40)). All subjects were therefore above the age and sex adjusted 97th BMI percentile, which defines obesity according to the Italian BMI charts (Cacciari *et al.* 2002) and had an age range of 8-18 yrs. This study was approved by the Ethics Committee of the Italian Institute and informed consent was obtained from all subjects and their parents. All subjects underwent an oral glucose tolerance test (1.75 g/kg, up to a maximum of 75 g glucose in 250 ml of water) following an overnight fast. Plasma samples were drawn at baseline, after 30 min and 120 min, for determination of plasma glucose and insulin concentration. Categorisation of glucose tolerance status was made using the WHO criteria (Alberti *et al.* 1998). The impaired fasting glucose was defined by fasting glucose levels ≥5.6 mmol/l (Genuth *et al.* 2003). Blood samples were drawn for measurement of resistin, endotoxin, adiponectin, markers of inflammation and cardiovascular disease (CVD). Blood pressure measurements were taken as previously described (Invitti *et al.* 2003). Insulin resistance was measured by HOMA-IR (fasting insulin x fasting glucose/22.5) (Matthews *et al.* 1985). No data was available in relation to smoking status.

5.2.2 Biochemical Measurements.

Resistin concentrations were determined using ELISA (R&D Systems Europe Ltd., Abingdon, UK), with a sensitivity of 10 pg/ml and an intra- and interassay CV of 4.7% and 8.4%, respectively. Serum endotoxin levels were assayed using a Chromogenic Limulus Amebocyte Lysate test (Cambex, New Jersey, USA), with a sensitivity of 0.1 EU/ml and an intra- and interassay CV of 3.9±0.46 and 9.6±0.75 respectively. Previous

studies further confirmed the specificity of the endotoxin assay (Creely *et al.* 2006). This study utilised Linco multiplexed CVD biomarker immunoassays (Linco Research, Missouri, USA) to examine the levels of the following inflammatory and CVD risk markers: TNF-α, PAI-1, CRP, sICAM-1, soluble vascular cell adhesion molecule-1 (sVCAM-1), matrix metalloproteinase-9 (MMP-9), myeloperoxidase (MPO), vascular endothelial growth factor (VEGF) and adiponectin. This immunoassay had a sensitivity of 16-50,000 pg/ml for MMP-9, MPO and PAI-1 and further, a sensitivity of 80-250,000 pg/ml for sICAM-1, and adiponectin; with an intra- and interassay CV of 4.5-12.3% and 8.5-16.3%, respectively. Plasma glucose was measured using an automated glucose analyser (Roche Diagnostics, Mannheim, Germany). Serum insulin levels were measured by a chemiluminescent assay (DPC, Los Angeles, USA) with a sensitivity of 14.3 pmol/l and intra- and interassay CV of 3.7% and 6.7%, respectively.

5.2.3 Statistical Analysis.

All analyses were performed using statistical software (SPSS, version 14; Woking, UK). Variables that were not normally distributed were log transformed. Differences between groups were calculated using a Student's t-test for independent samples. A Pearsons' correlation analysis was used to analyse bivariate relationships between variables such as resistin, endotoxin and, markers of inflammation and vascular injury. Data were expressed as mean±SD. A p-value <0.05 was considered statistically significant.

5.3 Results.

5.3.1 Sexual Dimorphism of Serum Resistin Levels in Childhood & Adolescent Obesity.

Analysis of total resistin levels with regards to gender, revealed that girls demonstrated significantly higher resistin concentrations than boys (girls: 28.85±22.65 ng/ml resistin, n=74; boys: 20.66±14.39 ng/ml resistin, n=40; $p<0.05$), as demonstrated in **Figure 5.3.1** below.

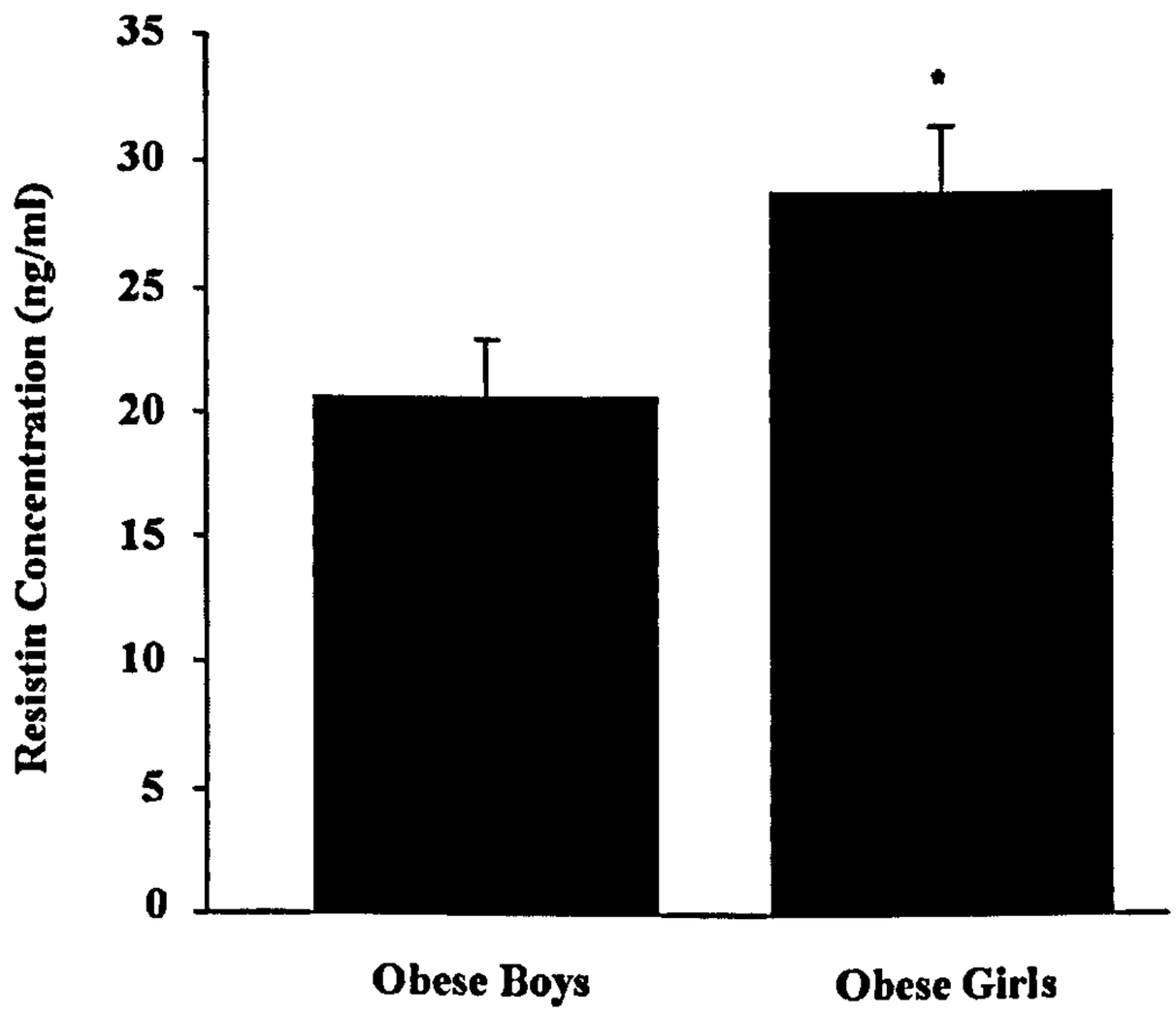


Figure 5.3.1 Comparison of circulating resistin levels (ng/ml) in obese girls (n=74) and obese boys (n=40).

In this cohort of subjects, the average circulating resistin levels were determined as 26.40±20.79 (SD) ng/ml (n=114). Further examination of resistin levels in sub-groups within this cohort, revealed that resistin concentrations were significantly higher in subjects with obesity only (45.06±23.49 ng/ml; n=30), in comparison to subjects with insulin resistance (25.80±15.24 ng/ml; $p<0.001$; n=42), high triglyceride and low LDL levels (11.47±6.26 ng/ml; $p<0.001$; n=24) or subjects with MS (16.61±16.97 ng/ml; $p<0.001$; n=18).

5.3.2 Clinical & Biochemical Characteristics: Gender-differences in Adiponectin & Bacterial Endotoxin Levels.

From the cohort, further biochemical analysis was performed on 55 subjects; of which, 21 male subjects and 34 BMI and age-matched female subjects were analysed. Clinical and biochemical characteristics of male or female obese subjects are provided in **Table 5.3.2** below.

Clinical or Biochemical Characteristic	Male Subjects (±SD)	Female Subjects (±SD)	p value
Age, years	14±3	14±2	-
BMI, kg/m^2	34.0±5.1	35.6±5.3	-
Fasting glucose, mmol/l	4.4±0.3	4.5±0.5	N/S
2 h glucose, mmol/l	6.0±1.0	5.7±1.0	N/S
HOMA-IR	2.6±1.2	3.3±2.1	N/S
Endotoxin, EU/ml	10.1±5.4	5.3±3.7	$p<0.01$**
Adiponectin, μg/ml	13.4±5.8	16.7±5.4	$p<0.05$*
CRP, μg/ml	7.5±8.3	9.0±7.5	N/S
TNF-α, pg/ml	5.0±2.5	5.9±5.8	N/S
PAI-1, ng/ml	27.0±10.7	26.0±15.7	N/S
sICAM-1, ng/ml	110.4±37.4	103.1±47.9	N/S
Systolic BP, mmHg	121.0±10.8	119.1±8.5	N/S
Diastolic BP, mmHg	74.0±11.2	72.7±8.5	N/S

Table 5.3.2 Clinical and biochemical characteristics for obese, BMI and age-matched male (n=21) and female (n=34) subjects. Data are expressed as mean±SD. Any significant differences in data between male and female subjects are highlighted. *, $p<0.05$, **, $p<0.001$.

Serum concentration data demonstrated that male subjects had significantly higher levels of endotoxin when compared with female subjects (Males: 10.1±5.4 EU/ml; Females: 5.3±3.7 EU/ml; $p<0.01$). In contrast, female subjects had significantly higher levels of adiponectin in circulation than male subjects (Males: 13.4±5.8 μg/ml; Females: 16.7±5.4 μg/ml; $p<0.05$). No significant differences were observed in HOMA-IR, blood pressure or several markers of inflammation and CVD between the sexes.

5.3.3 Correlation of Resistin with Adiponectin, Markers of Inflammation & CVD in Childhood & Adolescent Obesity.

Further analysis revealed that circulating resistin levels did not significantly correlate with any markers of inflammation (CRP: $p=0.751$, $r=-0.044$; TNF-α, $p=0.111$, $r=-0.221$; $n=55$) vascular injury (PAI-1: $p=0.233$, $r=-0.163$; sICAM-1, $p=0.166$, $r=-0.189$; $n=55$), insulin resistance index (HOMA-IR: $p=0.220$, $r=-0.171$; $n=55$) or adiponectin ($p=0.369$, $r=-0.123$; $n=55$). Similar results were observed when further examining resistin levels in relation to such parameters in specific sub-groups within this cohort: obesity only, insulin resistance only, high triglyceride and low LDL levels and, MS only. Furthermore, in terms of gender, resistin levels did not significantly correlate with any of the above mentioned markers of inflammation, vascular injury or insulin resistance index in either male subjects ($n=21$) or female subjects ($n=34$).

5.3.4 Correlation of Endotoxin with Markers of Inflammation & CVD in Childhood & Adolescent Obesity.

In this study, bacterial endotoxin was investigated as a potential mediator/marker of inflammation in childhood and adolescent obesity. Circulating endotoxin levels were shown to correlate with several parameters of inflammation and vascular injury; these

included TNF-α (p=0.020, r=0.327; n=50), PAI-1 (p<0.001, r=0.529; n=52), sICAM-1 (p=0.004, r=0.397; n=52), MMP-9 (p<0.001, r=0.457; n=52), MPO (p=0.016, r=0.331; n=52) and VEGF (p=0.003, r=0.419; n=48) (**Figure 5.3.4A-F**). However, no significant correlation was noted between circulating endotoxin levels with CRP (p=0.744, r=-0.046; n=52), sVCAM (p=0.623, r=0.070; n=52) or adiponectin (p=0.110, r=-0.224; n=52) levels. With regards to gender, endotoxin levels did not significantly correlate with the above mentioned markers of inflammation, vascular injury or insulin resistance index in male subjects or female subjects separately (data not shown).

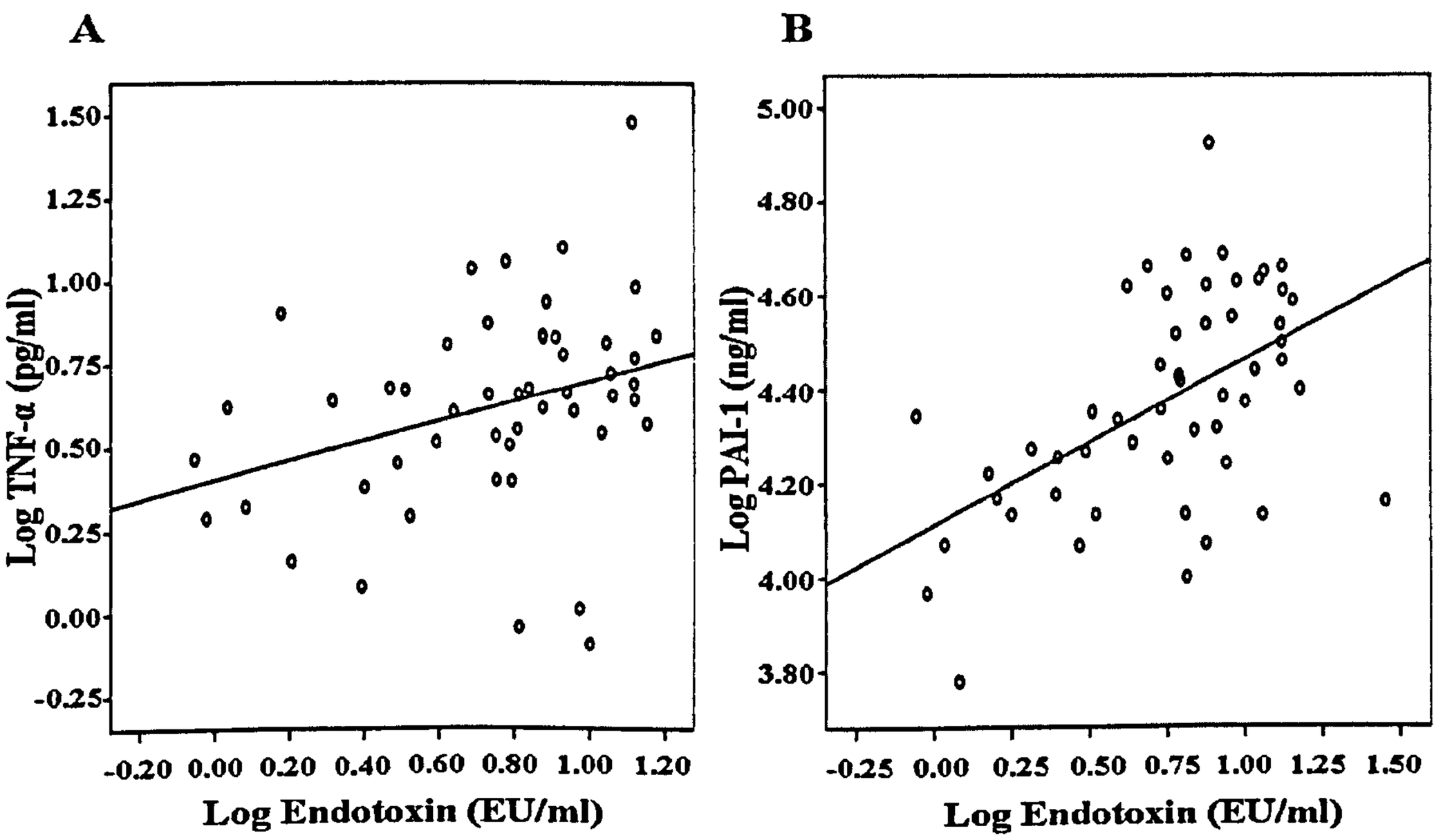


Figure 5.3.4 Correlation between log endotoxin (EU/ml) and (A) log TNF-α (pg/ml) p=0.020, (B) log PAI-1 (ng/ml) p<0.001.

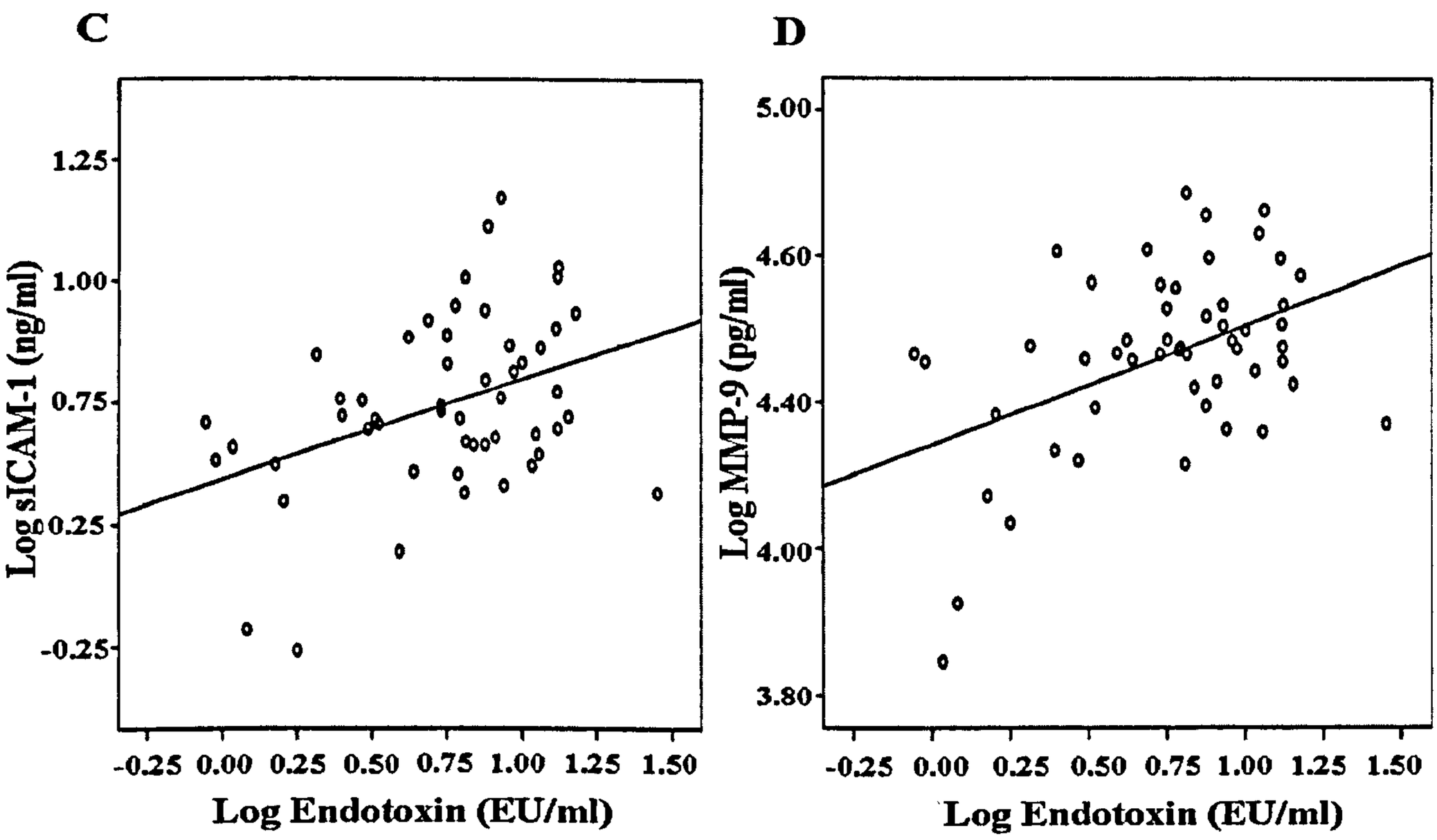


Figure 5.3.4 Correlation between log endotoxin (EU/ml) and (C) log sICAM-1 (ng/ml) p=0.004, (D) log MMP-9 (pg/ml) p<0.001, (E) log VEGF (pg/ml) p=0.003 and, (F) log MPO (pg/ml) p=0.016 in 55 obese BMI and age-matched children and adolescents.

5.4 Discussion.

This study firstly demonstrated that circulating resistin levels in childhood and adolescent obesity are subjected to sexual dimorphism. Such gender differences in resistin levels coincide with previously documented observations; girls were shown to have higher resistin concentrations than boys in two independent studies (Gerber *et al.* 2005; Reinehr *et al.* 2006). Furthermore, such gender regulation of resistin is in accordance with studies carried out in adults (Yannakoulia *et al.* 2003). This study further demonstrated that resistin concentrations did not significantly correlate with any markers of inflammation or CVD, HOMA-IR or adiponectin in any of the sub-groups within this cohort, suggesting that resistin alone may not play a significant role in childhood or adolescent obesity. On the other hand, such results may have arisen due to all the subjects in this cohort being obese, with additional MS risk factors subdividing them. Therefore, as all subjects were at a heightened MS risk, the differences noted in resistin serum levels may be minimal. Furthermore, serum resistin concentrations in this cohort of subjects were high in comparison to previously documented resistin levels (McTernan *et al.* 2003; Gerber *et al.* 2005). Such high serum resistin levels may be a consequence of the additional risk factors associated with subjects in this cohort; alternatively, the sensitivity of resistin assays may affect results. Although no studies to date have reported resistin in relation to markers of inflammation or CVD in childhood obesity, similar findings were reported whereby circulating resistin concentrations did not correlate with any parameters of insulin resistance (Gerber *et al.* 2005; Reinehr *et al.* 2006). Such findings suggest that resistin may only have a relevant function in adult human obesity, as highlighted by several studies (McTernan *et al.* 2002; Azuma *et al.* 2003; Degawa-Yamauchi *et al.* 2003; McTernan *et al.* 2003; Valsamakis *et al.* 2004; Pagano *et al.* 2005). Alternatively, the regulation of resistin may differ considerably during puberty in comparison to adulthood.

It is now considered that chronic inflammation is a common pathophysiological basis for insulin resistance, MS and atherosclerotic CVD disease (Willerson *et al.* 2004; Dandona *et al.* 2005); this has recently been recognised in childhood obesity (Weiss *et al.* 2005; Meyer *et al.* 2006). Results from this study demonstrated that bacterial endotoxin levels exhibited significant and positive correlations with the inflammatory marker, TNF-α. Furthermore, male subjects demonstrated higher levels of endotoxin and lower levels of adiponectin than female subjects, despite both cohorts being of similar age and BMI. This study, therefore, suggests that an initiator of sub-clinical inflammation, circulating commensal bacterial endotoxin, potentially derived from the gut, as previously suggested (Cornell 1985; Bauer *et al.* 2002), may serve as a source, or even mediate such inflammatory responses in childhood and adolescent obesity. Such potential *in vivo* inflammatory properties of endotoxin have previous been demonstrated in adult insulin resistant, type 2 diabetic and cirrhotic patients (Fogelstrand *et al.* 2004; Creely *et al.* 2006). Collectively, these studies suggest an endotoxin-mediated mechanism of sub-clinical inflammation in childhood and adolescent obesity.

Elevated circulating levels of PAI-1 have been associated with central obesity (Mavri *et al.* 2004), insulin resistance (Juhan-Vague *et al.* 1991; Bastard *et al.* 2000) and an increased risk of CVD (Juhan-Vague *et al.* 2000). Additionally, systemic PAI-1 concentrations have been shown to decrease in obese individuals following weight loss (Alessi *et al.* 1997). Other studies have reported elevated concentrations of ICAM in obese children with sleep-disordered breathing, in comparison to non-obese children (O'Brien *et al.* 2006). Moreover, endothelial activation has been associated with adiposity in obese children and adolescents (Garanty-Bogacka *et al.* 2005). In this study, several markers of vascular injury, including PAI-1, sICAM-1, MMP-9, MPO

and VEGF, significantly and positively correlated with circulating endotoxin concentrations. This suggests that such endotoxin-stimulated inflammation in childhood obesity, may consequently further contribute to an accelerated risk of cardiovascular morbidity.

In conclusion, although resistin may not be a crucial player in obesity-related inflammatory, bacterial endotoxin and several inflammation/CVD markers, on the other hand, are all associated with childhood and adolescent obesity. Furthermore, even at such an early age in the obese state, girls demonstrate a more favourable metabolic profile in terms of endotoxin concentrations, adiponectin levels and risk markers of CVD. This may manifest in later life as delayed CVD mortality and morbidity for female subjects when compared with male subjects. Furthermore, among the inflammatory markers evaluated in this study, endotoxin has potential to serve as an indicator or possible mediator of sub-clinical inflammation in childhood and adolescent obesity.

Chapter 6

Adiponectin Complexes in Human Cerebrospinal Fluid: Distinct Complex Distribution from Serum

6.1 Introduction.

Adipose tissue secretes a vast array of proteins that can exert multiple effects through the CNS and peripheral tissues to modulate energy homeostasis (Ahima *et al.* 2000). Leptin is one of these adipokines that has been extensively studied in this context. It serves as a potent satiety signal to the brain and regulates adiposity by suppressing appetite, increasing insulin sensitivity and influencing other anorexigenic hormones that mediate energy expenditure (Friedman *et al.* 1998; Elmquist *et al.* 1999; Ahima *et al.* 2000). Translocation of leptin across the BBB is modulated through a saturable transport system, consistent with a role of BBB in regulating behavior and maintaining CNS homeostasis (Ahima *et al.* 2000; Zlokovic *et al.* 2000).

Adiponectin is an adipocyte-specific secretory protein involved in numerous aspects of energy homeostasis (Scherer *et al.* 1995). Many studies have reported a strong correlation between increased circulating levels of adiponectin and improved insulin sensitivity (Berg *et al.* 2001; Yamauchi *et al.* 2001; Tsao *et al.* 2002; Pajvani *et al.* 2003). Furthermore, circulating adiponectin levels are reduced in states of obesity-induced insulin resistance, whilst weight reduction triggers an increase in adiponectin levels (Tsao *et al.* 2002; Combs *et al.* 2003). Systemic adiponectin treatment or transgenic adiponectin overexpression in rodents improves metabolic status, enhancing the ability of insulin to suppress hepatic glucose production (Berg *et al.* 2001; Yamauchi *et al.* 2001; Tsao *et al.* 2002; Pajvani *et al.* 2003; Yamauchi *et al.* 2003). In contrast, adiponectin-null mice exhibit hepatic insulin resistance (Nawrocki *et al.* 2006). Collectively, this suggests that the primary *in vivo* process targeted by adiponectin action in the context of insulin sensitisation is hepatic glucose homeostasis. Consistent with these *in vivo* observations, primary hepatocytes exposed to recombinant

adiponectin respond by reducing cellular glucose output as a function of adiponectin even at very low insulin concentrations. However, several recent papers have highlighted the need for both peripheral and central insulin action on hepatic glucose output (Cherrington 2005; Okamoto *et al.* 2005). Similarly, adiponectin may exert its action both peripherally and centrally.

Adiponectin consists of an N-terminal collagenous domain and a C-terminal globular domain (Tsao *et al.* 2002; Pajvani *et al.* 2003) that shares homology to types VIII and X collagen, in addition to complement factor C1q (Tsao *et al.* 2002; Pajvani *et al.* 2003). When secreted, adiponectin circulates in serum in distinct stable forms, as trimers and LMW hexamers, or HMW multimeric complexes, comprising of 12-18 subunits (Berg *et al.* 2002; Pajvani *et al.* 2003). A critical cysteine residue in position 39 is the key mediator of oligomerisation beyond the basic trimeric building block (Pajvani *et al.* 2003).

Previous studies have documented a characteristic sexual dimorphism in adiponectin levels in humans, with female subjects having significantly higher circulating levels than male subjects (Arita *et al.* 1999). This sexual dimorphism is also reflected in differential levels of the various circulating adiponectin complexes. The hexamer is the more prevalent form of adiponectin in male subjects, whereas female subjects have higher circulating levels of the HMW complex; since the HMW form has been implicated as the key complex in the context of insulin sensitisation, this may be the source of increased insulin sensitivity of female subjects compared to male subjects (Pajvani *et al.* 2003).

The adiponectin Cys-39 mutant with its destabilised homotrimeric structure shows greater bioactivity *in vivo* than the native oligomeric complexes in terms of its potency to reduce serum glucose levels (Pajvani *et al.* 2003); additionally, the recombinant globular, collagenous stalk-free form of adiponectin influences lipid oxidation in peripheral skeletal muscle tissues (Yamauchi *et al.* 2003), with limited effects on hepatic gluconeogenesis (Fruebis *et al.* 2001). However, the globular form of the protein has not yet been identified under normal physiological conditions *in vivo*. The native full-length adiponectin complex exerts more potent effects on the liver. Previous studies have demonstrated that transgenic mice overexpressing endogenous full-length adiponectin display substantial improvement in hepatic insulin sensitivity (Combs *et al.* 2004). It is also recognised that the ratio between HMW to LMW oligomeric forms of adiponectin measured by the adiponectin sensitivity index (S_A), rather than total circulating levels of adiponectin, is a critical determinant of TZD-mediated improvements in hepatic insulin sensitivity (Pajvani *et al.* 2004). Diabetic patients have decreased S_A values, which improve with TZD-treatment (Pajvani *et al.* 2004). The HMW oligomers are thus established as important adiponectin forms with regard to hepatic insulin sensitivity.

A direct functional impact of adiponectin on metabolism, affected by enhancing insulin sensitivity is therefore well established. However, recent studies have shed light on a central role for adiponectin, demonstrating profound effects of adiponectin on energy homeostasis in the brain. Intracerebroventricular (i.c.v) administration of globular, full-length or Cys-39Ser mutant adiponectin into wild-type and *ob/ob* mice decreased body-weight; effect being mediated via increased energy expenditure (Qi *et al.* 2004). Furthermore, peripherally administered adiponectin caused an increase in CSF

adiponectin, suggesting serum-to-CSF transport (Qi *et al.* 2004). However, these studies measured the adiponectin levels by RIA in mice only and lacked a more detailed examination of the specific complex distribution of adiponectin. Central treatment with adiponectin proved more potent than systemic treatment of adiponectin in wild-type and *ob/ob* mice, further highlighting the brain as an important target for adiponectin action (Qi *et al.* 2004). Additionally, adiponectin was shown to induce Fos protein immunostaining and increase hypothalamic corticotrophin-releasing hormone (CRH) synthesis in the paraventricular nucleus (PVN), suggesting the activation of hypothalamic sympathetic circuits (Qi *et al.* 2004). Further evidence suggesting a role for adiponectin in the CNS originates from several studies reporting the expression of adiponectin receptors (ADIPOR) 1 and 2 in the brain (Yamauchi *et al.* 2003) and, more specifically, in mouse hypothalamus and on brain endothelial cells of the BBB (Spranger *et al.* 2006); the latter may potentially serve as a transport system for receptor-mediated transcytosis of adiponectin across the BBB. However, a more specific role for these receptors remains to be defined. These studies suggest that adiponectin may act centrally to exert metabolic actions. However, the question whether adiponectin serves a role centrally under normal physiological conditions is yet to be resolved. One recent study failed to detect the presence of adiponectin in human CSF (Spranger *et al.* 2006) and, with another, reported that exogenous adiponectin does not cross the BBB in mice (Pan *et al.* 2006; Spranger *et al.* 2006), concluding that direct effects of adiponectin on CNS pathways may not be relevant.

The focus of this study was to firmly establish the presence of adiponectin in human CSF and subsequently to establish the distribution of the various oligomers by means of sub-fractionation and quantitative Western blot analysis of CSF samples.

6.2 Subjects, Materials & Methods.

6.2.1 Subjects.

A total of 22 subjects (all of whom were Caucasian) were analysed in the study (BMI: 28.0±4.7 kg/m^2; age: 58-81 yrs). Half of the subjects were male (BMI 28.1±3.8 kg/m^2; age range 58-80 years) and half were female (all post-menopausal; BMI 28.0±5.7 kg/m^2; age range 62-81 years). Of the 22 subjects, 2 subjects were smokers, 6 subjects used to be smokers and 14 subjects were non-smokers. CSF and matched serum samples were obtained from these consenting subjects undergoing either elective liposuction surgery of thigh, hip or knee (n=19), general orthopaedic surgery (arthroscopy) (n=2) or gynaecological surgical procedures (n=1) (all non-malignant). Prior to surgery, subjects received spinal anaesthesia injection. All samples were acquired in accordance with the Ethics Committee of the Birmingham Heartlands Hospital (Birmingham, UK) and analysed at the Albert Einstein College of Medicine (Bronx, NY, USA) under a protocol approved by the Institutional Review Board. Patients with malignancy, acute and chronic renal or liver disease, neurological disorders, on immunosuppressants, current or recent systemic high dose corticosteroids, antibiotics or weight altering medication were excluded from the study. All subjects were fasted overnight before examination. The sampling of CSF was performed according to standardised procedures with the examined subject in a lateral recumbent position and lumbar puncture at the L3–L4 or L5–L6 interspace using a standard needle. Prior to spinal anaesthesia injection, a clear volume of CSF was extracted. CSF samples were passed through a 0.2 micron syringe filter (Whatman, Florham Park, NJ, USA), aliquoted, flash frozen and stored at −80°C until analysis. A fasting blood sample (5 ml) was also taken at the time of venous cannulation. Samples were immediately centrifuged at 5,000 x g for 20 min, flash frozen and stored at -80°C.

6.2.2. Sample Preparation & Size-fractionation of Adiponectin Complexes using Fast Protein Liquid Chromatography (FPLC).

This technique is an adaptation of the FPLC procedure detailed in the General Materials & Methods section (**Chapter 2.10**).

Analysis of serum samples: Once thawed, 30 μl of each serum sample was spun at 10,000 x g for 10 min. Next, 20 μl of Column Buffer (25 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$; pH 8) was added to each serum sample. The sample mixture was then injected into a GL column (Superdex 200 10/300; GE Healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA).

Analysis of CSF samples: Firstly 20 μl of adiponectin knockout mouse serum (kindly donated by Nawrocki A, Albert Einstein College of Medicine of Yeshiva University, New York, USA) was added to 200 μl of CSF sample, as a carrier. The mixture was then spun at 10,000 x g for 10 min before injected undiluted on to the column. In total, 200 μl gradient-fractions were sequentially retrieved for each CSF and serum sample. For CSF fractions, 200 μl of each fraction was vacuum concentrated for 1 hr (SpeedVac Plus, Savant Instruments, Holbrook, NY, USA). Fractions were analysed by quantitative Western blot analysis as described below.

6.2.3 Detection & Visualisation of Adiponectin Oligomers in Sub-fractionated Samples using Western blot.

This developed protocol is a modified version of the western blotting procedure outlined in the General Materials & Methods section (**Chapter 2.4**). In brief, for serum fractions, 20 μl of 5X Laemmli loading buffer (**Appendix I**) was added to 60 μl of each

fraction then heated at 95°C for 20 min. Lyophilised CSF fractions were reconstituted in 25 μl Sample Buffer then heated at 95°C for 5 min. Separation of proteins and immunoblotting were performed as previously described (**Chapter 2.4.2 and 2.4.3**). Briefly, 20 μl of each serum or CSF sample mixture was loaded on a pre-cast 4-12% Bis-Tris gel (Bio-Rad Laboratories, Hercules, USA) and electrophorectically ran at 180V for 1 hr.

Following SDS-PAGE, proteins were transferred onto a polyvinylidene fluoride membrane (Millipore Corp., Billerica, Massachusetts, USA) for 1 hr as previously described (**Chapter 2.4**) then blocked using 4% non-fat dry milk in Tris-buffered Saline (TBS) containing 0.1% Tween 20 (TBS-T) for 1 hr. Primary antibodies specifically raised against the human N-terminal hypervariable region of adiponectin (DQETTTQGPGV) and the human C-terminal region of adiponectin were diluted (1:200) in TBS-T; these antibodies recognise a single band of 30 kDa by Western blot analysis and have equal affinity for all oligomeric forms of adiponectin. Membranes were washed 4 times for 5 min in 0.1% TBS-T. CSF blots were additionally probed with a biotin-conjugated goat anti-rabbit antibody (Invitrogen Corp., Carlsbad, California, USA) (1:500).

To allow for visualisation of bands, CSF blots were decorated with a fluorescent streptavidin-conjugate secondary antibody (Invitrogen Corp., Carlsbad, California, USA) (1:5000), whilst serum blots were decorated with a fluorescent dye-conjugated anti-rabbit secondary antibody (Rockland Immunochemicals Inc., Gilbertsville, USA) diluted (1:5000) in 0.1% TBS-T. Fractions 1 to 8 (HMW adiponectin), 9 to 16 (LMW adiponectin) and 16 to 25 (adiponectin trimer) from sedimentation were visualised at

700 nm for CSF blots and 800 nm for serum blots using a Phosphor Imager (LI-COR Biosciences, Lincoln, Nebraska, USA), then quantified using Odyssey Licor System software (LI-COR Biosciences, Lincoln, Nebraska, USA).

6.2.4 Detection & Visualisation of 'Total' Unfractionated Adiponectin Levels by Western blot.

Briefly, 20 μl of loading buffer was added to 100 μl of CSF sample; whereas 20 μl loading buffer was added to 1 μl of serum. Sample mixtures were then heated at 95°C for 20 min prior to loading onto a pre-cast 4-12% Bis-Tris gel (Bio-Rad Laboratories, Hercules, USA). Detection and visualisation of total adiponectin oligomers was performed using the Western blot procedure as detailed above in (**Chapter 6.2.3**).

6.2.5 Assessment of 'Total' Adiponectin, Insulin & Glucose Levels using RIA & a Glucose Analyser.

Total serum and CSF adiponectin levels were determined by RIA (Linco Research Inc., St Charles, Missouri, USA). This RIA utilises ^{125}I-labeled murine adiponectin and a multispecies adiponectin rabbit antiserum to determine the level of adiponectin in serum or CSF. The lower limit of sensitivity of human adiponectin that can be detected by this assay was 1 ng/ml for a 100 μl sample size; whereas the upper limit of sensitivity for this assay is 200 ng/ml for a 100 μl sample size.

Glucose levels were determined using a glucose analyser (YSI-2300 STAT PLUS; Yellow Springs Instruments, Yellow Springs, Ohio, USA) in accordance with the manufacturers' instructions. Calculation of homeostasis model assessment of insulin

resistance (HOMA-IR) (Matthews *et al.* 1985) was performed using a HOMA-IR calculator obtained from: www.dtu.ox.ac.uk/index.html?maindoc=/homa/.

6.2.6 Statistical Analysis of Results.

The SPSS statistical program, version 14.0 for Windows (SPSS, Woking, UK) was used to analyse data. Results are expressed as percentage or mean ± standard deviation. A two-tailed Student's t test was used to assess differences between serum and CSF adiponectin oligomers in male and female patients as these were Gaussian in distribution. A bivariate Pearson's correlation coefficient was used to analyse associations between the following variables: (1) log Serum adiponectin, (2) log CSF adiponectin, (3) BMI and (4) HOMA-IR. A p-value of <0.05 was considered statistically significant.

6.3 Results.

6.3.1 'Total' Matched Serum & CSF Protein Expression Levels of Adiponectin by Western blot Analysis.

To date, adiponectin levels in CSF have only been reported as RIA measurements. Using Western blot analysis, this study demonstrates that a single 30 kDa band cross-reactive with highly specific anti-adiponectin antibodies can be found in human CSF. In parallel, a corresponding serum sample from the same patient was analysed (**Figure 6.3.1.1**).

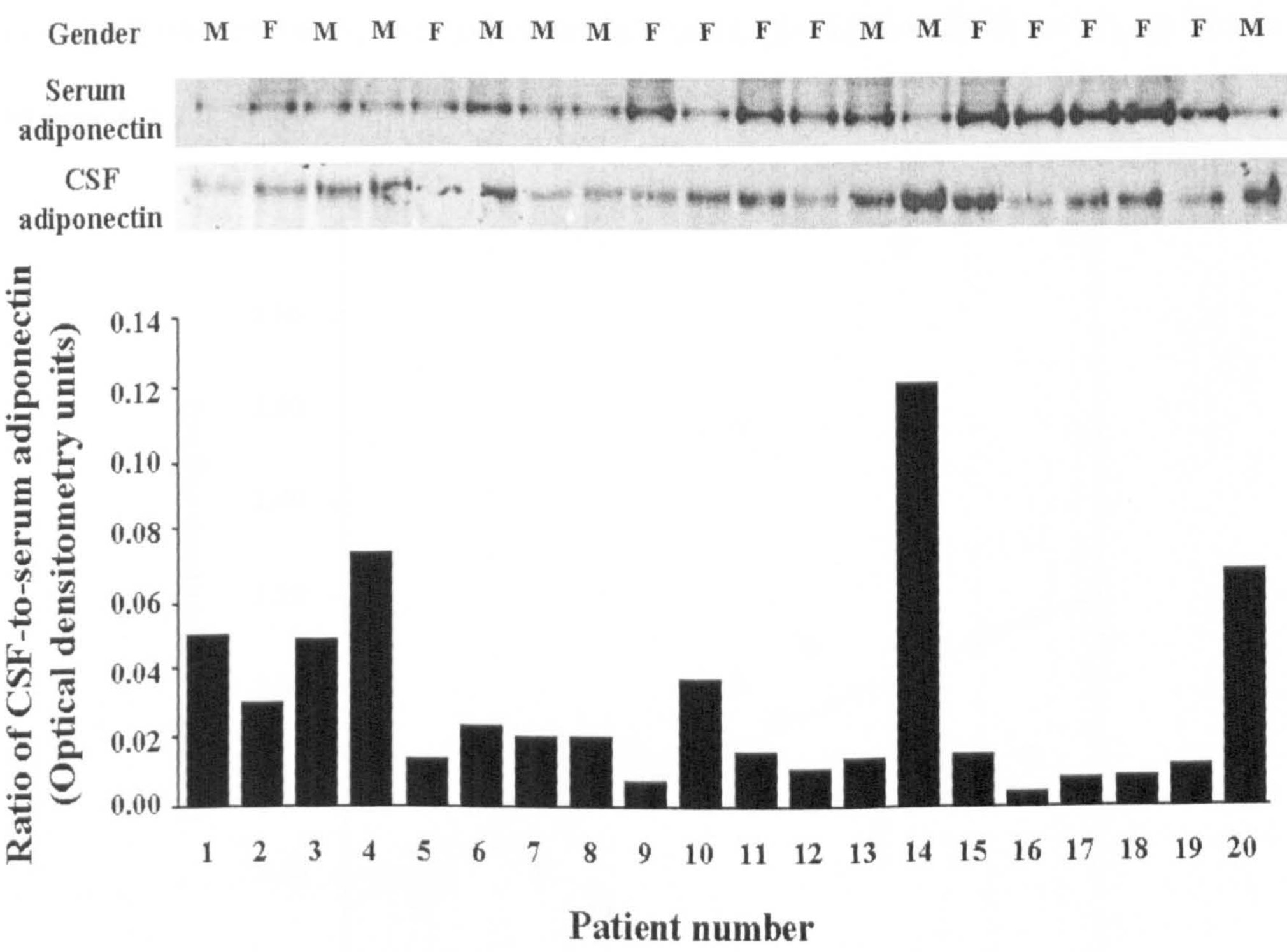


Figure 6.3.1.1 Total adiponectin in serum and matched CSF by Western blot analysis. The CSF-to-serum ratio of adiponectin is given for each patient analysed (n=20). Representative Western blots are shown for total serum adiponectin and corresponding total CSF adiponectin for each patient. Patient gender: M, male patient; F, female patient.

Note that the intensities should not be directly compared between the two Western blots. The relative ratio of CSF-to-serum of adiponectin expression is given for each patient based on the Western blot quantitation. This ratio varies from patient to patient, suggesting that the levels in CSF are actively controlled. The serum and CSF levels by RIA were subsequently measured. In absolute terms, the abundance of adiponectin is much lower in CSF, namely approximately 0.1% of the levels found in serum (serum=14.0±5.7 [mean±SD] μg/ml; CSF=11.9±18.8 ng/ml [n=22]). RIA analysis further revealed that generally, higher levels of adiponectin in serum tend to be associated with higher levels of adiponectin in CSF; this correlation was significant in men (p=0.044; r=0.615; n=11), but not in women (p=N.S; r=0.263; n=11), as shown in **Figure 6.3.1.2** below.

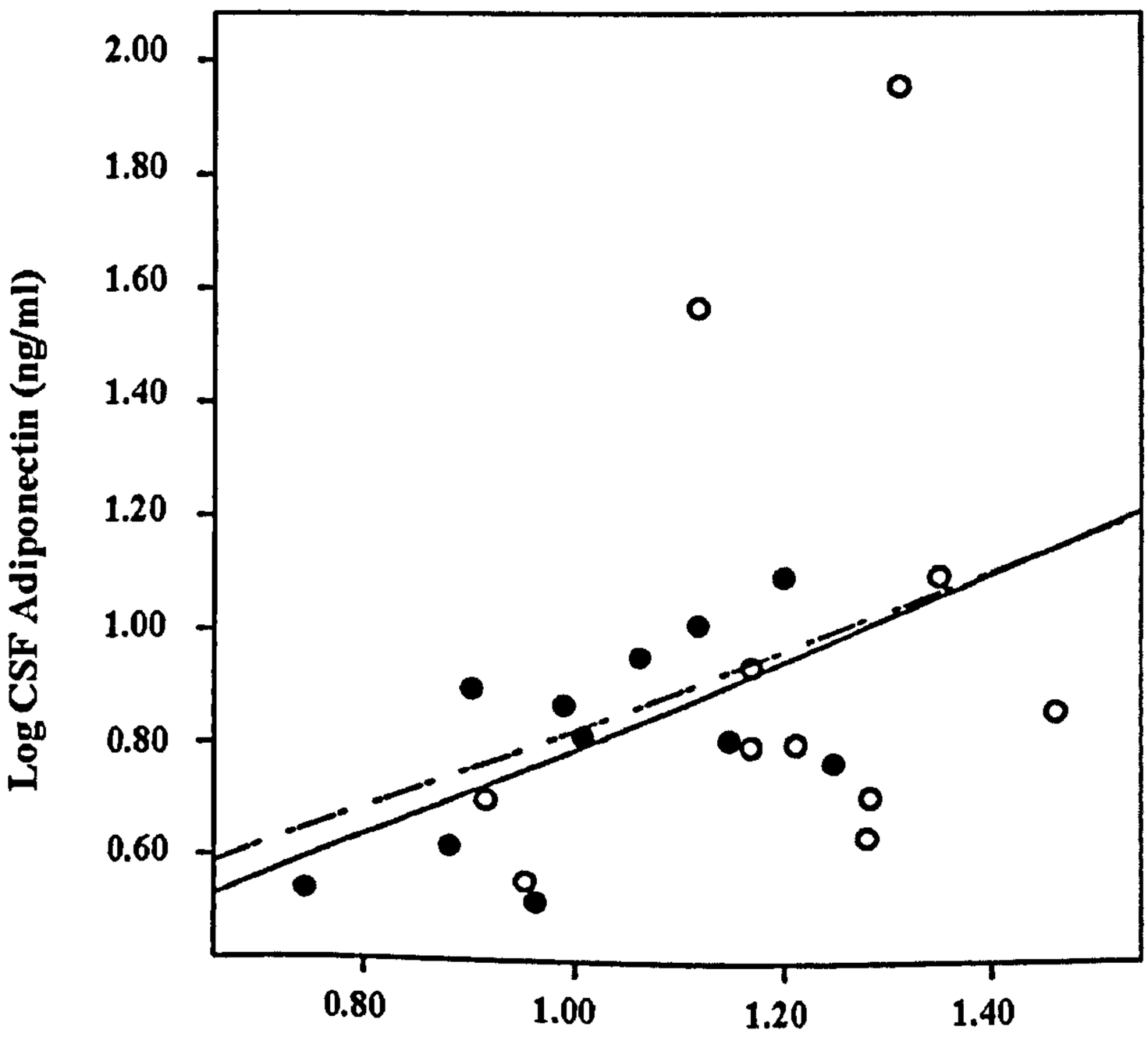


Figure 6.3.1.2 Total CSF adiponectin levels in male patients (solid symbols) (p=0.044; r=0.615; n=11) and female patients (open symbols) (p=N.S; r=0.263; n=11) were measured by RIA. Solid line, trendline for male patients; dashed line, trendline for female patients.

6.3.2 Trimer & LMW Hexamer Forms of Adiponectin are the Predominant Complexes Found in Human CSF.

Following sub-fractionation of CSF by gel-filtration chromatography and subsequent quantitative Western blotting, the distribution of adiponectin oligomers in human CSF was visualised (**Figure 6.3.2.1**); only the adiponectin LMW hexamer, and more prevalently, the trimer are found in CSF.

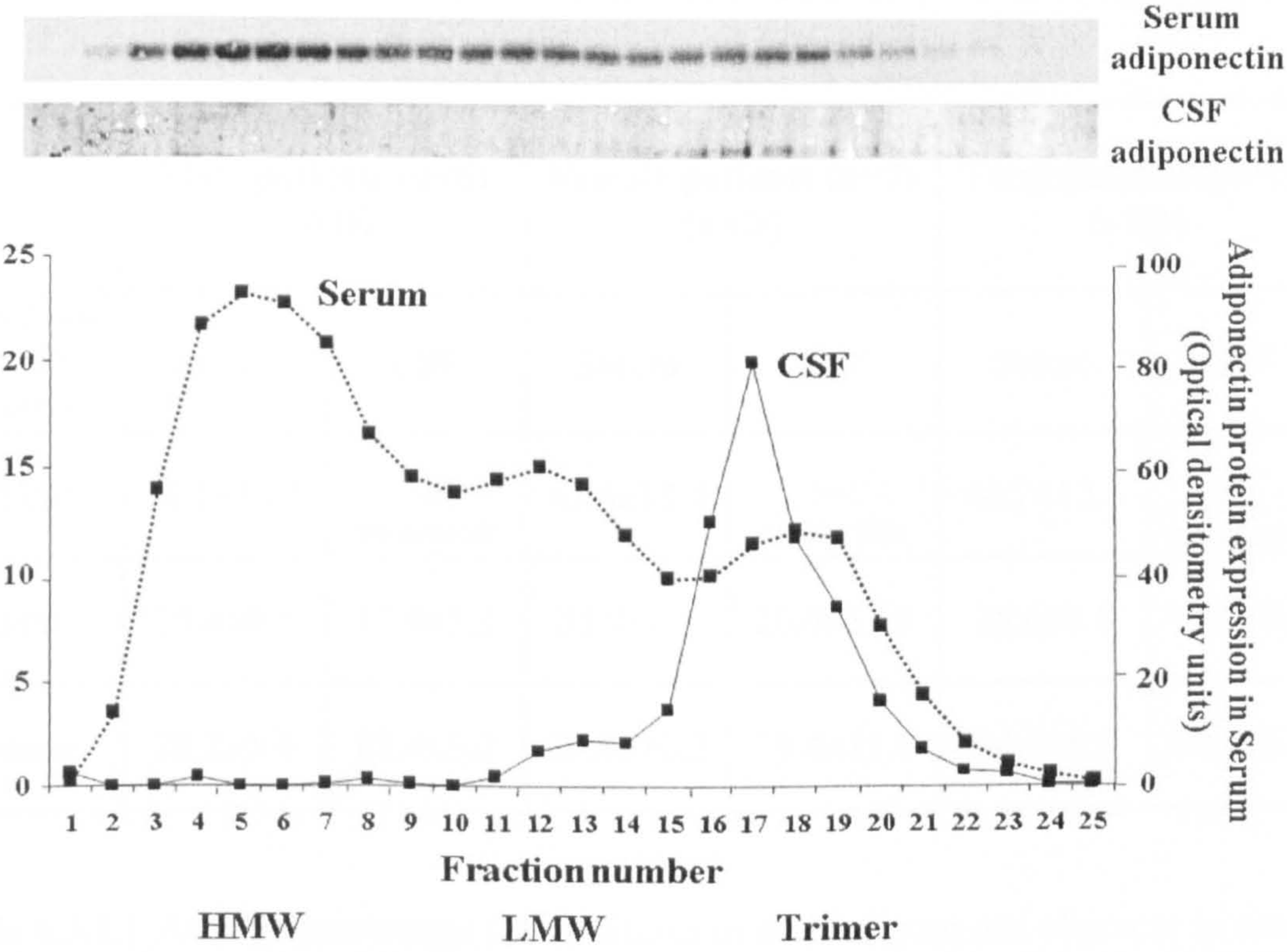


Figure 6.3.2.1 The distribution of adiponectin oligomers in CSF (solid line) in comparison with that in matched serum (dotted line) from one female patient (n=13). The average percentage composition of each adiponectin oligomer in serum was HMW, 40.2%; LMW, 35.6%; Trimer, 24.2% (n=13). The average percentage composition of adiponectin trimer and LMW hexamer in CSF were LMW, 19.2%; Trimer, 80.8%. Representative Western blots from the same female patient following size-fractionation highlight serum and CSF adiponectin oligomer distribution.

A comparison of serum and CSF adiponectin oligomer distribution revealed that adiponectin displays a diverse oligomeric complex distribution of oligomers in CSF, when compared with matched serum within the same patient. Serum showed the

expected distribution of HMW complexes, LMW hexamers and the adiponectin trimers. CSF, in contrast, lacked the HMW form prominent in serum. While a single representative example is shown in **Figure 6.3.2.1,** the analysis was expanded to an additional 13 samples. For serum analysis, the fraction of each adiponectin oligomer was calculated relative to the total adiponectin levels. The average percentage composition of adiponectin oligomers in serum across a range of patients in the study was HMW, 40.2±12.5%; LMW, 35.6±6.8%; Trimer, 24.2±8.9% (n=13) (**Table 6.3.2.1**).

	Male patients (n=6) (±SD)		**Female patients (n=7) (±SD)**		**Total patients (n=13) (±SD)**	
% Adipo-nectin Oligomer	Serum	CSF	Serum	CSF	Serum	CSF
HMW	36.5±11.3	Not measurable	43.4±13.4	Not measurable	40.2±12.5	Not measurable
LMW	35.4±9.1	17.6±5.2	35.9±4.7	20.6±11.8	35.6±6.8	19.2±9.1
Trimer	28.2±5.4	82.4±5.2	20.8±10.2	79.4±11.8	24.2±8.9	80.8±9.1

Table 6.3.2.1 Average percentage compositions of each adiponectin oligomer in serum and CSF in male patients (n=6), female patients (n=7) and in both combined. No statistical differences in HMW, LMW or trimeric adiponectin were reached between male patients and female patients.

The analysis of CSF revealed: no detectable HMW; LMW 19.2±9.1%; Trimer 80.8±9.1%. The lack of the HMW form of adiponectin enabled exclusion of the possibility that CSF could have been contaminated by serum.

6.3.3 Sexual Dimorphism of Total Adiponectin & Oligomeric Complex Distribution.

Further analysis of total adiponectin levels in serum compared with CSF using Western blot, revealed that male subjects have a significantly higher CSF-to-serum ratio of adiponectin than female subjects ($p<0.05$; $n=20$) **(Figure 6.3.3.1)**.

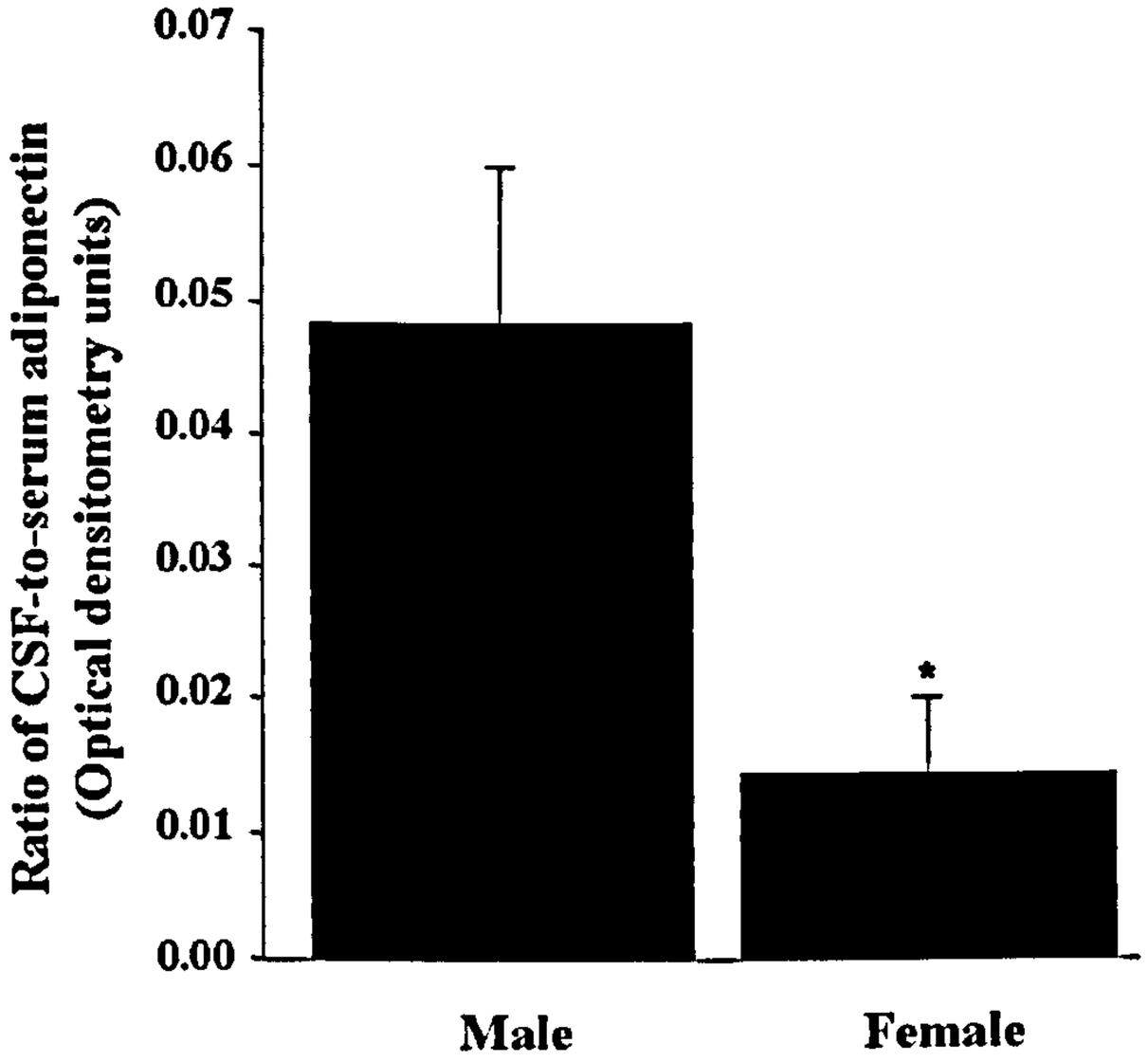


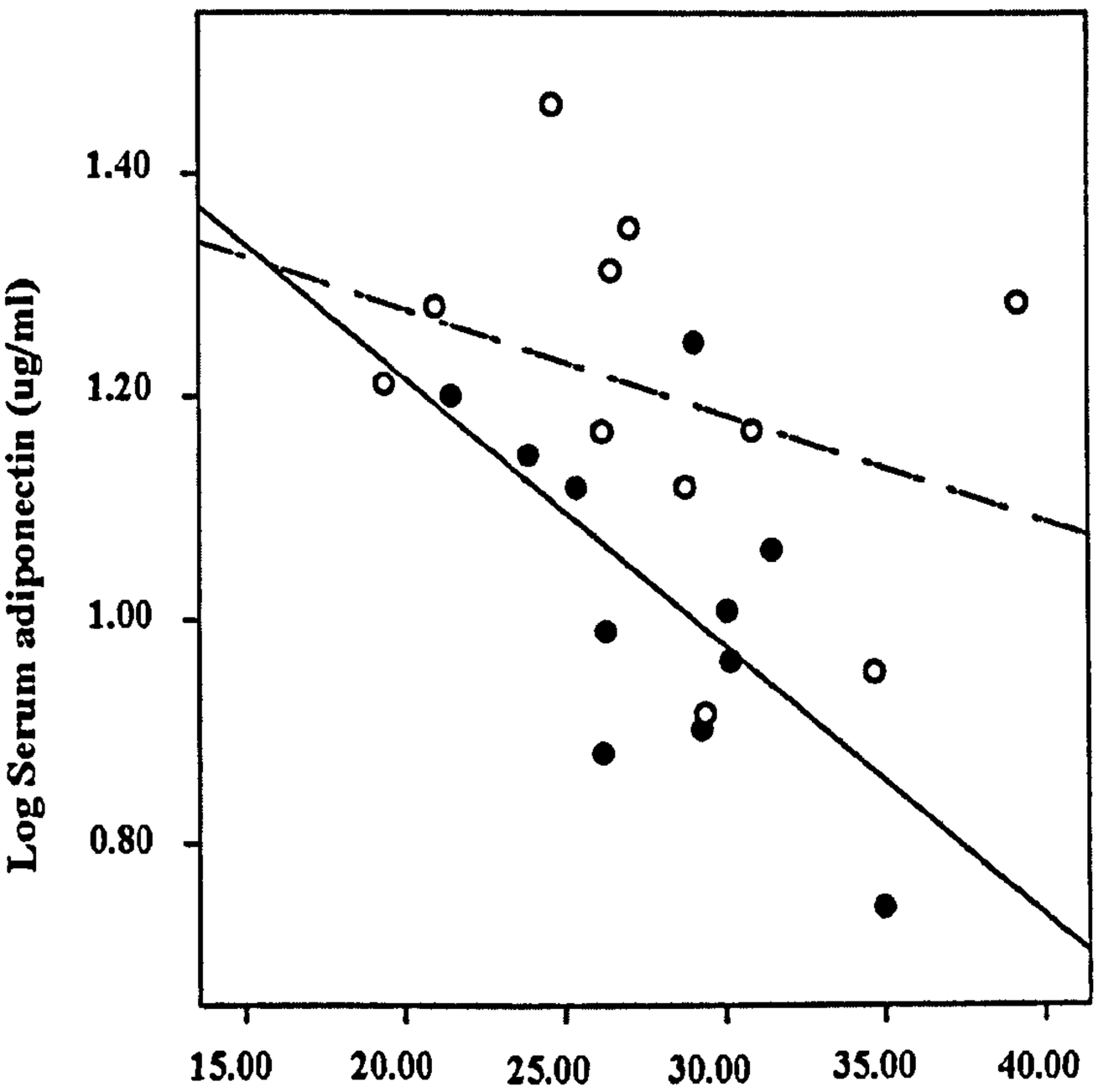
Figure 6.3.3.1 The ratio of total CSF-to-serum adiponectin in male patients compared with female patients by Western blot analysis. *, $p<0.05$; $n=20$.

It is acknowledged that women have higher levels of HMW adiponectin in serum than men. In contrast, men have slightly higher levels of trimeric adiponectin in serum than women. Although **Table 6.3.2.1** demonstrates no significanct gender-related differences in serum HMW and trimeric adiponectin (p=N.S; men, n=6; women, n=7), further analysis using a larger cohort may substantiate significance. No significant differences were detected at the level of LMW adiponectin hexamer (p=N.S) **(Table 6.3.2.1)**. Furthermore, although men displayed slightly higher levels of adiponectin trimer in CSF than women, this did not reach statistical significance (p=N.S; men, n=6; women, n=7) **(Table 6.3.2.1)**. Additional studies will therefore be required to see whether these

trends can be confirmed in larger cohorts. Potentially higher levels of trimeric adiponectin levels in men may reflect the general diverse adiponectin oligomeric distribution between the sexes. Previous studies have established that females have higher levels of HMW adiponectin (Pajvani *et al.* 2003), whereas males exhibit higher levels of the adiponectin hexamer, which, either by potential androgen regulation, may yield higher levels of trimeric adiponectin.

6.3.4 Correlation with BMI & HOMA-IR.

As in many previous studies, the levels of total adiponectin in serum were inversely related to BMI. Furthermore, examining such a correlation in this cohort using RIA analysis, revealed that total serum adiponectin significantly correlates with BMI in male patients (p=0.047; r=-0.609; n=11) (**Figure 6.3.4.1**); however, this was not the case in the female patients in this cohort (p=N.S; r=-0.329; n=11) (**Figure 6.3.4.1**). Additionally, when examining the correlation between CSF adiponectin and BMI in men and women, analysis revealed a trend, albeit not significant, towards an inverse relationship of CSF adiponectin with BMI (male patients, p=0.067; r=-0.571; n=11; female patients, p=N.S; r=-0.116; n=11) (**Figure 6.3.4.2**). Non-significant correlations in terms of adiponectin levels, gender and BMI may in part, stem from the sample size of the study.



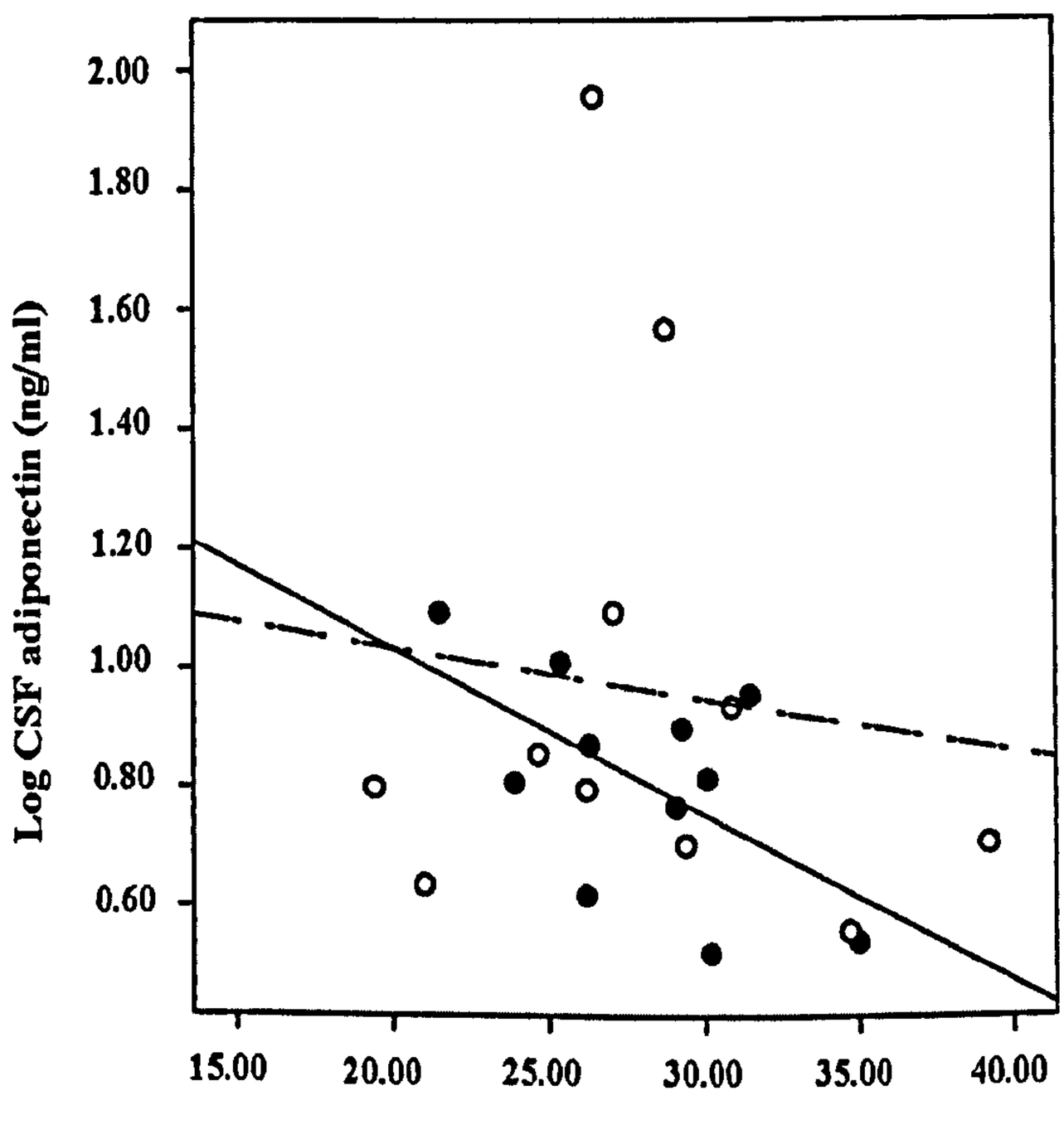


Figure 6.3.4.1 (Top) Correlation of total serum adiponectin with BMI in male patients (solid symbols) (p=0.047; r=-0.609; n=11) and female patients (open symbols) (p=N.S; r=-0.329; n=11) by RIA. **Figure 6.3.4.2 (Bottom)** Correlation of total CSF adiponectin with BMI in male patients (p=N.S; r=-0.571; n=11) and female patients (p=N.S; r=-0.116; n=11) by RIA.

Within this cohort of patients, glucose and insulin concentrations were 6.0±1.7 mM and 9.4±13.3 μU/ml (n=22) respectively. When examining insulin resistance index, serum adiponectin levels were shown to inversely correlate with HOMA-IR index in male patients only (male patients, p=0.014; r=-0.744; n=10; female patients, p=N.S; r=-0.504; n=11). Similarly, CSF adiponectin demonstrated a significant correlation with HOMA-IR in male patients, although not in female patients (male patients, p=0.037; r=-0.663; n=10; female patients, p=N.S; r=-0.330; n=11).

6.4 Discussion.

This study firstly established the presence of adiponectin in human CSF, confirming the ability of adiponectin to traverse the BBB and further identify its potential to function centrally under normal physiological conditions. A key finding from this study was the diverse oligomeric distribution between CSF and serum within matched samples from the same patients. While all forms of adiponectin were present in serum, only the trimer and LMW hexamer were present in CSF; in particular, the adiponectin trimer was substantially more prevalent in CSF than the LMW form. Interestingly, male subjects displayed a higher CSF-to-serum ratio of total adiponectin expression in comparison to female subjects. Sub-fractionation analysis of serum further revealed that men have slightly more adiponectin trimer than women; although not significant, men also had slightly higher levels of adiponectin trimer in CSF compared to women. As in many previous studies, the LMW hexamer does not seem to be regulated in any significant fashion.

This study therefore highlights that the trimer is the primary form of adiponectin in human CSF. This further supports previous observations that i.c.v. injection of either wild-type, Cys39Ser mutant or globular adiponectin had a central effect, whilst the collagenous tail domain lacking the globular head domain was ineffective (Qi *et al.* 2004). I.c.v. administration of adiponectin proved to have a more potent effect on energy homeostasis than systemic administration of equivalent amounts of adiponectin (Qi *et al.* 2004).

A number of recent papers highlight the relevance of the HMW form for the peripheral functions of adiponectin (Waki *et al.* 2003; Pajvani *et al.* 2004; Tonelli *et al.* 2004;

Fisher *et al.* 2005; Wang *et al.* 2005; Hara *et al.* 2006; Lara-Castro *et al.* 2006). In light of the very large size of this complex (>500 kDa), it is not surprising that the HMW form is very inefficiently translocated across the BBB and not found in any measurable quantities in CSF. The HMW form, despite its relevance for peripheral adiponectin action, does not seem to play a major physiological role for central adiponectin action. In contrast, the trimeric form of adiponectin seems to be most relevant for the central action of this protein.

The BBB plays a major dynamic regulatory role in the passage of circulating peptides involved in energy homeostasis (Zlokovic *et al.* 2000). How, exactly, the adiponectin trimer crosses the BBB is currently unknown. Adiponectin receptors (ADIPOR1 and ADIPOR2) have been detected in the brain (Yamauchi *et al.* 2003) and on BBB endothelial cells (Spranger *et al.* 2006), suggesting that a regulated receptor-mediated transport system may be the favourable mechanism of entry. However, the identification and kinetic properties of adiponectin transporters remains to be determined.

Leptin concentrations in CSF are positively related to plasma level and BMI, but the CSF-to-plasma leptin ratio is reduced at the highest plasma leptin level, suggesting a saturable brain transport mechanism (Caro *et al.* 1996; Schwartz *et al.* 1996). The decrease in CSF-to-plasma leptin among obese patients is consistent with leptin resistance (Caro *et al.* 1996; Schwartz *et al.* 1996). CSF leptin levels in children account for 5% of plasma levels, but unlike plasma level, which is higher in girls, the CSF leptin concentration is similar between girls and boys (Wiedenhoft *et al.* 1999). Therefore, the CSF-to-plasma leptin ratio is lower in girls and, this may underlie the propensity towards excess body-fat (Wiedenhoft *et al.* 1999). A nocturnal plasma leptin surge

entrained to meal timing has been demonstrated in lean and obese humans (Sinha *et al.* 1996; Schoeller *et al.* 1997). However, this is not accompanied by an increase in CSF leptin (Wong *et al.* 2004). As leptin transport across the BBB is fully saturated at higher serum leptin concentrations, it is possible that the BBB serves as a rate-limiting step to prevent increases in CSF leptin concentrations. The BBB could thus contribute to 'leptin resistance'; such a notion has been reported in obese subjects, where the increases in plasma leptin were not followed by parallel increases in the CSF leptin levels (Caro *et al.* 1996; Schwartz *et al.* 1996). How the lack of a nocturnal increase in CSF leptin relates to the periodicity of energy balance and neuroendocrine axis is unknown (Wong *et al.* 2004). Similar mechanisms may apply for adiponectin. Indeed, Kim and colleagues recently reported that a mouse model expressing a dominant negative version of the IGF-1 receptor transgenically in muscle displayed complete adiponectin resistance (Kim *et al.* 2006). Future experiments will have to address more specifically the mechanism of adiponectin BBB transport under different physiological conditions.

ADIPOR1, preferential for globular adiponectin and trimeric adiponectin, is abundantly present in skeletal muscle, mediating AMPK activation to increase glucose uptake and lipid oxidation (Yamauchi *et al.* 2003). ADIPOR2, on the other hand, favours full-length adiponectin, is more highly enriched in the liver, mediating hepatic glucose homeostasis (Yamauchi *et al.* 2003). Interestingly, higher levels of ADIPOR1 than in ADIPOR2 are found in the brain (Yamauchi *et al.* 2003), consistent with the findings in this study, that the adiponectin trimer is the predominant CSF form. Whether BBB endothelial cells specifically express higher levels of ADIPOR1 is, however, uncertain.

The relatively low overall protein concentrations in CSF and the low abundance of adiponectin in CSF make the biochemical analysis challenging. In addition, the concentrations found in CSF may or may not be sufficiently high to bind and activate the known ADIPORs, given the affinity constants reported for the receptors. However, it is difficult to rule out the possibility that other mechanisms are in place to increase the local concentrations of the adiponectin trimer to levels that would effectively trigger receptor activation. Alternatively, additional, yet-to-be identified receptors may exist that display higher affinities.

Within this study, antibodies that specifically recognise human adiponectin and do not show any cross-reactivity with murine adiponectin were used. To avoid introducing an exogenous source of adiponectin altogether, serum from adiponectin null mice was used as a carrier during the biochemical sub-fractionation of the CSF samples, a step that proved to be essential for the efficient detection of CSF adiponectin.

Putative transport systems for both leptin and TNF-α have been identified or postulated to exist (Zlokovic *et al.* 2000); these transport systems should be able to independently facilitate receptor-mediated transcytosis of peptides across the BBB (Pan *et al.* 2004). Whether such a scenario exists for adiponectin transport is currently unknown. Once across the BBB, the influence of adiponectin on peripheral energy expenditure may be mediated by the hypothalamus. Adiponectin induces c-*fos* expression in the PVN (Qi *et al.* 2004) and increases the synthesis of hypothalamic CRH (Qi *et al.* 2004), suggesting activation of adiponectin-responsive neurons via the melanocortin pathway. Agouti $A^{y/a}$ mice, incapable of melanocortin signalling (Dinulescu *et al.* 2000), are resistant to central administration of adiponectin (Masaki *et al.* 2003), further indicating that the

melanocortin pathway is essential for the anorexigenic effects of adiponectin on energy expenditure. Leptin *ob/ob* mice are particularly sensitive to CNS and systemic adiponectin treatment (Berg *et al.* 2001; Qi *et al.* 2004), suggesting perhaps a compensatory role for adiponectin to counteract reduced leptin signalling in the melanocortin pathway. Furthermore, mice lacking leptin receptors only in pro-opiomelanocortin (POMC) neurons have a modest increase in body weight (Balthasar *et al.* 2004). Future studies examining mice lacking adiponectin receptors in POMC neurons may prove illuminating and may establish whether leptin and adiponectin act in concordance to activate such neurons.

Western blot analysis of total adiponectin revealed that male subjects have a higher CSF-to-serum ratio than female subjects. Sexual dimorphism in terms of total systemic adiponectin, with women having higher levels of adiponectin than men, has been reported (Nishizawa *et al.* 2002; Cnop *et al.* 2003; Combs *et al.* 2003); Although this was preserved in CSF (Qi *et al.* 2004), such findings were achieved using assay-based measures. As it is currently unknown whether available RIAs and ELISAs are biased towards some adiponectin oligomers, total adiponectin data should be interpreted with care. Although this study demonstrated that the adiponectin oligomeric distribution was not significantly different in serum and CSF with regards to gender, further analysis using a larger cohort of subjects for sub-fractionation would be beneficial and may further establish significance.

The three oligomeric adiponectin complexes have differential secretion rates (Xu *et al.* 2005), possibly dependent on the status of energy expenditure and on peripheral and hepatic insulin sensitivity. The rate of HMW adiponectin secretion from adipocytes is

slower than that of the other two oligomeric complexes (Xu *et al.* 2005). Testosterone has been shown to selectively impede the secretion of the HMW form of adiponectin from adipocytes, possibly by activating intracellular sequestering molecules (Xu *et al.* 2005). Interestingly, testosterone elevates the relative levels of trimeric and hexameric adiponectin forms at the expense of the HMW form in circulation (Xu *et al.* 2005), consistent with the increased susceptibility of men to hepatic insulin resistance. Androgens may therefore serve as one of many factors modulating the pattern of adiponectin oligomeric complex distribution within target tissues. Consequently, healthy women displaying higher levels of HMW adiponectin also display improved hepatic insulin sensitivity compared to men. Additionally, post-menopausal women may exhibit a more androgenic setting of adiponectin oligomers in comparison to young pre-menopausal women. Future examination of menopausal status in terms of adiponectin oligomer distribution in serum and CSF may further define the role of hormonal factors as modulators of adiponectin.

A recent paper reported negative data regarding the presence and/or transport of adiponectin into CSF (Spranger *et al.* 2006). It is unclear why these authors were unable to detect adiponectin in CSF. However, given the negative data reported for the transport of bacterially produced globular trimer in these studies, it is possible that the collagenous stalk or some additional post-translational modification associated with it may be important for the transport process.

Several key questions remain outstanding with regards to central adiponectin action. What factors determine the distribution of adiponectin forms between CSF and the periphery? Is this distribution altered in obese or insulin resistant patients, i.e. is there a

specific defect in the BBB translocation machinery under some pathological conditions? Does the nutritional status affect this distribution? Future studies examining adiponectin oligomeric distribution in CSF in diabetic or in chronically obese patients, as well as pre-and post peroxisome proliferator-activated receptor-γ agonist intervention treatment, may clarify the mechanisms underlying central adiponectin action. In conclusion, adiponectin is present in human CSF, predominantly in the trimeric form. More specifically, CSF adiponectin employs a distinct oligomeric pattern to that of serum and may be further modulated by gender.

Chapter 7

Discussion

The molecular mechanisms underlying the link between obesity, inflammation, insulin resistance and the progression to T2DM are still only partially understood. At the centre of these conditions however, is the 'adipocyte cell' itself. It is now recognised that adipocytes integrate and respond to numerous autocrine, paracrine and endocrine signals (Rajala *et al.* 2003; Scherer 2006), by secreting a vast array of adipokines (Tilg *et al.* 2006). It is also suggested that inflammation, particularly within obesity, employs an enhanced activation of innate immunity (Dandona *et al.* 2004), through a series of complex interactions amongst components of the innate immune signalling pathway and the insulin signalling cascade, which results in abnormal systemic levels of adipokines. Consequently, dysregulation of such signalling processes may therefore alter glucose homeostasis and lipid metabolism, thus contributing to insulin resistance and the progression to T2DM (Wellen *et al.* 2005; Shoelson *et al.* 2006). Principally, this thesis investigated obesity-associated inflammation in human adipose tissue, followed by the potential central actions of adipokines in human CSF; by assessing the expression, secretion and functionality of two major adipokines, resistin and adiponectin.

7.1 The Role of Resistin in Human Obesity & Inflammation.

This thesis firstly explored resistin in relation to human obesity, as previous studies have shown contradiction (Azuma *et al.* 2003; Degawa-Yamauchi *et al.* 2003; Lee *et al.* 2003; Heilbronn *et al.* 2004). More specifically, real-time PCR analysis revealed a correlation between resistin and increasing adiposity in AT. Subsequent, Western blot analysis established that resistin was expressed at higher levels in obese AbSc AT. As such, both mRNA and protein data complied with previous observations highlighted elevated resistin levels in obesity (McTernan *et al.* 2002; McTernan *et al.* 2003).

Resistin has been identified in many cell-types in the humans, including adipocytes, pre-adipocytes and macrophages (**Table 1.4.1.2**). Within adipose tissue, the majority of studies to date have reported that the predominant source of human resistin is the macrophage (Kaser *et al.* 2003; Patel *et al.* 2003); this study established that human resistin is also expressed and secreted from isolated adipocytes. It could be suggested that, as adipose tissue mass accumulates during obesity, increased levels of resistin enter into circulation to potentially exert a significant impact on various inflammatory and metabolic signalling systems.

Numerous studies have suggested an inflammatory role for adipokines in conjunction with the insulin signalling cascade (Hotamisligil 2003; Arkan *et al.* 2005; Hotamisligil 2005; Shoelson *et al.* 2006). This thesis therefore explored the role of human adipose tissue as a potential inflammatory target. Subsequent studies identified elevated expression levels of key intermediates of the innate immune system, in particular, MyD88, TRAF-6 and NF-κB, in obese and type 2 diabetic adipose tissue. This firstly implicated human adipose tissue as a site for an innate immune response and secondly, suggested that in states of severe increased adipose tissue biomass and T2DM, there is an enhanced level of activation of innate immunity. Such enhanced innate immunity has resulted in adipose tissue itself being considered as 'inflamed' (Lehrke *et al.* 2004). This inflammatory state may consequently propagate and recruit circulating macrophages into the inflammatory site, and, in conjunction with adipocytes, produce an immense level of inflammatory mediators. Elevated systemic levels of such inflammatory mediators may contribute to a state of insulin resistance and accelerate the progression to T2DM.

This thesis further addressed the adipocyte itself as site for an innate immune response in human obesity and T2DM. In particular, studies examined the duality in function of resistin on components of the innate immune pathway and insulin signalling cascade in isolated adipocytes. *In vitro* analysis of resistin revealed that antigenic stimuli were capable of increasing resistin levels from isolated adipocytes. Additionally, human recombinant resistin itself increased the level of inflammatory cytokine secretion from adipocytes; this affect was enhanced by insulin and further, suppressed by the insulin sensitiser, RSG. Previous studies have suggested NF-κB and JNK as central inflammatory and metabolic regulatory kinases in innate immunity and insulin signalling, respectively (Hirosumi *et al.* 2002; Arkan *et al.* 2005; Wellen *et al.* 2005). Resistin was shown to stimulate the expression of these central kinases; suggesting that resistin may influence more than one mechanism to stimulate inflammatory cytokine release from human isolated adipocytes; potentially via the integration of both inflammatory and metabolic signalling systems, as highlighted in **Figure 7.1.**

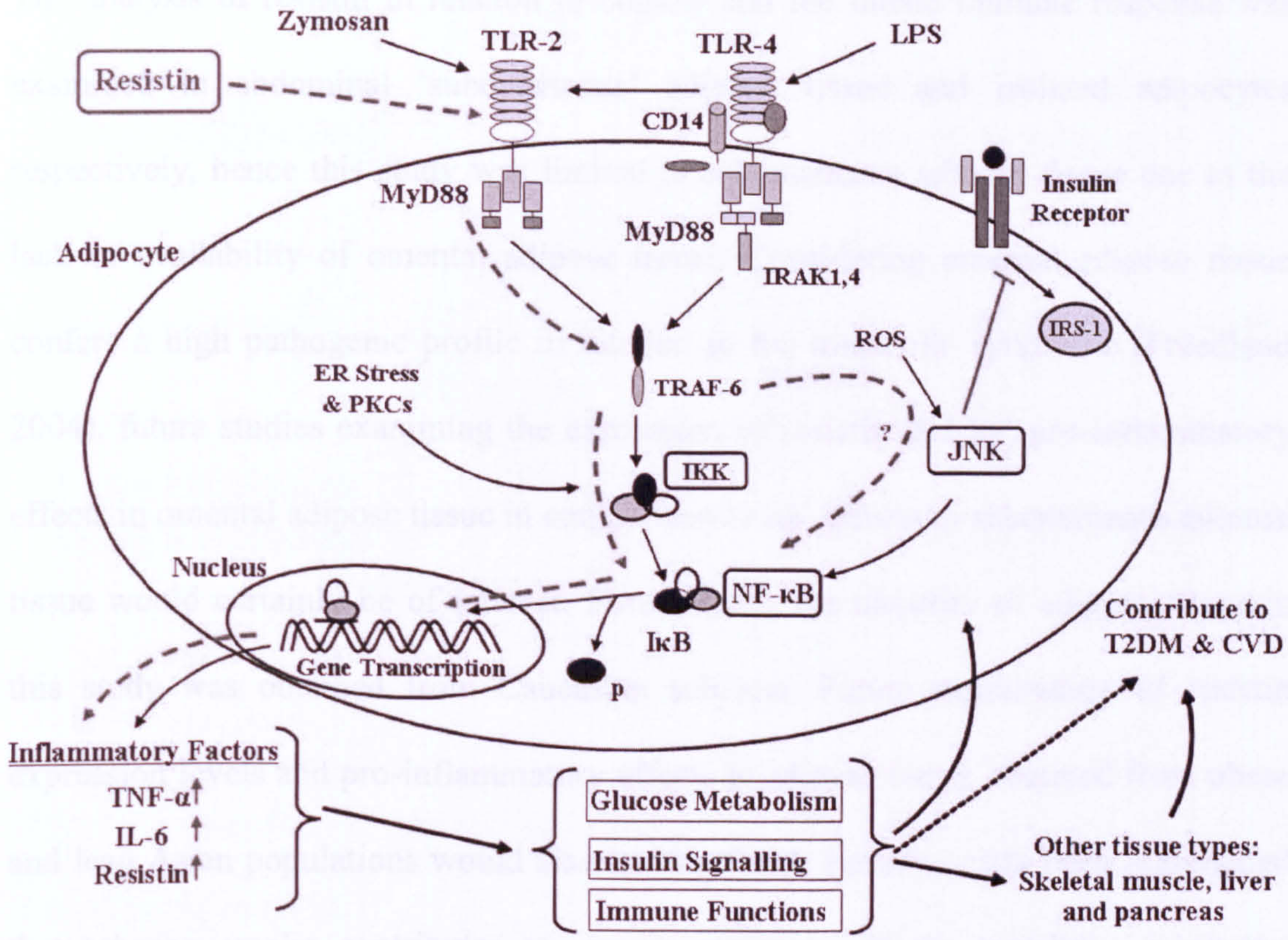


Figure 7.1 Illustration of the hypothesised model of resistin action within the adipocyte. Resistin affects several key components of the innate immune signalling pathway and factors of the insulin signalling cascade, therefore altering the production of inflammatory mediators. Consequently, resistin may indirectly influence the progression of T2DM and CVD.

Several studies have highlighting the importance NF-κB in the regulation of inflammatory processes that may affect insulin sensitivity (Shoelson *et al.* 2003; Cai *et al.* 2005; Shoelson *et al.* 2006). To further analyse the significance of resistin in both NF-κB and JNK signalling pathways, subsequent studies included inhibition of both signalling systems. Results identified both NF-κB and JNK as potential master regulators of resistin secretion from adipose tissue; this is paralleled by other studies demonstrating reduced pro-inflammatory cytokine secretion when such key regulators are pharmacologically inhibited (Creely *et al.* 2006). Collectively, these studies suggest NF-κB and JNK as potential therapeutic targets for the treatment of inflammatory-associated insulin resistance and T2DM.

The analysis of resistin in relation to obesity and the innate immune response was examined in abdominal 'subcutaneous' adipose tissue and isolated adipocytes respectively, hence this study was limited to subcutaneous adipose tissue due to the lack of availability of omental adipose tissue. Considering omental adipose tissue confers a high pathogenic profile in relation to the metabolic syndrome (Freedland 2004), future studies examining the expression of resistin and its' pro-inflammatory effects in omental adipose tissue in comparison to its' effects in subcutaneous adipose tissue would certainly be of interest. Furthermore, the majority of adipose tissue in this study was obtained from Caucasian subjects. Future examination of resistin expression levels and pro-inflammatory effects in adipose tissue obtained from obese and lean Asian populations would also be beneficial. Finally, it has been highlighted that tobacco smoke contributes to insulin resistance in the metabolic syndrome (Wietzman *et al.* 2005; Strauss *et al.* 2001). Future studies evaluating the association of environmental tobacco smoke exposure and active smoking, as measured by circulating cotinine concentrations, in relation to resistin would further be of interest.

7.2 A Central Role for Adiponectin.

In contrast to resistin, adiponectin reduces insulin resistance and thrombogenic risk and, is expressed and secreted from adipocytes at higher levels in healthy lean subjects (Scherer *et al.* 1995; Bouskila *et al.* 2005; Trujillo *et al.* 2005). Paradoxically, an increase in adipose tissue biomass results in decreased levels of adiponectin mRNA and protein (Arita *et al.* 1999; Hara *et al.* 2003; Reinehr *et al.* 2004; Fernandez-Real *et al.* 2005). **Figure 7.2** highlights that adiponectin can be influenced by several conditions.

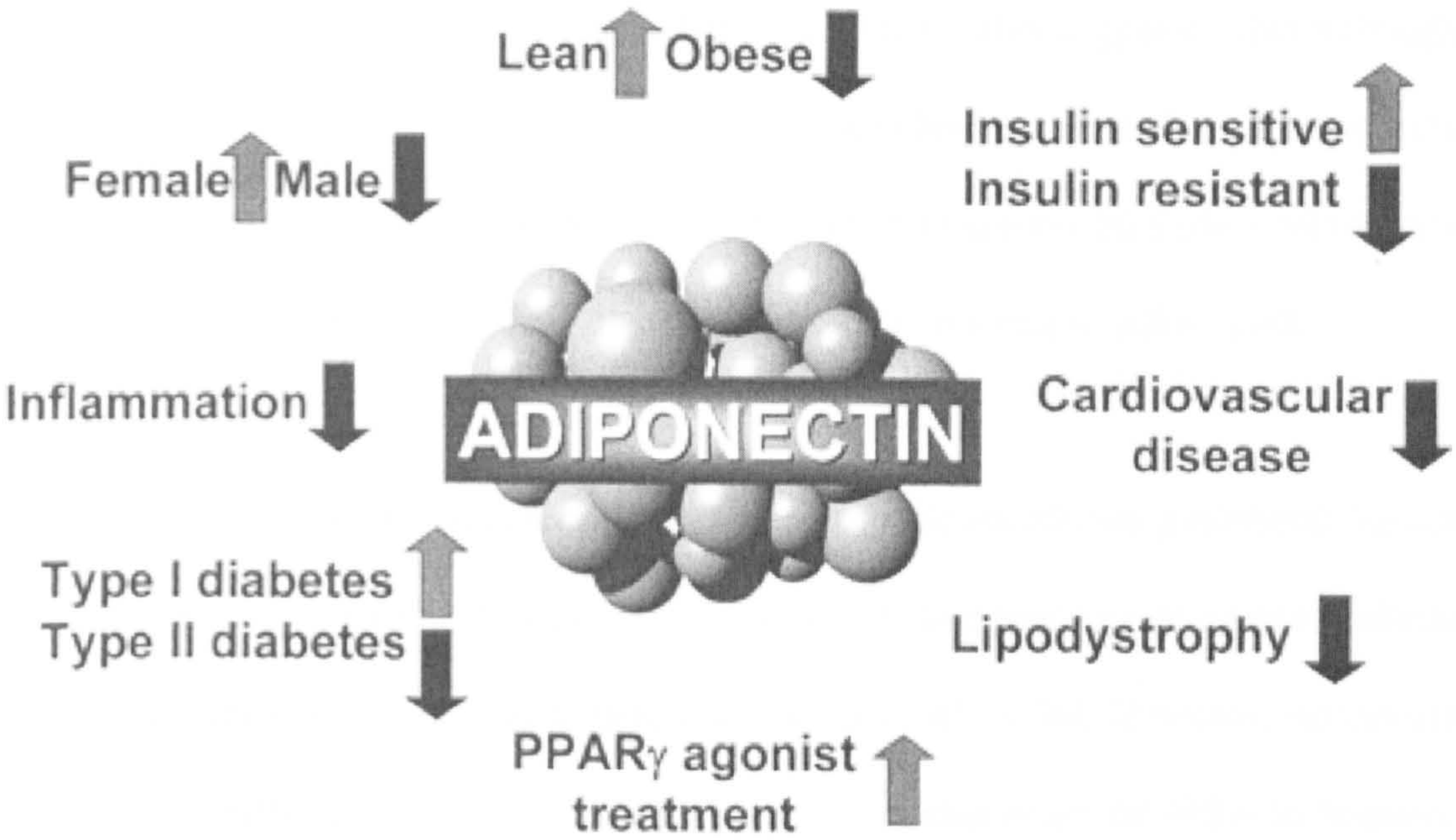


Figure 7.2 Summary of the most significant factors and disease states that result in an up-regulation or down-regulation of adiponectin in adipose tissue. Figure obtained from (Trujillo *et al.* 2005).

In serum, adiponectin circulates in several different complexes, each of which have predominant functions (Berg *et al.* 2002; Pajvani *et al.* 2003). Several studies have demonstrated that pharmacological treatment with recombinant adiponectin affects both glucose and lipid metabolism (Fruebis *et al.* 2001; Tsao *et al.* 2002; Yamauchi *et al.* 2002; Yamauchi *et al.* 2003). More specifically, administration of a recombinant proteolytic fragment of adiponectin (globular adiponectin) into mice, decreases plasma glucose, without altering food-intake (Fruebis *et al.* 2001). Although the potent effects of the pharmacologically generated recombinant globular adiponectin have been described in relation to lipid oxidation and glucose uptake in muscle (Fruebis *et al.* 2001; Yamauchi *et al.* 2002); it has been suggested that such affects are unlikely to be a reflection of the function of the endogenous protein, as no molecular equivalent to the globular form to date, has been identified *in vivo* (Rajala *et al.* 2003; Nawrocki *et al.* 2004). The studies in this thesis have, for the first time, identified the

trimeric form of adiponectin *in vivo*. Other studies have also suggested that although globular adiponectin is known to circulate in human plasma at low abundance of ~1% of total adiponectin, levels of adiponectin are ~100-fold greater than other adipokines (Fruebis *et al.* 2001), thus highlighting the importance of trimeric adiponectin.

In addition to studies demonstrating the effects of adiponectin on peripheral insulin sensitisation, recent rodent studies have revealed that adiponectin can exert its effects on energy homeostasis via central mechanisms (Qi *et al.* 2004). However, no studies to date have fully established whether adiponectin is able cross the BBB in humans. The studies in this thesis determined that the adiponectin trimer is the most predominant oligomer expressed in human CSF. Such a result may have major implications for adiponectin in energy homeostasis. The globular form of adiponectin is implicated to function predominantly by regulating skeletal muscle insulin sensitisation and energy expenditure (Fruebis *et al.* 2001), by promoting lipid catabolism and glucose uptake. This study suggests that such effects may occur via the CNS; therefore proposing a secondary anorexigenic function for adiponectin to hepatic insulin sensitisation in humans. The globular form of adiponectin may function in a similar manner to leptin; signalling from adipose to peripheral skeletal muscle tissues, via the hypothalamus; serving as an adipogenic hormone with neurogenic properties. Such a mode of action for trimeric adiponectin may be subsequently altered by gender. Western blot analysis revealed sexual dimorphism of adiponectin levels, in that males have a higher CSF-to-serum ratio of total adiponectin; this was only partly reflected by sub-fractionation data. Although not significant, male subjects express slightly higher levels of trimeric adiponectin in serum and CSF. Further adiponectin FPLC studies using a larger cohort of subjects

could be beneficial and may establish significant differences in adiponectin oligomeric structures in relation to gender. Such results also suggest androgens as potential contributory factors in the alteration of adiponectin complex distributions, thus influencing peripheral insulin sensitivity.

7.4 Future Perspectives for Resistin & Adiponectin.

Recent studies have shed light on differences between resistin in rodents and humans, indicating the diversity of resistin action in these species. However, further studies are required to establish the relevance of resistin to human T2DM, in particular its effects on the CNS and β-cell function. Further work is also required to understand the basis for formation of different HMW and LMW oligomers of resistin in circulation and, their functionally effects. Recent work has determined that resistin undergoes post-translational modification, leading to the molecule being secreted as two major oligomers (Patel *et al.* 2004). Moreover, HMW forms of resistin have recently been identified in both rodents and humans serum (Patel *et al.* 2004; Gerber *et al.* 2005). In contrast, HMW forms of adiponectin, rather than total amounts, have been shown to correlate better with TZD-mediated improvements in insulin sensitivity (Pajvani *et al.* 2004); a similar scenario may well apply for resistin. As it is unknown which resistin oligomer is more associated with increased disease risk, development of assays specific for the different molecular weight forms of resistin, rather than absolute measurements, would prove beneficial. Furthermore, future studies examining the oligomeric distribution of resistin in serum and matched CSF using FPLC, would certainly be interest. It should also be noted that resistin-like molecules, such as the RELM-β, may have relevance to pathological states, which may need further

investigations. Additionally, identification of a receptor for resistin would be a major advance to a clearer understanding of its function and signalling mechanisms.

With regards to adiponectin, whilst this study determined the presence of trimeric and LMW forms of adiponectin in human CSF; future studies using FPLC to examine the adiponectin oligomeric distribution in serum and matched CSF from T2DM subjects' pre and post-TZD treatment or in relation to pre-and post-prandial insulin levels, may further elucidate the central properties of adiponectin in the brain. Additionally, deducing whether the human trimeric or LMW hexameric forms of adiponectin exert similar effects on energy expenditure as those observed in rodents would be of interest (Qi *et al.* 2004). Furthermore, determining what factors maintain the equilibrium between globular adiponectin in central and peripheral energy homeostasis *versus* HMW full-length adiponectin in hepatic insulin sensitisation would prove beneficial. In addition to this, establishing how exactly adiponectin signals through anorexigenic POMC neurons in the arcuate nucleus and paraventricular nucleus (PVN) to maintain energy expenditure would further define the central actions of adiponectin in humans. Finally, as receptors for adiponectin have been identified in the PVN (Qi *et al.* 2004), exploring the expression of adiponectin receptors in the hypothalamus in various pre- and post-fasted states and explore the possibility of other high-affinity receptors within the hypothalamus would also prove illuminating.

In summary the results of this thesis have shown that resistin and adiponectin play an important role in the obesity-related inflammation and energy homeostasis respectively. It is apparent that alteration in the production of adipokines via chronic

activation of the innate immune system may promote obesity-related T2DM. Maintaining such an adipokine balance is therefore critical to the health of the individual. However, the growing knowledge of the role of inflammatory molecules generated within adipose tissue and, their link to T2DM, means that interventions that reduce the production of pro-inflammatory cytokines, for instance NF-κB or JNK inhibitors, may have therapeutic potential.

APPENDICES

APPENDIX I

AI.1 WESTERN BLOTTING SOLUTIONS.

1.1 Sodium Dodecyl Sulphate (SDS) (4%)

10 ml 20% SDS solution
50 ml dH_2O
Solution stored at room temperature (RT)

1.2 Loading buffer

625 μl Tris-HCl (pH 6.8) 125 mM
500 μl SDS 4%
1 ml Glycerol
200 μl Dithiothreitol (DTT)
125 μl Bromophenol Blue
250 μl Distilled H_2O

1.3 5X Laemmli Loading buffer

0.5 M DTT in 2 M Tris HCl pH 8.8
10% SDS
50 % v/v glycerol
10% w/v bromophenol blue
dH_2O

Electrode Buffer for SDS-PAGE Electrophoresis		
REAGENT	**FINAL CONCENTRATION (X5)**	**QUANTITY (DILUTED IN 1L)**
Tris	1.24×10^{-1} M	15 g
Glycine (Biorad, Hercules, CA, USA)	9.6×10^{-1} M	72 g
SDS	20% (v/v)	25 ml

Transfer Buffer for Electrophoretic Transfer		
REAGENT	**FINAL CONCENTRATION (1X)**	**Quantity (DILUTED IN 4L)**
Tris	25 mM	15.15 g
Glycine	192 mM	72.0 g
Methanol	100%	1 L

1.4 Phosphate Buffered Saline (PBS) (pH 7.6)
PBS 120 mM
NaCl 2.7 mM
KCL, 10 mM

1.5 PBS-Tween (PBS-T) (1.0%)
1 L PBS (prepared as above)
1 ml Phosphate Buffered Saline (PBS) ('Tween 20' (0.1% (v/v), Sigma UK).
Solution stored at RT.

1.6 PBS/PBS-T solution for antibody preparation (0.5%)
X quantity 1.0% PBS-T (prepared as above)
X quantity PBS (prepared as above)

1.7 Tris-buffered Saline-Tween (TBS-T) (10X): 0.5M Tris Base, 9% NaCl, pH 7.6
61 g Trizma base
90 g NaCl
1 L dH_2O
Solution mixed to dissolve and pH adjusted using HCl. Solution stored at RT.

1.8 TBS-T (1X)
TBS-T (10X) diluted (1:10) with dH_2O

1.9 Blocking Solution for Millipore® filters (20%)
20 g non-fat milk solution (Marvel Milk Powder, UK)
200 ml PBS 0.5% PBS (Tween 20 (0.1% (v/v), Sigma UK)

AI. 2 GENERAL CELL-CULTURE SOLUTIONS.

2.1 Lysis buffer

Ammonium Chloride (NH_4Cl)	0.154 mol/l
Potassium Bicarbonate ($KHCO_3$)	10 mmol/l

2.2 Phenol red-free medium

Dulbecco's minimal essential medium (DMEM/F-12) Phenol red free
1% transferrin (see below)
Penicillin (100 U/ml) and streptomycin (100 mg/ml) added.
Medium was stored at 4°C.

2.3 Transferrin

Transferrin binding-protein found in serum, is responsible for the transfer of iron to cells. Transferrin binds Fe^{2+} and prevents its oxidization to Fe^{3+}, preventing loss of iron from the medium. The stimulatory activity of transferring is proposed to be associated with its iron binding properties and, it is further capable of binding other metal irons in the medium at concentrations which are toxic (Barnes *et al.* 1980).

AI. 3 BUFFERS & SOLUTIONS USED IN RT-PCR PROCEDURES.

3.1 DN*ase* Treatment

DNase I	REACTION BUFFER	STOP SOLUTION
1 U/μl in 50% Glycerol 10 mM Tris-HCl (pH7.5) 10 mM $CaCl_2$ 10 mM $MgCl_2$	200 mM Tris-HCl (pH 8.3) 20 mM $MgCl_2$	50 mM EDTA

3.2 Reverse Transcription (RTn) Buffer

100 mM Tris-HCl (pH 9.0 at 25°C)
500 mM KCl
1% Triton® X-100

3.3 Taq DNA polymerase stored in RTn Buffer

50 mM Tris-HCl (pH 8.0)
100 mM NaCl
0.1 mM EDTA
1 mM DTT
50% Glycerol

AI 4 FPLC SOLUTION

Column Running buffer
25 mM HEPES
150 mM NaCl
1 mM $CaCl_2$
Solution stirred to dissolve contents then adjusted to pH 8.0
Solution was stored at RT

APPENDIX II

RTn & REAL-TIME PCR

2.1 mRNA sequence.

Within any gene, the deoxyribonucleic acid (DNA) or genetic code that encodes the protein structure is divided into introns (non-coding) and exons (coding) regions. When a gene is transcribed from the DNA strand inside the nucleus by RNA polymerase, messenger ribonucleic acid (mRNA) is produced, which comprises of only the exons. Each gene can consist of a varied number of exons. It is these regions that will ultimately describe the specific polypeptide chain that will be assembled within the ribosomes located in the cytoplasm of cells. Therefore, quantitative analysis of mRNA will give an accurate analysis of the 'blueprint' prepared for protein synthesis.

2.2 RT-PCR.

The initial materials for PCR are reliant on complementary DNA (cDNA) to serve as a template; DNA must therefore be synthesised from the extracted RNA. This process is termed reverse transcription (RTn) and subsequently allows the synthesis of cDNA. Such a process involves 'reading' mRNA sequences to then assemble a cDNA chain, that comprises the complimentary bases to that on the mRNA strand (guanine bonds with cytosine and, adenine to thymine) (**Figure AII.2.1**).

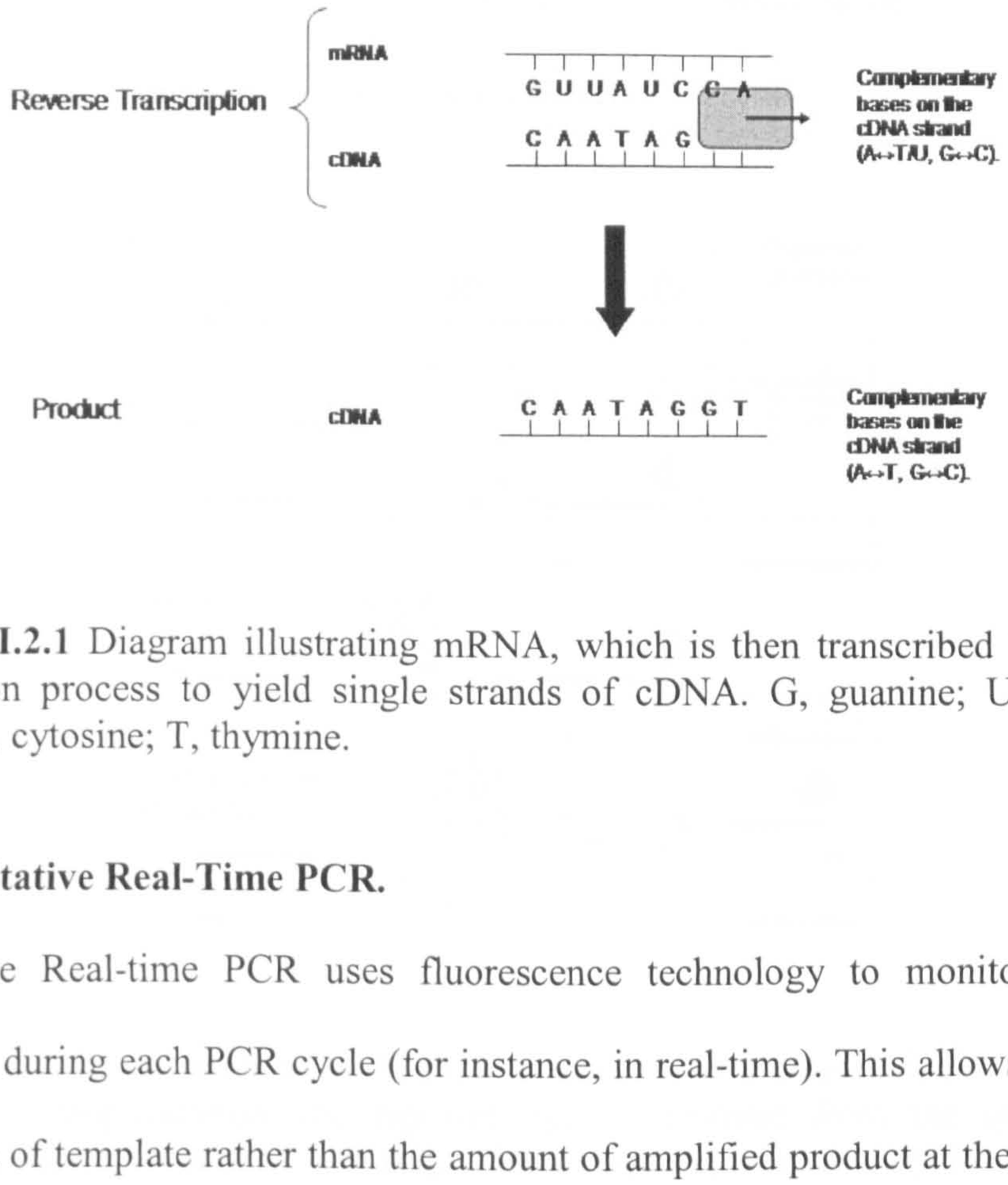


Figure A1I.2.1 Diagram illustrating mRNA, which is then transcribed in a reverse transcription process to yield single strands of cDNA. G, guanine; U, uracil; A, adenine; C, cytosine; T, thymine.

2.4 Quantitative Real-Time PCR.

Quantitative Real-time PCR uses fluorescence technology to monitor amplicon production during each PCR cycle (for instance, in real-time). This allows analysis of the amount of template rather than the amount of amplified product at the endpoint of the reaction. In this study, the mRNA levels were analysed using an ABI 7700 Sequence Detection system, which utilises TaqMan chemistry for highly accurate quantification of specific mRNA levels (**Figure AII.2.4**). TaqMan probes contain a fluorescent reporter dye usually on the 5' base and a quenching dye typically on the 3' base, which suppresses the reporter. Due to the close proximity of the two, the quencher prevents emission of any fluorescence while the probe is intact. If the gene of interest is present, the probe anneals between the forward and reverse primer sites within the PCR product. When the Taq DNA polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This removes the reporter from the proximity of the quencher, resulting in a florescent signal that accumulates with each cycle. The fluorescent signal yielded can be

quantitatively measured by a laser and charged coupled device (CCD) camera, enabling real time detection of cDNA amplification.

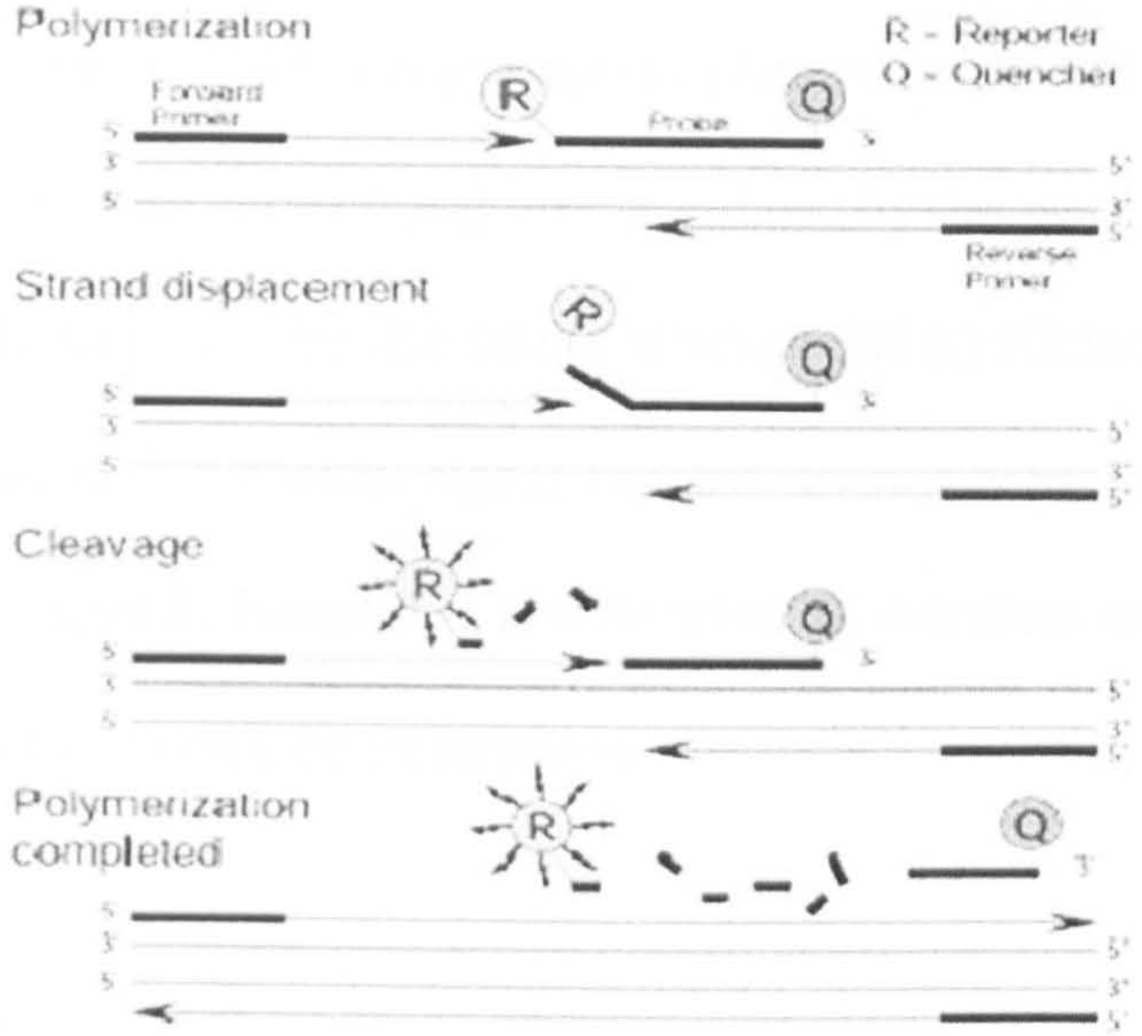


Figure AII.2.4 Diagram showing TaqMan chemistry. When probe is cleaved from the DNA during amplification, the reporter dye is removed from the quencher and fluoresces. This increases in intensity with every amplification cycle, allowing for real-time assessment of the reaction.

APPENDIX III

WESTERN BLOTTING

3.1 Calculation of Protein Content of Samples for Western blot Analysis.

Protein samples were analysed using a spectrophotometer at 655 nm. Conversion of optical densities to protein content (μg) was calculated by the construction of a standard curve (**Figure AIII.3.1**) using bovine serum albumin (BSA) diluted in dH_2O, each time samples were assayed. No protein signal (optical density) was detected in a mixture containing only Reagent S, Reagent A and Solution B, therefore excluding interference with calculated protein sample concentrations.

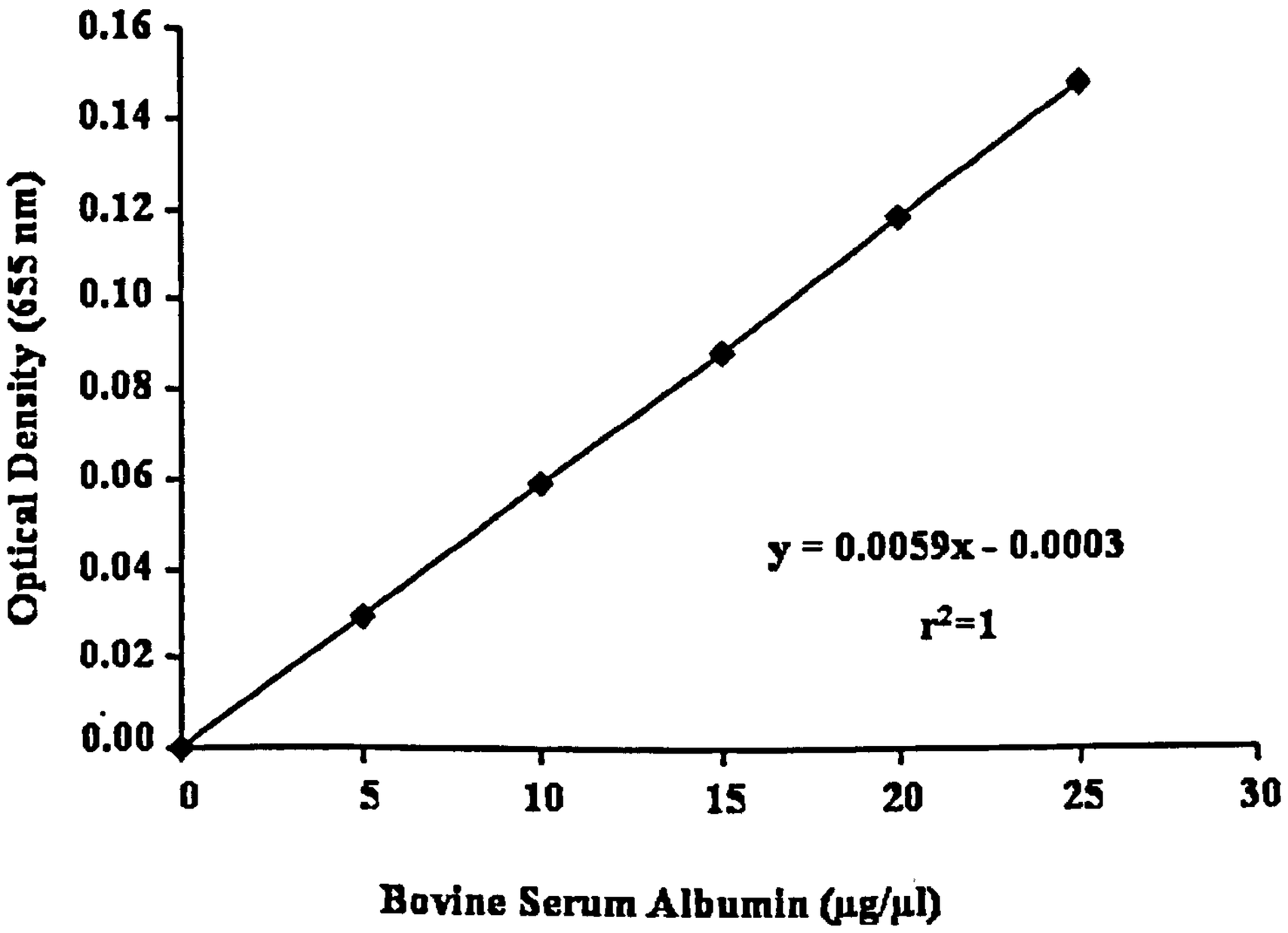


Figure AIII.3.1 Graph to show standard curve used as a reference to calculate protein content in samples of proteins extracted from adipose tissue and isolated adipocytes. Bovine serum albumin was diluted in dH_2O to known concentrations and absorbance read at 655 nm on a spectrophotometer.

3.2 Rainbow Marker for Protein Size Comparison in Western blot Analysis.

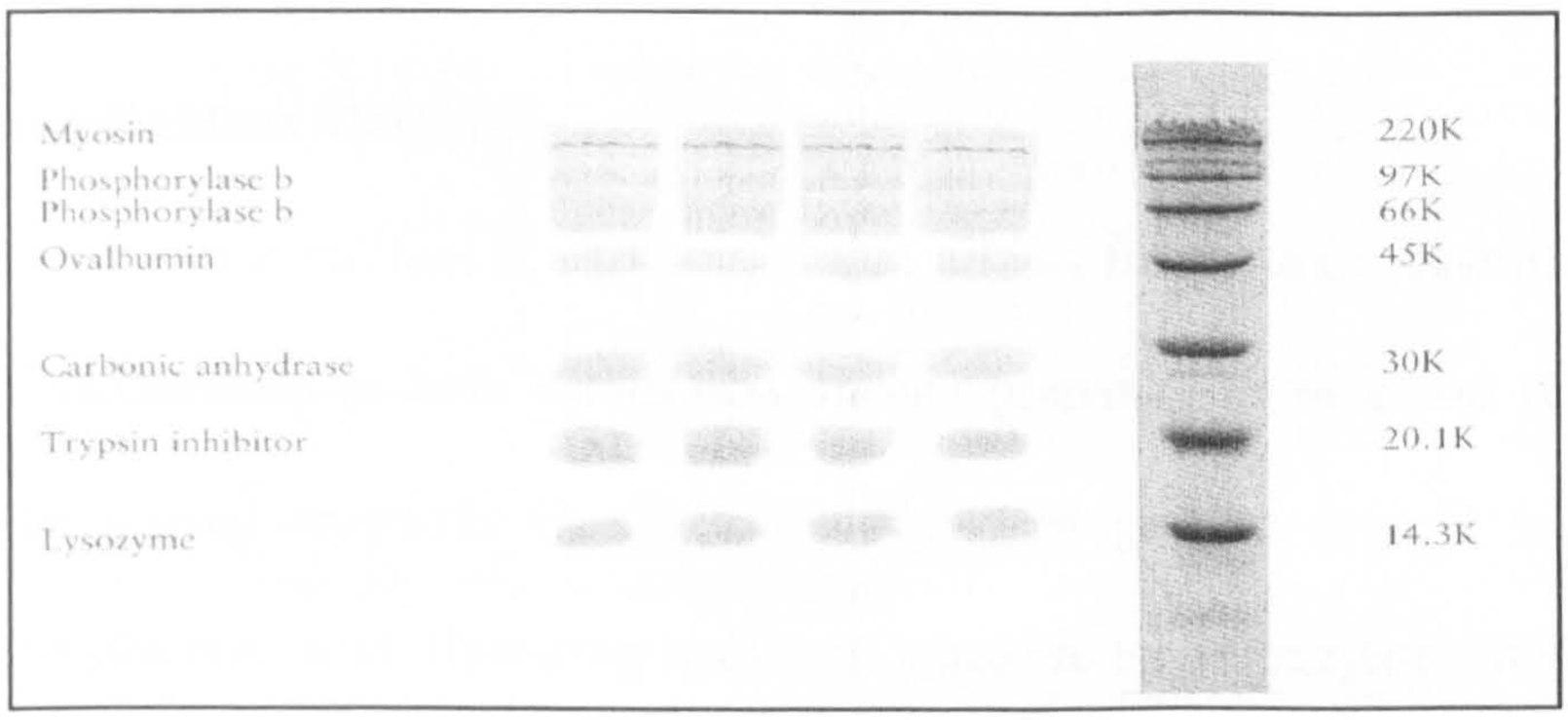


Figure AIII.3.2 A ladder of proteins used as a marker (Amersham Pharmacia Biotech, Buckinghamshire, UK), which is resolved on a 8-15% SDS-PAGE gel (Laemmli 1970). Black bands represent the same gel exposed to photographic film (Hyperfilm™ β-max).

APPENDIX IV

DEFINING PLASMA GLUCOSE LEVELS & HOMA INDEX

4.1 Glucose Oxidase Method.

The glucose oxidase method is a technique that allows for accurate quantification of glucose concentration in both serum and plasma preparations of blood (Lott *et al.* 1975). The procedure works on the knowledge that glucose present in blood is oxidized to gluconic acid, liberating hydrogen peroxide by an enzyme called glucose oxidase. The liberated hydrogen peroxide is converted to water and oxygen, by peroxidase. The level of converted oxygen can be measured using an oxygen accepter termed 4 aminophenazone, which takes up oxygen to form a pink covered chromogen, which is then detected. Plasma glucose levels remain stable at RT for up to 6 hr.

4.2 The Homeostasis Model Assessment (HOMA) Index.

The HOMA model evaluates insulin sensitivity and β-cell function as a percentage of a normal reference population. This index works on the premise that blood insulin and glucose levels are determined by a feedback loop between the liver and pancreatic β-cell. As described in this thesis, high glucose levels are accounted for by a compensatory mechanism that increases insulin secretion from the β-cell. Thus the proportion of insulin and glucose should be directly reflective of insulin sensitivity. A mathematical model was derived to estimate the amount of β-cell function and insulin resistance which would equate to steady plasma levels of insulin and glucose in each individual. The formula used in this thesis to interpret HOMA was as follows: (Fasting insulin (μU/l) x Fasting Glucose (mmol/l))/ 22.5 = HOMA index. Alternatively, a HOMA-IR calculator can be downloaded from the following website: www.dtu.ox.ac.uk/index.html?maindoc=/homa/.

CHAPTER 8

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