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THE HLA-B ASSOCIATED TRANSCRIPT 1 (BAT1) EXPRESSION IN HUMAN ADIPOSE TISSUE; BAT1 MODULATION WITH INCREASING ADIPOSITY AND DIABETES

By

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Dedicated to

my family

my dearest friend Dr Saif Alhusaini

my mentors Professor Sudhesh Kumar & Dr George Valsamakis

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DECLARATION

I declare that this thesis is an accurate record of my results obtained by myself within the labs at University of Warwick, Clinical Science Research Institute and, the data that has arisen is detailed in this thesis. All sources of support and technical assistance have been stated in the text of the acknowledgments. None of the work has been previously submitted for a higher degree. All sources have been specifically acknowledged by means of reference.

SYNOSPIS

Obesity and type 2 diabetes (T2DM) are both inflammatory disorders with parallel escalating epidemics. Novel insights provided by the new biology suggest common pathways by which several pathogenic components of obesity affect glucose metabolism and cellular responsiveness to insulin leading eventually to the development of T2DM; inflammation is considered critical for the development of the above metabolic disorders and is directly influenced by weight gain. Adipose tissue (AT), particularly the abdominal fat depot is currently considered source of inflammatory agents that fuel whole body's low grade inflammatory state. The HLA-B Associated Transcript 1 (BAT1) is a cellular member of the DExD/H-box RNA-helicases with essential role for cellular mRNA export, that also attains anti-inflammatory properties, as it was shown by studies investigating monocytes and T-cell lines. Furthermore, BAT1 polymorphisms were linked to predisposition to immunopathologic disorders including type 1 diabetes. These findings suggest a potential protective role of BAT1 against the obesity-associated low-grade inflammatory state that contributes to T2DM development. The role of BAT1 in the adipocytes has not been investigated so far.

Therefore, this thesis examined BAT1 expression and regulation within specific human AT depots and the adipocyte itself. Initial studies indicated BAT1 expression in ex vivo human AT but also the repressing effect of increasing adiposity and T2DM on BAT 1 expression. Remarkably, there was no difference in BAT1 expression between obese subjects and patients with T2DM indicating that BAT1 becomes suppressed with

increasing adiposity and remains suppressed through to the development of T2DM and thereafter; this could in turn reduce the capacity to response to the inflammatory insults.

As human AT contains many different types of cells besides adipocytes, including fibroblasts, macrophages, lymphocytes, pre-adipocytes and endothelial cells, some of which actually increase with increasing adiposity (*e.g.* macrophages and lymphocytes) subsequent studies determined the expression of BAT1 particularly in isolated human primary pre-adipocytes and mature adipocytes; the human pre-adipocyte cell line Chub-S7 was used for this purpose. It was shown that BAT1 (mRNA and protein) was expressed in both cell types with maximum expression in mature (lipid accumulating) adipocytes. At the stage of complete maturation, the effects of nutrients and inflammatory factors on BAT1 expression were examined. Both glucose and non-esterified fatty acids (NEFA) were shown to repress BAT 1; these findings were in keeping with the *ex vivo* data determined in terms of AT from obese and T2DM subjects. Furthermore, these studies indicated a synergistic action of both JNK and NFκB when used in combination to reduce BAT1 expression, indicating interconnectivity between JNK and NFκB pathways, as noted in other human AT studies examining other molecules. Regarding NEFA however, the JNK pathway seemed to mediate its repressing effect on BAT1. These studies also showed that the potent inflammatory agent lipopolysacharide (LPS) also significantly reduced BAT1 expression which was again in keeping with the previous *ex vivo* AT data since LPS is raised in conditions of metabolic disease. Finally, the investigation of the paracrine influences of leptin and resistin on differentiated primary adipocytes highlighted BAT 1 repression whilst adiponectin appeared to have no significant effect alone to alter BAT 1 expression or inhibit LPS-induced BAT1

repression. Taken together, BAT1 was more susceptible to the repressing effects of nutritional factors (glucose and NEFA) in excess than paracrine inflammatory or anti-inflammatory adipokines. The fact that several factors modulate BAT1 expression may suggest that BAT1 represents a first line, non-selective, cellular protective agent, which is therefore influenced by several different factors through common inflammatory pathways. Thus, BAT1 suppression may be an early key event in the pathogenesis of a low chronic inflammatory state. As such BAT1 could represent an important target to manipulate to combat the low chronic inflammatory state observed in both obese and T2DM patients.

ABBREVIATIONS

AbSc	Abdominal Subcutaneous
AbSc AT	Abdominal Subcutaneous Adipose Tissue
Akt	also known as Protein Kinase B (PKB)
ACCORD	Action to Control Cardiovascular Risk in Diabetes
AIDS	Acquired immune deficiency syndrome
AMPK	AMP-activated Protein Kinase
ANG II	Angiotensinogen II
ANOVA	Analysis Of Variance
AP-1	Activator Protein - 1
AT	Adipose Tissue
ATMs	Adipose tissue macrophages
ATP	Adenosine tri phosphate
BAT1	HLA-B associated transcript 1
BMI	Body Mass Index
BSA	Bovine Serum Albumin
C	Control
cDNA	Complimentary DNA
CEBP-α	CCAAT enhancer-binding protein-α
CNS	Central nervous system
CRP	C-reactive Protein
Ct	Cycle Threshold
CVATT	Critical visceral adipose tissue threshold
CVD	Cardiovascular Disease
Da	Daltons
DC	Detergent Compatible ()
DCCT	Diabetes Control and Complications Trial
ΔCt	Delta Cycle Threshold
DGAT2	Diacylglycerol acyltransferase 2
dH2O	Distilled water
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DN*ase*	Deoxyribonuclease
dNTPs	Deoxynucleotides Triphosphates
dsRNA	Double-stranded RNA
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-linked Immunosorbant Assay

ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated protein kinase
FAM	RT-PCR Reporter Fluorochrome/Dye Label
FFA	Free Fatty Acid
FFAs	Free Fatty Acids
FTO gene	Fat mass and obesity-associated gene
GAD65	Glutamic acid decarboxylase$_{65}$
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCOS	GeneChip® Operating Software
gDNA	Genomic DNA
GIP	Gastric inhibitory peptide
GLP-1	Glucagon-like peptide
GLUT-4	Glucose-transporter-4
GRB2	Growth factor receptor-bound protein 2
GSK-3	Glycogen synthase kinase 3
GSK-3β	Glycogen synthase kinase 3β
HBSS	Hanks' balanced salt solution
HCl	Hydrogen chloride
HIV	Human immunodeficiency virus
HLAs	Human leukocyte antigens
H6PD	Hexose-6-phosphate dehydrogenase (
HPV-E7	Papillomavirus E7 oncoprotein
hr	Hour
HRP	Horseradish peroxidase
hTERT	Human telomerase reverse transcriptase
IBMX	3-Isobutyl-1-Methylxanthine
ICAM-1	Intercellular Adhesion Molecule-1
IDF	International diabetes federation
IFNγ	Interferon gamma
IgA	ImmunoglobulinA
IGT	Impaired Glucose Tolerance
IKK	Inhibitor of NF-κB Kinase
IKK	Inhibitor of NF-κB Kinase
IL	Interleukin
IL-1	Interleukin-1
IL-1ß	Interleukin-1 ß
IL-6	Interleukin-6
IL-10	Interleukin-10
IR	Insulin Resistance
IRS	Insulin Receptor Substrate

IRS-1	Insulin Receptor Substrate-1
IRS-2	Insulin Receptor Substrate-2
IRS-3	Insulin Receptor Substrate-3
JNK	c-Jun N-terminal Kinase
K_{ATP} channels	ATP-sensitive potassium channels
kDa	Kilodaltons
kg	Kilogram
L	Litre
LPS	Lipopolysaccharide
M	Molar
m^2	Meter squared
MAPK	Mitogen-activated Protein Kinase
M-CSF	Macrophage-Colony stimulating factor
MCP-1	Monocyte Chemotactic Protein-1
MetS	Metabolic Syndrome
µg	Microgram
mg	Milligram
MHC	Major histocompatibility complex
min	Minute (time)
µl	Microlitre
ml	Millilitre
mM	Millimolar
µM	Micrololar
mRNA	Messenger Ribonucleic acid
mTOR	Mammalian target of rapamycin
N	Number
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified Fatty Acid
NEFAs	Non-esterified Fatty Acids
NFkB	Nuclear Factor-κB
ng	Nanogram
NHANES III	National Health and Nutrition Examination Survey III
N_2	Nitrogen
NK	natural killer
nM	Nanomoler
NO	Nitric oxide
ODU	Optical density units
Om	Omental
Om AT	Omental Adipose Tissue

PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate-buffered Saline
PBS-T	Phosphate-buffered Saline containing 0.1% Tween 20
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate Carboxylase
PI3K	Phosphoinositide-3 Kinase
PKB	protein kinase B
PKC	Protein Kinase C
PPAR-γ	Peroxisome Proliferator Activated Receptor-γ
p-value	Probability value
PVDF	Polyvinylidene-fluoride
PYY	Peptide YY
QRT-PCR	Quantitative Real-Time Polymerase Chain Reaction (
RBP-4	Retinol binding protein-4
RFLP	Restriction fragment length polymorphism
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Room Temperature
RT-PCR	Real-time PCR
s	Second (time)
SAPK	Stress-activated protein kinase
Sc	Subcutaneous
Sc AT	Subcutaneous Adipose Tissue
SD	Standard Deviation
SEM	Standard Error of the Mean
SnRNP	Small nuclear ribonucleoproteins
SOCS	Suppressor of cytokine signaling
SOS	Son of Sevenless
SOS study	Swedish Obese Subjects Study
SREBP-1c	Steroid regulatory element-binding protein-1c
Taq	Thermus Aquaticus (DNA polymerase)
TBS	Tris-buffered Saline
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 Diabetes Mellitus
TEMED	N, N, N’, N’-Tetramethylethelenediamine
TERTs	Human telomerase reverse transcriptases
TG	Triglyceride
TLR	Toll-like Receptor
TLR-4	Toll-like Receptor-4

TLRs	Toll-like Receptors
TNF-α	Tumour Necrosis Factor-α
TOR	Target of rapamycin
UCP2	Uncoupling protein2
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volts
WHO	World Health Organisation
yrs	Years

Chapter 1

Introduction

1.1 **Obesity**

1.1.2 Definition - Classification - Risk Status Assessment

Obesity is a serious growing global health problem affecting more than 400 million people worldwide. It is associated with more than 45 comorbidities and a cluster of atherogenic disorders that compose the metabolic syndrome; the latter is recognized by the International Diabetes Federation guidelines as a progressive condition that contributes to the development of diabetes increases the risk of adverse cardiovascular events and mortality from all causes. WHO, has defined obesity, as a 'disorder of body composition in that there is an abnormal, absolute or relative proportion of body fat in relation to lean body mass, to the extent that health is impaired' (WHO 2011).

According to the National Health and Nutrition Examination Survey III (NHANES III), the morbidity and mortality rates are closely related to the degree of obesity making the classification of the weight status imperative, as this enables health practitioners to stratify individual's health-risk and thus to modify the level of intervention accordingly. Body Mass Index (BMI) and waist circumference correlate well with total adiposity (Marc-Andre Cornier *et al*. 2002; Lemieux *et al*. 1996) and are used in clinical practice for the estimation of weight status. Increasing BMI and increasing waist circumference have been both associated with increasing risk of death from cardiovascular disease and mortality from all causes (Despres *et al*. 1990), while the presence of any obesity-related disorders increases further the overall mortality risk (Yusuf *et al*. 2002; Webster *et al*. 1984).

BMI is derived by dividing the body weight (kg), by the square of the height (m). It is considered a relatively accurate marker of total body fat mass. Individuals with BMI

between 18.5 and 24.9 kg/m^2 are classified as normal weight, while those with BMI 25-29.9 kg/m^2 as overweight. Patients with BMI 30-34.9 kg/m^2 are classified as obese-Class I, those with BMI 35 -39.9 kg/m^2 are classified as obese-Class II and finally those with BMI of 40 kg/m^2 or over are classified as extremely obese Class III. BMI values greater than 25 kg/m^2 increase the risk of morbidity and mortality for many diseases [6], including type 2 diabetes mellitus (T2DM), hypertension, heart disease, stroke and arthritis (Table 1.1.2).

Table 1.1.2 Increased Risk of Obesity Related Diseases with Higher BMI

Disease	**BMI ≤25 kg/m^2**	**BMI between 25 and 30 kg/m^2**	**BMI between 30 and 35 kg/m^2**	**BMI of ≥35 kg/m^2**
Arthritis	1.00	1.56	1.87	2.39
Heart Disease	1.00	1.39	1.86	1.67
Type 2 Diabetes	1.00	2.42	3.35	6.16
Gallstones	1.00	1.97	3.30	5.48
Hypertension	1.00	1.92	2.82	3.77
Stroke	1.00	1.53	1.59	1.75

Source: American Obesity Association; Centers for Disease Control. Third National Health and Nutrition Examination Survey. Analysis by The Lewin Group, 1999

1.1.3 Body Fat Distribution

Obesity is a heterogeneous condition with respect to regional distribution and biological properties of fat tissue (Bouchard *et al*. 1993; Vague 1947). In the 1950s, Vague (Vague 1947) first proposed that excess fat stored on the trunk could be metabolically more damaging than fat stored on the limbs. This theory was later proved to be true by Kissebah & Krakower in 1994 and Kahn and Flier in 2000, so that central adiposity to be considered nowadays an independent risk factor for the development of insulin resistance and T2DM later in life. Visceral adipose tissue refers to fat accumulation within omental and mesenteric fat depots and constitutes about 6-20% of total body fat tissue. It is less receptive to the anabolic effects of insulin and metabolically-lipolytically more active than the peripheral fat tissue which refers to subcutaneous fat accumulation and comprises 80% of total adipose tissue.

1.1.4 Obesity-associated morbidities

Many studies have shown increased morbidity and mortality among obese individuals (Bell *et al* 2001; Kopelman PG 2000; Berrios *et al* 1997) which actually begins to rise at BMI $\geq$25kg/m^2 and even more sharply when BMI exceeds 30kg/ m^2. Hypertension, hyperlipidaemia, obstructive sleep apnoea insulin resistance and T2DM are only some of the obesity-related diseases, while some types of cancer, such as breast, colon, prostate and endometrial cancer, are also more common in obese individuals (Fig 1.1.4). As a result, obesity is not considered only as a cosmetic problem but as a very serious disease which if left untreated increases T2DM, cardiovascular disease and mortality from all causes.

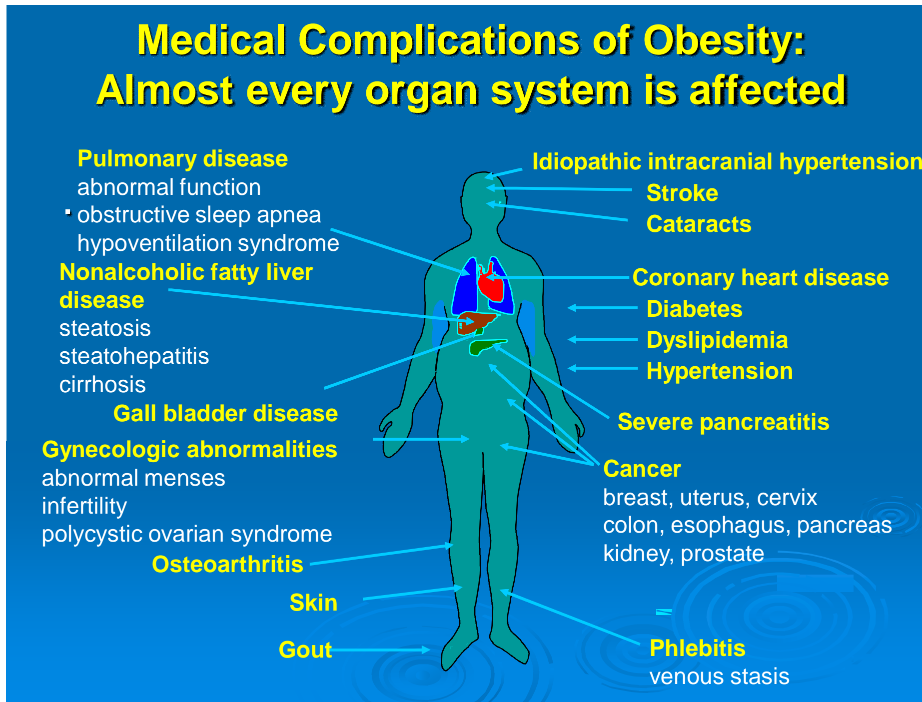


Fig 1.1.4 Obesity-associated morbidities

1.1.5 Obesity and T2DM relation - Diabesity

The prevalence of diabetes took a sharp and unexpected upward turn in the last couple of decades. Data from large epidemiologic studies reveal the parallel escalation of obesity and diabetes epidemics and suggest increased risk of T2DM with increasing obesity; it is estimated that 51-59% of patients with T2DM are obese (Fig 1.2) (Ogden *et al*. 2002; Barsh GS *et al*. 2000). Obesity and diabetes are both characterized by defects of insulin action; the term ‘diabesity’ express their close relationship to each other. Further to the degree of adiposity, the duration of obesity, the genetic susceptibility (Comuzzie 2002; Maes *et al*. 1997) and the central adiposity estimated in clinical practice with the use of waist circumference and waist to hip ratio seem to be better predictors of diabetes

development (Stunkard *et al.* 1990). Results of recent studies in obese subjects and patients with T2DM made the winds of change to blow in the treatment of diabetes. Action to Control Cardiovascular Risk in Diabetes (ACCORD) Trial which investigated the effect of intensive glycaemic control in patients with T2DM was stopped because of increased mortality in the tight controlled group, which had gained at the same time the more weight (Silventoinen *et al.* 2000). In The Swedish Obese Subjects (SOS) study where morbidly obese patients were followed for an average of 11 years after bariatric surgery it was demonstrated that weight loss reduced by >80% the diabetes mortality (Carmichael and McGue 1995). Furthermore, the Diabetes Prevention Program (DPP) showed 58% reduction in the incidence of diabetes following sustained moderate weight loss of just 5-10% of the initial body weight (Knowler et al. 2002). Similar were the findings of several other studies (Look AHEAD, Finnish Diabetes Prevention, Da Qing) while modern non-insulin injectable forms of diabetes treatment with incretin analogues and mimetics that reduce body weight appear to reduce the cardiovascular risk further, supporting a current belief of a shift from glucocentric to weight-centric management of diabetes. Up to date, several theories linking different pathogenic mechanisms that make obese individuals prone to develop diabetes have been suggested. Although a unifying hypothesis still remains elusive, most of them suggest insulin resistance and progressive pancreatic ß-cells failure as the underlying pathogenic mechanism.

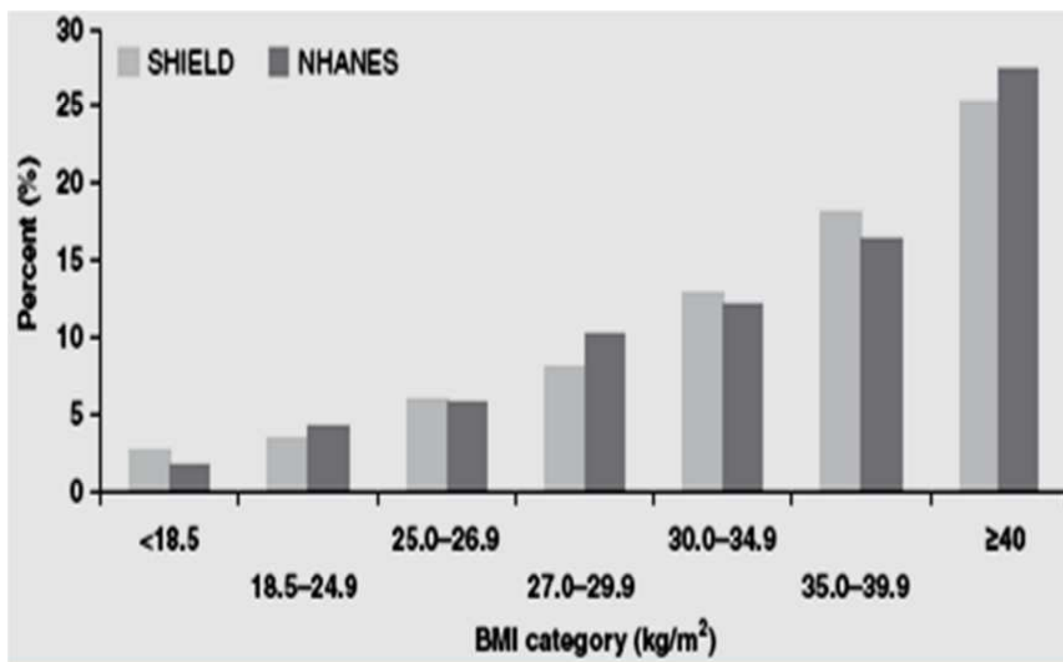


Fig 1.1.5 Prevalence of Diabetes (%) by BMI level as it is revealed by the epidemiologic studies SHIELD and NHAHES (Bays *et al.* 2007)

1.2 Obesity and impaired glucose metabolism

1.2.1 Physiologic Actions of Insulin and the Insulin-Signaling

Insulin is an important metabolic hormone released by the pancreatic β-cells in response to elevated levels of glucose and other nutrients in the blood, which also attains anabolic properties. It facilitates the uptake of glucose, fatty acids and amino acids by several tissues including liver, adipose tissue and muscle and promotes the storage of these nutrients in the form of glycogen, lipids and protein, respectively. Insulin plays principal role in the regulation of glucose homeostasis in fasting state and prostprandially (Woerle *et al.* 2004; Woerle *et al.* 2003; Mitrakou *et al.* 1992). T2DM ensues by the combination of peripheral resistance to insulin's effects because of defects in the insulin

signaling pathway plus the pancreatic β-cell failure to properly secrete adequate amounts of insulin to meet the metabolic needs.

In respect to glucose metabolism, insulin exerts its effects by binding to its receptor. The latter is composed of two extracellular a-subunits and two transmembrane b-subunits linked together by disulphide bonds (Figure 3) (Bevan 2001). Binding of insulin to the extracellular a-subunit results in receptor activation and autophosphorylation of a number of tyrosine residues present in the b-subunit (Van Obberghen *et al*. 2001) which are in turn recognized by specific phosphotyrosine-binding domains of adaptor proteins including the insulin receptor substrates (IRS-1, IRS-2 and IRS-3) (Saltiel and Kahn 2001; Lizcano and Alessi 2002). The phosphorylation of tyrosine residues on IRS proteins by the activated insulin receptor, allows them to associate with the regulatory subunit of phosphoinositide 3-kinase (PI3K). Once activated, the catalytic subunit of PI3K phosphorylates phosphoinositides at the 3′ position of the inositol ring or proteins at serine residues. Among substrates that are phosphorylated by PI3K is the phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P2) leading to Ptd(3,4,5)P3 formation. This signal pathway results in the activation of the 3-Phosphoinositide-dependent kinase 1 (PDK1) which phosphorylates the activation loop of a number of protein serine/threonine kinases of the AGC kinase super family, including protein kinase B (PKB; also called Akt). The latter deactivates glycogen synthase kinase 3 (GSK-3), thus promoting glycogen synthase and glucose storage as glycogen while it also activates the translocation of GLUT-4 vesicles from their intracellular pool to the plasma membrane (Asnaghi *et al.* 2004), thus cellular glucose uptake. Saltiel and Kahn (2001) also described another PI3K-independent pathway by

which GLUT4 is recruited to the plasma membrane. This pathway starts with insulin binding to its receptor, followed by phosphorylation of Cbl (which is associated to its adaptor protein CAP). The Cbl-CAP complex then translocates to the plasma membrane where via Crk, C3G, TC10 and other adaptor molecules, it promotes GLUT4 translocation to the plasma membrane.

Further to glucose metabolism, insulin is also involved in protein metabolism as it promotes cellular amino acid uptake and protein synthesis (Saltiel and Kahn 2001) while at the same time it inhibits protein degradation. The suggested pathway is that of PI3K-PDK1- Akt which deactivates GSK-3 thus facilitating protein synthesis and the storage of amino acids (Lizcano and Alessi 2002). In addition, Akt activates the mammalian target of rapamycin (mTOR), which enhances protein synthesis (Asnaghi *et al*. 2004).

Lipid homeostasis provides another metabolic pathway in which insulin is implicated. Exerting anabolic effects, insulin promotes the cellular uptake of fatty acids and the synthesis of lipids (via the steroid regulatory element-binding protein; SREBP)-1c) (Shimomura *et al.* 1999), whilst inhibiting lipolysis (Kitamura *et al.* 1999). In addition, insulin also attains mitogenic properties; MAPK cascade is the leading pathway to its mitogenic responses (Ogawa *et al.* 1998) which may be activated via two different pathways. The first cascade starts with IRS phosphorylation followed by activation of GRB2 and SOS and the second one (IRS- independent pathway) starts with the phosphorylation of SHC followed by GRB2 activation; both end up with MAPK cascade stimulation.

Remarkably, negative feedback mechanisms characterize the insulin activated pathways thus modulating insulin transduction molecules activity and insulin

effectiveness. For example, the insulin/insulin receptor complexes are internalized into endosomes within which they are subsequently dissociated and degraded, providing a mechanism by which insulin receptors are downregulated in hyperinsulinaemic states as in obesity thus aggravating IR. In addition, insulin promotes the production of SOCS (suppressor of cytokine signaling) proteins that block the insulin signaling circuitry (Emanuelli *et al.* 2000; Krebs *et al.* 2000; Endo *et al.* 1997; Matsumoto *et al.* 1997). Similarly, the GSK-3β activation by Akt/PKB exerts negative feedback on insulin signaling by phosphorylating IRS-1 at serine 332 which blocks the insulin response by inhibiting insulin receptor-mediated tyrosine phosphorylation of IRS-1 (Hotamisligil 2006); as GSK-3β also modulates some of the anti-inflammatory effects of insulin, it suggests a molecule that mediates both insulin resistance (IR) and inflammatory responses (Dugo *et al.* 2006 and 2007)

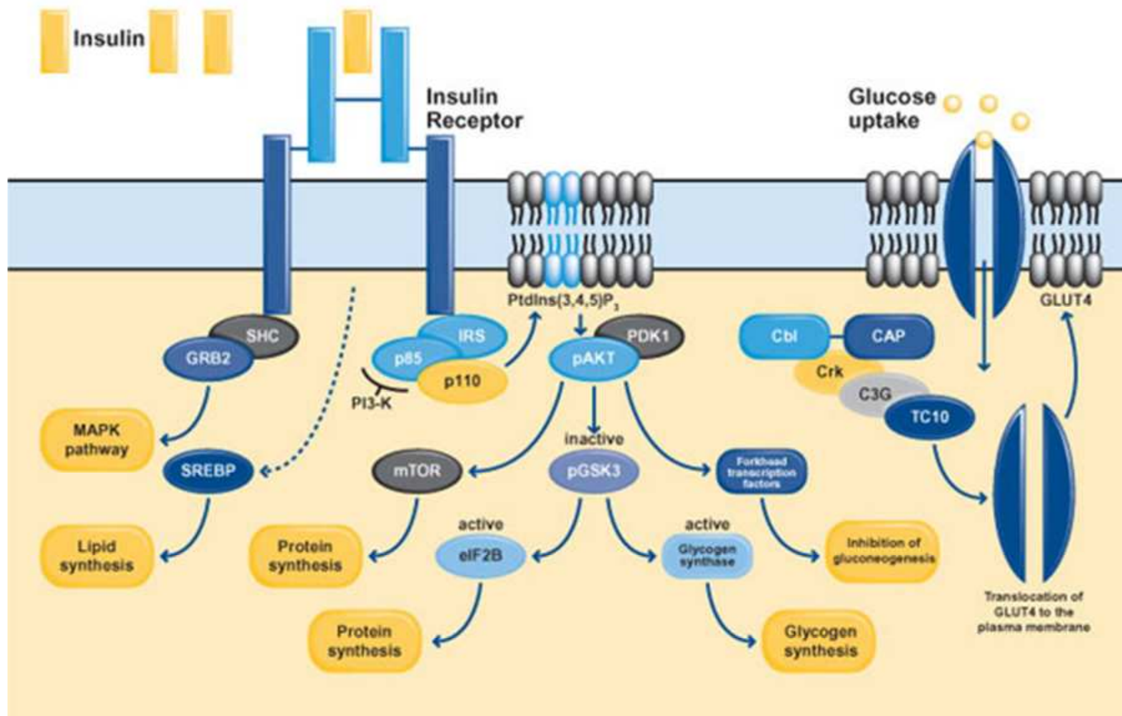


Fig 1.2.1 Insulin signaling pathway. Activation of the insulin receptor evokes increased transcription of SREBP and the phosphorylation of members of the IRS family, SHC and Cbl. Upon tyrosine phosphorylation, these proteins interact with signaling molecules through their SH2 domains, which results in the activation of a variety of signaling pathways, including PI 3-kinase signaling, MAPK activation and the activation of the Cbl/CAP complex. These pathways act in a coordinated manner to regulate glucose, lipid and protein metabolism (adopted by Bevan P 2001).

1.2.2 Modulation of insulin signaling in Obesity; Etiologic Factors and Molecular Mechanisms

A principal mechanism for the maintenance of glucose homeostasis is the appropriate secretion of insulin in response to increased glucose levels plus the effective action of insulin in stimulating glucose uptake and metabolism in peripheral tissues. T2DM ensues by the combination of peripheral insulin resistance and pancreatic beta

cells failure to secrete sharply adequate amounts of insulin to meet the metabolic needs. Regarding reduced response to insulin effect, a number of defects in insulin signal transduction pathway have been described in human subjects with insulin resistance and T2DM (Krook *et al.* 1998; Kerouz *et al.* 1997; Goodyear *et al.* 1995; Caro *et al.* 1987) however, the detailed molecular basis for insulin resistance is not well understood in every case.

In obesity-associated impaired glucose metabolism, the increased glucose levels in the blood result from elevated glucose production in the liver (gluconeogenesis and glycogenolysis) and decreased glucose uptake by muscle (Wellen and Hotamisligil 2005; Saltiel and Kahn 2001). The traditional views on metabolic derangements of diabetes have been largely "glucocentric", considering hyperglycemia the main underlying cause. However, the recognition that obese individuals who usually suffer from hyper- or dys-lipidaemia develop insulin resistance and diabetes much more frequently than lean people and also that people with T2DM almost invariably manifest serious breakdown in lipid dynamics, reflected by elevated levels of circulating non-esterified fatty acids (NEFAs) and triglycerides (TG) (Zimmet *et al.* 2001; Baumann *et al.*2000; Maddux *et al.* 1993), led researchers to investigate the potential role of altered lipid metabolism in the pathogenesis of T2DM. Two theories mainly explain the close relationship between fat excess and impaired glucose metabolism, the *"Randle cycle"* that provides the reciprocal relationship between fatty acid oxidation and glucose oxidation and the *"Ectopic Fat Storage Hypothesis"* according to which the impaired insulin effect is due to deposition of lipids within insulin-target tissues. Recently, two more hypotheses were added to the suggested theories by which obesity may lead to T2DM; first, the identification of

adipose tissue as an endocrine organ that produces and metabolizes multiple bioactive factors, which may potentially impair glucose metabolism and second, change of adipose tissue phenotype due to a *low-grade inflammatory state* that impairs insulin effectiveness. Although these theories provide metabolic mechanisms that seem to be different in origin and nature, the underlying trigger factor may be related to the effect of substrate excess relative to what adipose tissue has the genetically determined capacity to store. As a result, obese individuals develop insulin resistance, which is initially compensated for by hyperinsulinemia, through which normal glucose tolerance is preserved. However, over time further deterioration of glucose metabolism, either by increased insulin resistance or by decreased compensatory insulin secretory responses or by both, accelerates the progression to impaired glucose tolerance and eventually to overt T2DM. Chronic hyperinsulinemia per-se has been also demonstrated by White *et al* (2003) to exacerbate insulin resistance and contribute directly to beta-cell failure and diabetes.

1.2.3 'Randle's Glucose-Fatty Acid' Hypothesis - Oxidative stress

In 1963 Philip Randle (1963) first proposed one set of metabolic pathways by which carbohydrate and fat metabolism interact. He called it a "cycle" because it describes a series of events that interlink glucose and fat utilization within the cells. Its basic outline is simple; carbohydrates, when available (postprandially), are preferred as fuels than lipids, which are stored in adipose tissue for future use. Insulin plays a primary role in this regulation, facilitating glucose peripheral uptake and utilization, while at the same time it inhibits NEFAs mobilization from the adipose tissue, inactivating hormone-sensitive lipase and thus it removes the competition for substrate utilization in peripheral

tissues. In contrast, when carbohydrates are decreased (fasting state), the serum insulin levels fall permitting lipolysis and NEFA mobilization, which then become the major fuel for peripheral tissues. Randle went a step further, demonstrating the inhibitory effect of enhanced NEFA oxidation on glucose metabolism. This particular metabolic effect takes place in states with lipid excess, as in obesity and is considered pathogenic in the development of insulin resistance and eventually T2DM in obese individuals. This theory was later confirmed by following studies showing that adipocytes decrease glucose uptake in peripheral tissues by the release of free fatty acids (Boden 1997) as even short periods of lipid excess decrease PI3K activity and insulin-stimulated glucose uptake in muscle (Jacobson *et al*. 2002; Dresner *et al*. 1999). It is suggested that by mass action, the increased plasma free fatty acids (FFAs) augment their cellular uptake and induce their mitochondrial β-oxidation. As a result, the cellular metabolism may be altered at the level of substrate competition, intermediates accumulation, enzyme regulation, intracellular signaling and/or gene transcription, affecting among others, glucose metabolism. Clinical studies in healthy volunteers, in which acute elevation of plasma NEFAs resulted in whole body insulin resistance, confirmed the proposed metabolic model (Dresner *et al*. 1999). Obese individuals have two main sources of plasma NEFA excess, the meal-derived fatty acids (high-calorie diets) and the adipose tissue lipolysis especially the visceral adipose tissue one.

In this direction is the theory regarding pathogenic role of *oxidative stress* in insulin resistance and β-cell dysfunction in obese individuals. It refers to a state in which imbalance between oxidant generation and antioxidant protection or repair of oxidative damage exists. Mitochondrial respiratory chains represent a major source of ATP but also

of cellular ROS production (Newsholme *et al*. 2007; Turrens 2003; Lenaz 2001), while plasma or subcellular membrane-associated NADPH oxidases are secondary sources of intracellular ROS (Newsholme *et al*. 2009 and 2007). Normally, during the aerobic cellular metabolic processes, several reactive oxygen species (ROS) are produced. In normal concentrations, these elements act as necessary messengers in biological systems (Redox signaling) (Rhee 2006; Dröge 2002). In beta-cells in particular, the mitochondrial-produced ATP and ROS seem to be involved in maintaining normal glucose responsiveness (Leloup *et al*. 2009; Pi *et al*. 2007; Bindokas *et al* 2003). In states of prolonged excessive fuel load such as fats, the excessive mitochondrial fat oxidation results in excessive production of ROS (Li *et al*. 2008; Aikawa *et al*. 2002; Djuric *et al* 2001; Inoguchi *et al*. 2000). When abnormally high, they become cytotoxic and damage cellular structures and organelles (including mitochondria) resulting in defective cellular metabolism and enhanced cellular apoptosis. This cytotoxic condition, triggers cellular inflammatory response; it has been demonstrated that the latter stimulates the inhibitory serine phosphorylation of IRS-1, thus disrupting insulin signal transduction causing insulin resistance (Wellen and Hotamisligil 2005). B-cells are considered very sensitive to oxidative stress as they are low in antioxidant enzymes (Robertson and Harmon 2007; Tonooka *et al*. 2007). It has been shown that oxidative stress in β-cells disrupts mitochondrial function and ATP production which is necessary for the K_{ATP} channels closure. As a result, β-cell secretory capacity is impaired (Zorov *et al*. 2006; Brady *et al*. 2004) which in accordance with the oxidative-stress induced β-cell apoptosis, contributes to beta-cell failure and diabetes.

1.2.4 Ectopic Fat Storage Hypothesis

Normally, the adipocytes take up lipids from fat-rich plasma lipoproteins, through the effect of fat cell-derived lipoprotein lipase and store almost pure triglycerides in quantities of up to 95% of their volume. This process called lipogenesis is enhanced by insulin (Vague 1956). In contrast, when energy is needed elsewhere in the body, the stored fat is mobilized (lipolysis) in the form of NEFAs by the hormone sensitive lipoprotein lipase, against the effect of insulin. However, when the diet-derived fat intake is increased as in obesity, fat storage within and around other tissues and organs which under normal conditions do not store lipids has been demonstrated, including liver, skeletal muscle and pancreatic β-cells (Ectopic Fat Storage Hypothesis) (Goodpaster and Kelley 1998; Shulman 2000). There is probably a critical visceral adipose tissue threshold (CVATT), after which fat deposition is diverted to extra-adipose tissues. It has been demonstrated that although initially the peripheral organs facilitate the storage-esterification of the surplus in the form of triglycerides, their limited triglyceride buffer capacity becomes saturated soon and the excess of lipids enter catabolic pathways. As a result, excessive mitochondrial production of toxic reactive lipid species ensues (oxidative stress), which mediate organ-specific oxidative damage and cellular dysfunction, leading progressively to the development of insulin resistance, impaired glucose metabolism and finally T2DM (Jacob *et al.* 1999).

1.2.5 Obesity as a state of chronic low-grade inflammation

In recent years, obesity has been considered as a state of chronic low-grade systemic inflammation, as it is suggested by the elevated levels of circulatory inflammatory agents including C-reactive protein (CRP) (Visser *et al*;1999), tumor

necrosis factor-α (TNF-α) (Dandona *et al*. 1998), interleukin-1 (IL-1) and -6 (IL-6) (Van Dielen *et al*. 2001; Yudkin *et al*. 2000; Visser *et al*, 1999) plus the increased plasma circulating mononuclear cells and lymphocytes (Perfetto *et al*. 2002) in otherwise healthy overweight and obese individuals. The low-grade inflammation has been implicated in the development of IR, β-cell dysfunction and T2DM (Laaksonen *et al*. 2004; Pradhan *et al*. 2002); IL-1ß for example induces nitric oxide (NO) production in pancreatic ß-cells, and may impair of insulin secretion (Wogensen *et al*. 1990). It was shown that adipose tissue produces certain bioactive substances that promote inflammation (*e.g*. interleukin - 6) (Cachofeiro *et al*. 2006) but also fat tissue *per se* is infiltrated by inflammatory cells including lymphocytes and macrophages which also secrete cytokines (*e.g*. IL-6 and TNF-α) contributing to the whole body inflammatory process (Lumeng *et al*. 2007); the degree of the participation is not clear. The pro-inflammatory cytokines increase the production of serine kinases such as MAPK (mitogen-activated protein kinase), TOR (target of rapamycin), PI3K and Jnk (Jun N-terminal kinase) (Prada *et al*. 2005; Ozcan *et al*. 2004; Hirosumi *et al*. 2002; Ozes *et al*.2001; Rui *et al*. 2001; De Fea *et al*. 1997) which in turn cause serine phosphorylation of IRS-1 thus IR (Rui *et al*. 2001). In addition, insulin as well as increased levels of pro-inflammatory cytokines enhance SOCS (suppressor of cytokine signaling) proteins production which blocks the insulin signaling circuitry (Emanuelli *et al*. 2000; Krebs *et al*. 2000; Endo *et al*. 1997; Matsumoto *et al*. 1997). TNF-α may also impair insulin secretion in pancreatic islet cells (Kwon *et al*. 1999) and may stimulate IL-6 production, which leads to ß-cell destruction (Pakala SV *et al*. 1999). Furthermore, high-fat diets in humans and animals (Shi *et al*. 2005; Turk *et al*. 2003), even the low-calorie ones (Rankin *et al*. 2007), as well as acute

high-fat meal challenges in both T2DM and healthy subjects have been shown to increase inflammation (Cani *et al.* 2008; Cani *et al.* 2007; Nappo *et al.* 2002), implying a direct effect of FFAs on inflammatory pathways stimulation. In addition, the stimulatory effects of monounsaturated fatty acids on TLR-4/NFκB pathway with respect to the secretion of adipokines and chemokines (including inflammatory molecules) from adipocytes, has been demonstrated (Schäffler *et al.* 2008), while both pharmacological and genetic intervention in pathways such as JNK and IKK improve glucose metabolism under conditions of obesity (Uysal *et al.* 1997; Hotamisligil *et al.* 1993). Putting together, a critical role of fats on inflammatory state generation and IR ensues. It has been shown that, similarly to inflammatory factors (*e.g.* TNF-α), elevated levels of free fatty acids (FFAs) stimulate the inhibitory serine phosphorylation of IRS-1, thus disrupting insulin signal transduction and inducing insulin resistance (Wellen and Hotamisligil 2005). The FFA-stimulated inflammatory serine/threonine kinases JNK, inhibitor of nuclear factor (NF)-κB kinase (IKK) and protein kinase C (PKC) (Schmitz-Peiffer and Biden 2008; Arkan *et al.* 2005; Hirosumi *et al.* 2002) seems to represent key intermediaries of the inflammatory pathways that mediate serine phosphorylation of IRS-1 thus block insulin action. These kinases also stimulate activator protein (AP)-1 complexes and NF-κB (Hotamisligil GS *et al.* 2006) thus enhancing the production of inflammatory mediators including TNF-α and IL-6 (Shoelson *et al.* 2003; Gao *et al.* 2002) providing link between FFA excess and whole body inflammation.

Recent studies explore the connection between gut microbiota, energy homeostasis and inflammation and its role in the pathogenesis of obesity-related disorders (Al-Attas *et al.* 2009; Baker *et al.* 2009; Miller *et al.* 2009; Shoelson and

Goldfine 2009; Creely *et al*. 2007). The concept of 'metabolic endotoxinaemia' suggests that toxins produced in the gut may play a key role in the pathogenesis of obesity-associated inflammatory state and that food ingestion affects plasma endotoxin levels. Several current studies provide potential pathogenic mechanisms by which gut microbiota may disrupt the energy balance equation (Bäckhed *et al*.2009; Turnbaugh *et al*. 2009; Martin *et al*. 2008; Turnbaugh *et al*. 2008; Dumas *et al*. 2006), alter fatty acid metabolism and composition in adipose tissue and liver (Cani *et al*. 2007 and 2008), modulate gut-derived peptides (PYY and GLP-1) (Cani *et al*. 2009; Samuel *et al*. 2008; Zhou *et al*. 2008; Bäckhed *et al*. 2007; Cani *et al*. 2006) and activate the lipopolysaccharide toll-like receptor-4 axis (Ghanim *et al*. 2009; Cani *et al*. 2008; Anderson *et al*. 2007; Cani *et al*. 2007), leading to obesity, insulin resistance, and diabetes in the host. Previous studies show that gut bacteria can initiate the inflammatory state of obesity and IR through the activity of lipopolysaccharide (LPS). LPS is a relatively large molecule consisting of a lipid and a polysaccharide joined by a covalent bond and is found in the outer membrane of gut derived Gram-negative bacteria. LPS has strong affinity for chylomicrons by which it crosses the GI mucosa, enters the circulation and exacerbates postprandial inflammatory reaction (Ghoshal *et al*. 2009) acting as toxin that binds to the CD14 toll-like receptor-4 (TLR-4) complex at the surface of innate immune cells, triggering inflammatory reaction (Shi *et al*. 2006). Remarkably, circulating LPS modulates adipose tissue metabolism provoking increased production of adipokines including TNF-α, IL-6 and resistin (Anderson *et al*. 2007) that exert detrimental effects on whole body metabolism.

1.2.6 Endoplasmic Reticulum Stress, Hyperglycemia, and Insulin Resistance

As indicated previously, chronic low-grade inflammation may cause IR by increasing the production of serine kinases such as MAPK, TOR, and Jnk which in turn cause serine phosphorylation of IRS-1 inhibiting insulin signal transition (Prada *et al.* 2005; Ozcan *et al.* 2004; Hirosumi *et al.* 2002; Ozes *et al.* 2001; Rui *et al.* 2001; De Fea, Roth 1997; Tanti *et al.* 1994) thus IR (Rui *et al.*, 2001). In addition, the proinflammatory cytokines enhance the production of SOCS proteins that block the insulin signaling circuitry (Emanuelli *et al.* 2000; Krebs *et al.* 2000; Endo *et al.* 1997; Matsumoto *et al.* 1997). An alternative theory has been proposed implicating endoplasmic reticulum (ER) stress as a key factor in obesity-associated chronic inflammation and IR (Hotamisligil 2006; Nakatani *et al.* 2005; Ozcan *et al.* 2004). Although ER stress response has naturally a protective role in cellular survival and is activated in states of glucose or nutrition deprivation (Scheuner *et al.* 2001) to protect against hypoglycemia and death (Tamatani *et al.* 2001) in fuel-excess states as in obesity it deranges glucose metabolism. It has been suggested that NF-κB and JNK pathways mediates ER-stress inhibitory effects on insulin signal transduction (Ozcan *et al.* 2006; Zhang *et al.* 2001).

1.3 Influence of glucose on insulin resistance.

1.3.1 Plasma glucose regulation in fasting and postprandial state

Normally, in *fasting state*, plasma glucose levels are maintained within the normal range by endogenous glucose release (glycogenolysis and gluconeogenesis); liver contributes by 80% to the endogenous glucose production and kidneys by 20% (Meyer *et*

al. 2002; Stumvoll *et al*. 1999). In contrast, *postprandially*, the glycogen breakdown and glucose release into the circulation are substantially reduced (Woerle *et al*. 2004; Meyer *et al*. 2002) while the hepatic glycogen synthesis increases, so that hyperglycemia to be avoided and replenishment of glycogen stores to be facilitated. These metabolic modulations follow changes in plasma insulin (increased) and glucagon (decreased) concentrations (Woerle *et al*. 2004; Woerle *et al*. 2003; Mitrakou *et al*. 1992) due to which hepatic glucose-6-phosphatase and phosphorylase are reduced, while glycogen synthase is activated. The *postprandial* glucose disposal involves taken up by tissues for energy production (glycolysis; about 2/3 of taken up glucose) or storage (about 1/3, either as glycogen or as triglycerides in the adipose tissue) (Woerle *et al*. 2003). The major tissues responsible for postprandial glucose disposal are liver (splanchnic), muscle, brain, and kidney (Meyer *et al*. 2002). Brain plays a substantial role in glucose disposal which is in brain's case insulin independent. Thus, only about 70% of postprandial glucose uptake is insulin dependent. About 2/3 of the glucose that undergoes glycolysis is oxidized, while the remainder is converted to gluconeogenic intermediates (*i.e.,* lactate, pyruvate, and alanine) which are then released back to circulation, enter the hepatic gluconeogenic pathway and after conversion into glucose-6-phosphate are by released 50% as free glucose back to the circulation and by 50% is incorporated in hepatic glycogen for future use.

1.3.2 Defects in Skeletal Muscle Control of Glucose Homeostasis in obesity

Skeletal muscle is the primary site of glucose disposition in the body (DeFronzo 1997) and resistance to the actions of insulin in skeletal muscle is a major pathogenic

factor T2DM. Lipid accumulation within skeletal muscle correlates quite well with insulin resistance in humans and is one of the suggested mechanisms implicated in the induction of IR in obesity (Phillips *et al.* 1996). It has been shown that excessive accumulation of lipids within the skeletal cells, activate at least in part via increased ceramide formation the protein kinase C and the JNK/SAPK pathway, which in turn block insulin signal transduction in muscle (Prada *et al*. 2005; Kim *et al*. 2004; Hirosumi *et al*. 2002).

1.3.3 Defects in Hepatic Control of Glucose Homeostasis in obesity

The liver plays a key role in obesity-induced hyperglycemia (Kahn, Hull 2006; Pilkis, Granner 1992). As mentioned above, the glucose production (glycogenolysis) by the liver is suppressed postprandially so that hyperglycemia to be avoided and replenishment of glycogen stores to be facilitated. Insulin plays principal role in modulation of hepatic glucose production as it promotes the synthesis of glycogen and represses hepatic glucose release. These effects are mediated by insulin-induced suppressed transcription of the phosphoenolpyruvate carboxykinase enzyme that controls gluconeogenesis and by the insulin-induced increased transcription of glucokinase and pyruvate kinase that promote glycolysis (Sutherland *et al.* 1996). Several hepatocellular transcription factors and cofactors such as peroxisome proliferators seem to mediate these effects (Li *et al*. 2007; Puigserver *et al*. 2003), modulated by the insulin-activated Akt/PKB pathway (Cantley 2006; Shaw, Taniguchi *et al*. 2006; Saltiel, Kahn 2001). In insulin resistant states, hepatic glucose production is not inhibited postprandially, so that hyperglycemic excursions to follow meal intake. Hepatic steatosis is a common finding

in obese individuals (Dixon *et al*. 2001; Matteoni *et al*. 1999; Ludwig *et al*.1980), while on the other hand recent findings relate liver fat accumulation with reduced hepatic insulin sensitivity (Seppälä-Lindroos *et al*. 2002; Schmitz-Peiffer 2000). Putting together, liver steatosis is a manifestation of IR in obesity (Ferre, Foufelle 2007; Patti *et al*. 2003). This association seems to be explained by the role of the transcription factor sterol regulatory-element-binding protein-1c (SREBP-1c) in the liver that induces the expression of a family of genes involved in glucose utilization (gluconeogenic genes) and fatty acid synthesis (lipogenic genes) and can be considered as a thrifty gene (Ide *et al*. 2004; Azzout-Marniche *et al*. 2000; Foretz *et al*. 1999). It has been shown that insulin at least in part via through the phosphatidylinositol 3-kinase pathway, increases the hepatocellular concentration of the transcription factor sterol regulatory-element-binding protein-1c (SREBP-1c). The subsequent SREBP-1c induced lipogenesis contributes to abnormally high hepatic content of fats (liver steatosis) which in turn reduce hepatic insulin sensitivity by mechanisms involving substrate competition, antagonism of insulin signaling or lipotoxicity (Seppälä-Lindroos *et al*. 2002; Schmitz-Peiffer 2000).

1.3.4 Central Nervous System Control of Glucose Homeostasis

Close hormonal and biochemical cross-talk between central nervous system (CNS) and peripheral tissues for glucose homeostasis is now well established (Gribble 2005). Leptin that is produced by the adipocytes in increased levels with increasing adiposity acts on the arcuate nucleus of the hypothalamus which contains high concentrations of leptin receptor and controls food intake (Morton *et al*. 2006). Rodents and humans with reduced levels of leptin (due to leptin gene mutation) or leptin receptor

demonstrate loss of satiety, increased appetite and severe obesity (Morton *et al*. 2006; Elmquist, Marcus 2003). In obesity, despite hyperleptinemia, appetite remains increased, implying attenuated response to leptin in obesity. This finding, along with the insulin sensitizing effects of leptin in peripheral tissues (Morton *et al*. 2006; Elmquist, Marcus 2003) may suggest a contributory effect of dysregulated leptin sensing pathway to obesity and diabetes development. Remarkably, it seems that neuronal connection between CNS and fat tissue exists which may actually play important role in appetite regulation. It has been shown that afferent-nerve signals from intra-abdominal adipose tissue modulate hunger and sense of satiety by enhancing the hypothalamic sensitivity to leptin (Yamada *et al*. 2006). Further to adipose tissue-derived hormonal (leptin) and neural factors that modulate hypothalamic control of appetite and peripheral insulin effectiveness, recent data suggest that metabolic agents (long-chain fatty acids) produced by the adipocytes may also regulate energy balance and metabolic homeostasis by altering the rate of lipid oxidation in selective hypothalamic neurons (Morton *et al*. 2006; Elmquist, Marcus 2003). This in turn alters the hypothalamic perception of metabolic balance and changes respectively the CNS-regulated efferent pathways responsible for fuel intake and utilization (Cota *et al*. 2007).

Within the last few years the role of gut-derived hormones on glucose metabolism and appetite gained a lot of attention and incretin based therapeutic approaches for the management of T2DM became popular. The glucagon-like peptide (GLP)-1 that comes from the L-cells of the distal small bowel and the GIP that is produced in the K-cells of proximal small intestine are low in patients with obesity and T2DM, while administration of therapeutic doses of GLP-1 analogues contributes not only to improved glucose

metabolism but also to decreased appetite and weight loss. Whether reduction of these agents represents a primary or secondary defect in obesity and T2DM is still unknown. What is known though is that these humoral circuits from gut do not function properly in obesity and diabetes implying the complexity of the multi-systemic modulation of energy and modulation via CNS and hypothalamus. Another gut hormone involved in energy homeostasis is ghrelin which levels increase with fasting and augments feeding (Cota *et al*. 2007; Morton *et al*. 2006; Yamada *et al*. 2006; Elmquist, Marcus 2003).

The sympathetic and parasympathetic innervation of adipose tissue has been well documented (Kahn *et al*. 2006; Boden, Hoeldtke 2003; Ikezu *et al*. 1999). By acting on the adiposal β3-adrenergic receptors, the sympathetic nervous system enhances lipolysis while the parasympathetic nervous system has been involved in lipogenesis. Both these function suggest the close cross-talk between CNS and adipose tissue. On the other hand, it is known the interrelationship between autonomous nervous system and immune system (*e.g.*, macrophages) (Flierl *et al*. 2007; Sternberg 2006). Putting together, adipose tissue cross-talks with immune system however how this is modified in obesity is still unknown.

Whilst previous discussion has noted the direct effect of insulin on hepatic glucose production, by promoting gluconeogenesis and repressesing glucogenolysis (Sutherland *et al*. 1996) studies also show that the CNS and the arcuate nucleus of the hypothalamus in particular are involved in the modulation of the hepatic glucose production (Pocai *et al*. 2005). Hypothalamus senses with specific proopiomelanocortin neurons-located ion channels called potassium adenosine triphosphate (K+ATP) channels the postprandial increase of insulin and glucose levels and via vagus nerve that represents

the efferent part of the circuit it decreases the hepatic glucose output. It has been shown that this loop is defective in obesity, contributing to enhance hepatic glucose production even in postprandial states (Parton *et al*. 2007).

1.4 The Role of Adipose Tissue in the Pathogenesis of T2DM

Obese individuals tend to be insulin resistant (Rexrode *et al*. 1996; Bonadonna *et al*. 1990). As previously detailed the insulin signaling pathway, as well as the physiologic role of CNS and organs, are disrupted in subjects with T2DM where normal glucose metabolism is altered in the liver and skeletal muscle. Previous studies have also referred to the obesity-related modulations of insulin action and signaling in these organs and provided current views for the etiologic factors and molecular mechanisms involved in insulin resistance generation and subsequent impaired glucose homeostasis and T2DM development in states with excessive fuel load as in obesity. Here we present the critical role of expanding adipose tissue in insulin signaling defects generation in obesity.

1.4.1 The Role of Adipose Tissue as Source of Free Fatty Acids in the Pathogenesis of Type 2 Diabetes

Findings from several studies have demonstrated that excessive release of free fatty acids decreases PI3K activity and insulin-stimulated glucose uptake in peripheral tissues (Dresner *et al*. 1999; Boden 1997). Obese individuals have two main sources of plasma FFA excess, the fat-rich diet and the adipose tissue (lipolysis). Insulin promotes triglyceride storage in the adipose tissue and inhibits lipolysis. However in obesity, fat depots become resistant to insulin (Foley *et al*. 1986; Stern *et al*. 1972;

Hirsch, Knittle 1970; Salans *et al.* 1968) so that the hormone sensitive lipase remains active, driving hydrolysis of the stored triglycerides with subsequent excessive FFAs release in the circulation. As a result, the circulating FFAs further to generating an atherogenic lipid profile, they are also taken up by peripheral tissues where they are either re-esterified and stored as triglycerides or used for energy production (oxidation). In the long term, the excessive 'ectopic' fat storage and oxidation generates oxidative stress that causes cellular damage and defects in cellular metabolism including IR (Li *et al.* 2008; Hotamisligil 2006; Aikawa *et al.* 2002; Djuric *et al.* 2001; Inoguchi *et al.* 2000). In addition, it also triggers cellular inflammation, resulting in serine phosphorylation of IRS-1, thus further blocking the insulin signal transduction (Wellen and Hotamisligil 2005).

1.4.2 The Role of Adipose Tissue as an ''Endocrine Organ'' in the Pathogenesis of Type 2 Diabetes

The traditional view of adipose tissue as a passive reservoir for energy storage is no longer valid. Studies highlight that human adipose tissue is a complex and highly active organ controlling energy balance, metabolism and the immune system via the expression and secretion of a variety of bioactive peptides, known as adipokines that act locally but also systemically as endocrine hormones (Fruhbeck *et al.* 2001; Ahima, Flier 2000). It has been demonstrated that adipose tissue actively communicates with liver, skeletal muscle, and the brain via secreted adipokines. The latter have diverse roles and adipose fat depot mass-modulated expression levels, indicating changes in adipose tissue mass and energy status and signal this information to organs that control fuel expenditure,

contributing to energy homeostasis regulation or peripheral insulin action. Current research has identified over 50 adipocyte-secreted bioactive factors (Table 1.4.2) with local (autocrine/paracrine) and systemic (endocrine) effects that are produced by the fat cells, and more are yet to be discovered. As individuals become obese and their adipocytes enlarge, adipose tissue undergoes molecular and cellular alterations and produces certain bioactive substances that promote inflammation including tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), IL-8 (Cachofeiro *et al*. 2006).

Initially, the adipocytes have been exclusively blamed for the adipose tissue-derived pro-inflammatory molecules. Recent studies however, suggest that obesity is associated with increased infiltration of macrophage (adipose tissue macrophages-ATMs) within the adipose tissue (Mozaffarian 2006; Sjöström *et al*. 1999; Stalmer *et al*. 1993; Hubert *et al*. 1983), which in turn secrete high concentrations of inflammatory compounds such as TNF-α and IL-6, that fuel further systemic inflammation (Lumeng *et al*. 2007) and induce the development of insulin resistance. Kintscher *et al* (2008) extended the original observations from ATMs to cells of adaptative immunity. They demonstrated increased T-lymphocytes accumulation within the adipose tissue of obese rodents mainly by using gene expression analyses and immunohistochemistry; more interestingly, the T-lymphocytes infiltration preceded that of the macrophages. Similar are the findings in human adipose tissue of morbidly obese patients as T-cell infiltration was demonstrated by Wu *et al* (2003) who also suggested that these T-lymphocytes may play a role in the development of IR during obesity. The accumulated T-lymphocytes could in turn produce interferon gamma (IFNγ) that stimulate monocyte chemoattractant protein-1 (MCP1) release from the adipocytes(Brake *et al*. 2006) (Weisberg *et al*. 2006).

Several hypotheses have been suggested to explain the accumulation of macrophages in fat tissue. The most widely accepted suggests that the enlarged adipocytes become 'fragile', leaking chemoattractants such as M-CSF and MCP-1 that activate and recruit macrophages in adipose tissue, even at the early stages of obesity (Takahashi *et al*. 2008).

1.4.3 Adipokines

TNF-α for example, is highly expressed in adipose tissues of obese subjects (Hotamisligil *et al.* 1995) and has been proposed as a link between insulin resistance, obesity, and diabetes (Sánchez *et al.* 2005). Supporting to this, is the finding that obese mice lacking either TNF-α or its receptors are protected against developing insulin resistance (Uysal *et al.* 1997), while direct exposure of isolated cells to TNF-α blocks insulin signaling transduction and induces a state of insulin resistance in several systems, including human primary adipocytes (Hotamisligil *et al.* 1996). TNF-α activates ERK and c-Jun N-terminal kinase (JNK) which in turn enhance serine phosphorylation of IRS-1 in adipocytes (Rui *et al*. 2001). It has been estimated that JNK activity is increased in obesity in several metabolically active sites including adipose tissue (Prada *et al*. 2005; Ozcan *et al*. 2004; Hirosumi *et al*. 2002), in response to various stress signals, including proinflammatory cytokines, FFAs, ER stress and reactive oxygen species (ROS). JNK in turn, not only increases the production of inflammatory molecules thus exaggerates inflammation (Shoelson *et al*. 2003) but also contributes to insulin resistance through direct serine phosphorylation of IRS-1. The abnormally Ser-phosphorylated IRS-1 inhibits insulin's intracellular signal progression causing IR (Pirola *et al* 2004; White

2003); this explains why inhibition of JNK decreases the development of obesity-associated IR (Hirosumi *et al*. 2002; Uysal *et al*. 1997; Hotamisligil *et al*. 1993).

Furthermore, data from studies in animal models have clearly linked resistin to IR (Satoh *et al*. 2004; Rangwala *et al*. 2004; Rajala *et al*. 2004; Banerjee, Lazar 2003; Rajala *et al*. 2003; Steppan *et al*. 2001). In addition, direct pro-inflammatory effects of resistin in animal adipose tissue have been shown (Senolt *et al*. 2007). In contrast to what happens in animal models though, the role of resistin in humans is less certain. Previous researchers have documented resistin's expression (mRNA and protein level) within both human pre-adipocytes and adipocytes and its expression in higher levels in abdominal fat (Sc and Om fat depots) compared to thigh and breast adipose tissue depots (McTernan *et al*. 2002). Other researchers demonstrated the synthesis and secretion of resistin by the adiposal macrophages (Curat *et al*. 2006); the extent of contribution of macrophages to the overall concentration of resistin in adipose tissue though, remains uncertain. Studies in humans have identified a strong link between resistin levels and obesity/IR/T2DM (McTernan *et al*. 2002; McTernan *et al*. 2002; Vidal-Puig, O'Rahilly 2001) while others failed to do so (Kielstein *et al*. 2003; Patel *et al*. 2003; Janke *et al*. 2002). This implies that resistin might not be a principal mediator of insulin resistance in humans. On the other hand, recent data from human studies suggest a very strong and consistent association between resistin and inflammatory molecules in morbidly obese individuals (De Luis *et al*. 2010; Kunnari *et al*. 2006; Iqbal *et al*. 2005) as well as in patients with inflammatory diseases (Senolt *et al*. 2007; Pang, Le 2006; Reilly *et al*. 2005; Lehrke *et al*. 2004); plasma resistin levels were positively correlated with inflammatory markers including TNF-α, IL-6 and CRP. In another study, (Lehrke *et al*. 2004) researchers

demonstrated TNF-α-induced resistin elevation, as a result of endotoxinemia. Furthermore, studies with thiazolidinediones (TZDs) that are insulin sensitizers show that TZDs reduce resistin levels and CRP values (Chu *et al*. 2002). Putting together, the lack of association between resistin and insulin resistance, with the documented presence of strong relationship between resistin and inflammatory markers may be explained by a direct stimulatory effect of resistin on inflammatory processes.

Leptin is another adipokine that is secreted by the adipocytes in direct proportion to adipose tissue mass. It serves as a metabolic signal of energy sufficiency (Friedman, Halaas 1998) and modulates the function of the hypothalamic-pituitary-adrenal/thyroid and -gonadal axes (Margetic *et al*. 2002; Flier *et al*. 2002; Hileman *et al*. 2000). Within the human adipose tissue, increased leptin concentration, as in obesity, exerts pro-inflammatory effects as it induces the release of TNF-a (Lappas *et al*. 2005) and the endothelial-derived MCP-1 production, facilitating the recruitment of macrophages into the fat tissue (Yamagishi *et al*. 2001). Furthermore, it has been demonstrated that leptin levels are rapidly increased by many acute phase cytokines, such as TNF-α and IL-6 (Kirchgessner *et al*. 1997; Sarraf *et al*. 1997) that are both present in excess in the adipose tissue of obese individuals (Fried *et al*. 1998; Hotamisligil *et al*. 1993). Putting together, there is an established interlink between leptin and inflammatory mechanism within the adipose tissue contributing to excessive production and release of inflammatory mediators.

There are other adipokines in which their role in glucose metabolism in human subjects is not clear yet or is positive. Chemerin for example is a recently discovered adipokine (Wittamer, *et al*. 2007) highly expressed in white adipose tissue (Bozaoglu, *et*

al. 2007). Its role in glucose metabolism remains unclear. There are animal data showing potent anti-inflammatory effects (Cash *et al*. 2008) and direct stimulatory effect of chemerin on adipocyte GLUT-4 and adiponectin expression via its own receptor (Bozaoglu *et al*. 2007; Goralski *et al*. 2007; Roh *et al*. 2007) plus enhancement of IRS-1 tyrosine phosphorylation, suggesting that insulin sensitizing effect of chemerin in adipose tissue (Takahashia *et al*. 2008). On the other hand however, the plasma chemerin levels were associated with BMI, triglycerides, and blood pressure in normal glucose tolerant subjects (Bozaoglu *et al*. 2007). Omentin is another protein synthesized and secreted by the visceral stromal vascular cells of the adipose tissue rather than the adipocytes. It seems that it improves insulin sensitivity in human subcutaneous and visceral adipose tissue as it increases Akt phosphorylation and glucose uptake by the adipocytes Kieffer, Habener 2000) while and its plasma levels have been positively related to adiponectin and HDL and inversely to obesity and IR in human subjects (de Souza Batista *et al*. 2007). Whether its role in glucose metabolism is important or not still needs to be determined.

Adiponectin which is more expressed in subcutaneous than visceral adipose tissue (Fain *et al*. 2004), is an adipokine for which a strong and consistent inverse relation to both inflammation and insulin resistance has been established (Chandran *et al*. 2003; Diez, Iglesias 2003); AMPK is suggested as mediator of its insulin-sensitizing effects (Diez, Iglesias 2003; Yamauchi *et al*. 2003; Yamauchi *et al*. 2002). Unlike other adipokines, serum levels of adiponectin are decreased in obesity and its associated medical complications (Trujillo 2005). Actually, it has been estimated that plasma levels of adiponectin decline before the onset of obesity and insulin resistance, therefore a

potential pathogenetic role of hypoadiponectinemia in the generation of these conditions (Hotta *et al.* 2001). Further to its involvement in metabolism, there is also an increasingly recognized inverse inter-relationship between adiponectin and inflammation. It has been shown that certain cytokines that increase within the adipose tissue with increasing adiposity *e.g.* TNF-α and IL-6, suppress adiponectin expression in adipocytes (Bruun *et al.* 2003), while on the other hand adiponectin levels are negatively associated with inflammatory molecules, including TNF-α (Zhou *et al.*2008; Park *et al.* 2006; Thakur *et al.* 2006; Xu *et al.* 2003), IL-6 and CRP, but positively related to anti-inflammatory cytokine IL-10 (Choi *et al.* 2007; Engeli *et al.* 2003). The attenuation of proinflammatory cytokine production by adiponectin is mediated in part by attenuating the translocation of NFκB to the nucleus (Wulster-Radcliffe *et al.* 2004; Ouchi *et al.* 2000), but also by inducing the expression of anti-inflammatory cytokine interleukin-1-receptor antagonist (Kumada *et al.* 2004; Wolf *et al.* 2004) and by suppressing the Toll-like receptor-4 (TLR-4) signaling pathway (Yamaguchi *et al.* 2005). Anti-oxidant effects have been also attributed to adiponectin (via upregulating the uncoupling protein 2 levels) by which it reduces oxidative stress and inhibits cellular inflammatory pathways activation (Negre-Salvayre *et al.* 1997). Finally, it has been recently demonstrated that adipose tissue may influence insulin sensitivity via non-hormone secretory factors such as retinol binding protein 4 (RBP4), which is the major transporter of retinoic acid in the body (Yang *et al.* 2005; Quadro *et al.* 1999). It has been shown that increased levels of RBP4 as in obesity and T2DM decrease the activity of PI3K and the phosphorylation of IRS-1, aggravating insulin resistance (Graham *et al.* 2006; Yang *et al.* 2005).

Table 1.4.2 Differences between adipocytes from subcutaneous (Sc) and visceral depots (Modified from original Source: Montague & O'Rahilly; 1998)

Factor	Regional Difference	Reference
Leptin mRNA & protein	Visceral < Sc	Lebreve AM *et al*, 1998; Harmalen VV *et al*, 1998; Montague CT *et al*, 1997
TNF-α	Visceral < Sc	Hube *et al*, 1999
IL-6	Visceral > Sc	Fried SK *et al*, 1998
PAI-1	Visceral > Sc	Shimomura I *et al*, 1996
Angiotensinogen mRNA	Visceral > Sc	Van Harmalen V *et al*, 2000
Resistin	Visceral = Sc	McTernan PG *et al*, 2002a
Adiponectin	Visceral < Sc	Fisher ffM *et al*, 2002
Androgen receptor mRNA	Visceral > Sc	Dieudonne M *et al*, 1998)
PPARγ	visceral = Sc	Montague CT *et al*, 1998
TZD stimulated pre-adipocyte differentiation	Visceral < Sc	Adams M *et al*, 1997
Lipolytic response to catecholamines	Visceral > Sc	Rebuffé-Scrive M *et al*, 1989
Antilipolytic effect of insulin	Visceral < Sc	Zierath J *et al*, 1998 Lefebvre A-M *et al*, 1998
β1 and β2-Adrenergic receptor binding and mRNA	Visceral > Sc	Hellmér J *et al*, 1992; Arner P *et al*, 1990
Dexamethasone-induced increase in LPL	Visceral > Sc	Fried SK *et al*, 1993
α2-Adrenergic receptor agonist inhibition of cAMP	Visceral < Sc	Vikman H-L *et al*, 1996
Insulin receptor affinity	Visceral < Sc	Zierath J *et al*, 1998
IRS-1 protein expression	Visceral < Sc	Zierath J *et al*, 1998
Insulin receptor	Visceral > Sc	Lefebvre A-M *et al*, 1998
Glucocorticoid receptor mRNA	Visceral > Sc	Rebuffé-Scrive M *et al*, 1990

1.5 Genetics of Obesity and T2DM

Alhtough there has been a sharp and unexpected upward turn in the prevalence of T2DM in the last couple of decades cannot be attributed to novel genetic defects, the genetic component to T2DM cannot be denied, given the high prevalence of the disease in particular ethnic groups (Knowler *et al*. 1990; Zimmet *et al*. 1983), the differences in incidence rates between monozygotic and dizygotic twins (Newman *et al*. 1987; Barnett *et al*. 1981) and the inheritance seen in families with rare monogenic diabetes (Gottlieb 1980). Several candidate genes have been proposed for human T2DM susceptibility including PPARγ (Barroso *et al*. 2006) KCNJ11 (Schwanstecher , Schwanstecher 2002), CAPN10 (Cox *et al*. 2004; Altshuler *et al*. 2000), HNF4A (Hara *et al*. 2006; Hara *et al*. 2004; , Silander *et al*. 2004; Damcott *et al*. 2004), TCF7L2 (Grant *et al*. 2006), IGF2BP2 (Huang *et al*. 2010), CDKAL1 (Ryoo *et al*. 2011), SLC30A8 (Kifagi *et al*. 2011) and HHEX (Ryoo *et al*. 2011). It is believed that each exerts only a partial contributing effect to the development of T2DM and that the disease ensues only when particular combinations of such 'thrifty' genes coexist with IR risk factors, such as obesity. This is exactly what happens with a specific polymorphism of IRS-2 which seems to increase susceptibility to T2DM, only among obese subjects (Bodhini *et al*. 2007). Interestingly, population studies and studies in family groups with evidence of childhood obesity led to the identification of specific genetic variants that predispose to both insulin resistance and obesity. In particular, further to leptin (Halaas *et al*. 1996), gene variants including ENPP1 (Meyre *et al*. 2005), RBP4 (Yang *et al*. 2005; Sivaprasadarao , Findlay 1988; Chambon 1996;), PEPCK (Rodgers *et al*. 2005; Moynihan *et al*. 2005), adiponectin (Vimaleswaran *et al*. 2008) and UCP2 (Dalgaard 2011) have shown strong link with the development of both obesity and insulin

resistance/T2DM, while in other cases such as in FTO gene, different polymorphisms predisposed to T2DM or obesity (Ramya *et al*. 2011).

Although a clear divide between type 1 diabetes mellitus (T1DM) and T2DM is suggested, the cross-over in terms of inflammatory pathways may blur those lines. T1DM is an autoimmune disease in which autoreactive cytotoxic (CD8+) T-cells recognize a number of antigenic determinants (insulin, IGRIP, GAD65, ICA512/IA-2, and I-A2ß) (Lieberman *et al*. 2003; Atkinson, Eisenbarth 2001; Wong *et al*. 1999) expressed in pancreatic ß-cells and progressively destroy them. Along with the cytotoxic T-cells, other components of the inflammatory responses including CD4+ T-cells, macrophages, natural killer (NK) cells and cytokines take part in insulinitis and ß-cell destruction. Remarkably, the fact that the CD8+ T-cells are the most abundant cells in T1DM-associated insulitis (Conrad *et al*. 1994; Bottazzo *et al*. 1985) suggests a potential crucial role of major histocompatibility complex (MHC) in the development of T1DM. In respect of T2DM studies, recent findings support a critical role of low grade whole body inflammation and subsequent IR development even in patients with T1DM (Chase *et al*. 2004); with a reduction in inflammation shown to restore response to insulin. Furthermore, gathering evidence appears to support that IR, a risk factor for progression to T1DM may also herald the onset of autoimmune diabetes (Razavi *et al*. 2006; Sherry *et al*. 2005; Betts *et al*. 2005; Fourlanos *et al*. 2004). Taken together, chronic inflammation may accelerate ß-cell death and lead to T1DM. Support for this has been noted through recent findings showing accelerated β-cell failure and apoptosis by inflammatory cytokines including TNF-α (Rui *et al*. 2001; Kwon *et al*. 1999), IL-1ß (Wogensen *et al*. 1990) and IL-6 (Pakala *et al*. 1999). Studies have also shown that

specific polymorphisms in the central human major histocompatibility complex (MHC) region class III of chromosome 6, where the HLA-B associated transcript 1 (BAT1) lies (Wong *et al*. 2003; Spies *et al*. 1989) predisposes to the development of autoimmune disorders including T1DM (Allcock *et al*. 1999). These findings appear related to previous experiments in monocytes and T-cell lines (Allcock *et al*. 2001) according to which BAT1 down-regulates inflammatory cytokines such as TNF-α, IL-1 and IL-6. As such this may suggest that BAT1 polymorphisms leads to suppressed BAT1 protein levels, leading to T1DM, via triggering inflammatory responses rather that autoimmune processes and if this is accurate, BAT1 inactivating mutations could herald the development of inflammation and subsequently IR and reduced β-cell secretory capacity in T2DM.

1.6 The protein encoded by the HLA-B associated transcript 1 (BAT1)

1.6.1 The major histocompatibility complex (MHC)

The major histocompatibility complex (MHC) is a large genomic region found on the short arm of chromosome 6 that encodes proteins involved in immune system processes including immune system response when foreign material presents inside a cell. This is achieved by displaying pieces of the foreign material or antigens (MHC:peptide) on the host cell's surface. Recognition of MHC:antigen complex by immune cells, such as T cells or natural killer (NK) cells activates the immune response against the presented antigen. Traditionally, MHC is divided into three classes of clustered genes according to their properties/functions. The class I and II MHC molecules perform the antigen presentation. In addition, they encode human leukocyte

antigens (HLAs) that are displayed on the cell surface and define an individual's tissue type. Class III MHC genes encode a group of soluble proteins found in the blood (complement system) that target foreign cells and breaks open their membranes, while close to them is a cluster of genes that control inflammation. Because of their role to defend against a great variety of foreign particles such as bacteria, MHC genes must be able to present a wide range of peptide antigens derived from such digested particles and are therefore highly polymorphic. It has been shown that specific MHC-related polymorphisms are associated with either increased or decreased susceptibility to a range of infectious diseases including tuberculosis, hepatitis and HIV/AIDS, while loss-of-function MHC gene mutations may lead to autoimmune disorders in which the body fails to recognize self-antigens for example multiple sclerosis, some forms of arthritis and diabetes, and inflammatory bowel disease (Cheong *et al*. 2001; Ota *et al*. 2001; Price *et al*. 1999).

1.6.2 Relation of BAT1 to immunopathologic disorders

The HLA-B associated transcript 1 (BAT1) gene is located in the central part of class III MHC genomic locus on chromosome 6, between TNF and HLA-B genes (Wong *et al*. 2003; Spies *et al*. 1989). Although it is difficult to study this region because of the presence of strong linkage disequilibrium within the MHC and the high gene density, it has been shown that the central part of class III MHC region contains genes that affect susceptibility to immunopathologic disorders (Cheong *et al*. 2001; Ota *et al*. 2001; Price *et al*. 1999). Thus, the ancestral haplotype 8.1 (HLA-A1, C7, B8, C4AQ0, C4B1, DR3, DQ2) for example of this region (HLA-DRB1) was shown to predispose to rapid

progression of HIV infection and to autoimmune disorders including TIDM, systemic lupus erythematosus, rheumatoid arthritis, myasthenia gravis, coeliac disease, common variable immunodeficiency, IgA deficiency and dermatitis herpetiformis (Allcock *et al.* 1999). These disorders are characterized by dysregulation of inflammatory cytokines hence they may be influenced by single gene. In addition several genes in the central MHC locus also have the potential to modulate immune or inflammatory responses in an antigen-independent manner, as is observed in studies of cultured cells from healthy carriers of the 8.1 ancestral haplotype however, only six genes (LTA, LTB, TNF, IKBL, AT6PG and BAT1) are conserved through modern human evolution (673) which means each constituent gene represents candidate for the observed genetic associations. Furthermore, antisense studies have shown that BAT1 protein can down-regulate inflammatory cytokines. Such studies indicate that monocytes and T-cells (Allcock *et al.* 2001) BAT1 may encode a negative regulator of the inflammatory cytokines TNF-α, IL-1 and IL-6 and may therefore influence immunological processes including the development of immunopathological diseases (Allcock *et al.* 2001). Taking this together studies indicate BAT1 may be directly responsible for the genetic association. Several other studies have shown an associated reduced BAT1 production (on a disease-associated haplotype) (Wong *et al.* 2003) with susceptibility to various autoimmune disorders. Thus, the restriction fragment length polymorphism (RFLP) of BAT1 has been associated with myasthenia gravis (Degli-Esposti *et al.* 1992), polymorphisms at positions -22 and -348 relative to the BAT1 transcription start site with T1DM (Price *et al.* 2004), BAT1 -348T polymorphism with rheumatoid arthritis (Quiñones-Lombraña *et al.* 2008), BAT1 polymorphisms in the promoter region at

positions -22C/G and -348C/T with chronic Chagas cardiomyopathy (Ramasawmy *et al.* 2006) and the haplotype block, NFKBIL1-ATP6V1G2-BAT1-MICB-MICA, with susceptibility to hepatitis C virus-associated dilated cardiomyopathy (Shichi *et al.* 2005).

1.6.3 Structure, biochemical function and cellular localization of BAT1

The first transcript from a protein coding gene that contains both introns and exons is called pre-mRNA. Current evidence suggests that, after the addition of a poly (A) tract to its 3' end, the pre-mRNA is modified within the nucleus or during transport to the cytoplasm to produce a mature mRNA molecule, before it can be used to produce a correct protein through translation (Tal *et al.* 1979; Goldenberg & Raskas 1979; Nevins and Darnell 1978; Lai and Khoury 1978). This RNA modification is called splicing and refers to removal of the introns to produce the mature mRNA molecule which contains only exons; the latter is then exported from the nucleus to the cytoplasm and translated. After splicing, the mature RNA is exported from the nucleus to the cytoplasm. The splicing and export processes are coupled. For pre-mRNA splicing to occur, a complex of five specialized small nuclear ribonucleoproteins (U1, U2, U4, U5, and U6; snRNPs) called spliceosome is necessary (Nilsen 2003; Hastings *et al.* 2001; Moore *et al.* 1993). The spliceosome (U1, U2, U4, U5, and U6) assembles on pre-mRNA in a stepwise manner, forming the E, A, B, and C complexes. Complex E (early complex) is formed by the binding of U1 SnRNP to the 5’ splice site while the binding of U2 snRNA to the branch point leads to the formation of complex A (pre-spliceosome complex). Then, the snRNPs U4/U5/U6 are bound to complex A, forming complex B which by structural rearrangement becomes active, able to catalyzes the removal of introns from the pre-

mRNA segment and the subsequent ligation of the flanking exons. (Shen *et al*. 2007 and 2006 and 2004). The splicing of the pre-mRNA is a two-step procedure. In the first step, the 5' splice site is cleaved to produce linear exon1 and lariat configured intron/exon2 followed by the second step, in which the 3' splice site is cleaved; then the two exons are ligated to produce mature RNA (Shin *et al*. 2004; Cartegni *et al*. 2002). This RNA structural rearrangements process requires RNA unwinding (Cordin *et al*. 2006; Linder 2006; Fairman *et al*. 2004; Laggerbauer *et al*. 1998; Raghunathan and Guthrie 1998) that is performed by a series of splicing factors that are members of the DEXD/H-box protein family (Sengoku *et al*. 2006; Rocak and Linder 2004; Silverman *et al*. 2003; Staley and Guthrie 1998), but also energy (ATP) with the exception of the formation of the E complex. Several DExD/H-box splicing factors possess an ATP-dependent RNA unwinding/helicase activity using the energy of ATP hydrolysis (Staley and Guthrie 1998); BAT1 is such a protein (Shen *et al*. 2008; Shen *et al*. 2007; Fleckner *et al*. 1997).

1.6.4 DEAD-box RNA helicases

The DEAD-box helicases are a family of proteins/enzymes that catalyze the unwinding of nucleic acids and are involved in various aspects of nucleic acids metabolism (de la Cruz *et al*. 1999) The DEAD-box RNA helicases in particular, have been implicated in every step of RNA metabolism, including RNA synthesis and pre mRNA splicing, but also ribosome biogenesis, mature mRNA export, translation, decay, and organellar gene expression (Johnson, McKay 1999; de la Cruz *et al*. 1999; Aubourg 1999); they contain 9–11 conserved helicase motifs (Cordin *et al*. 2006; Sengoku *et al*. 2006). The DE*X*D/H-box proteins do not have exactly the same properties and do not

necessarily exert exactly the same function. It has been shown that some attain RNA-dependent ATPase activity, while others unwind dsRNA *in vitro* (Yang and Jankowsky 2006; Sengoku *et al*. 2006; Tanaka and Schwer 2006; Rogers 2002; Wagner *et al*. 1998; Wang *et al*. 1998). In addition, a subset of DEAD-box RNA helicases actively disrupts misfolded RNA structures thus reverse stable nonfunctional secondary RNA formations and permit the occurrence of correct folding (Lorsch 2002; Mohr *et al*. 2002; Tanner NK, Linder P. 2001).

1.6.5 BAT1 structure/location

The HLA-B associated transcript 1 (BAT1) also called D6S81E or UAP56 is a human gene encoding a 56-kDa, 428 amino acid protein, member of the DEAD-box RNA helicases, with important role in the mRNA splicing and the export of the majority of mature mRNAs from the nucleus to the cytoplasm for translation. Similarly to most splicing factors, BAT1 is localized in the nuclear speckle (sub-nuclear structures that are enriched in pre-messenger RNA splicing factors) (Dias *et al*. 2010). The structure and crystallographic analysis of UAP56 protein are shown in table 3.1 and figure 3.3, respectively). The BAT1 protein contains nine conserved motifs that characterize the DEAD-box family of RNA binding proteins. Some of the motifs are responsible for binding to mRNA or ATP and others possess ATPase activity (Allcock *et al*. 2001; Schmid and Linder 1992). The UAP56's amino -terminal and carboxy-terminal domains are connected with a flexible liner (Shi *et al*. 2004; Zhao *et al*. 2004). They both have typical RecA-like fold with seven parallel β-strands surrounded by α-helices on both sides. The N-terminal domain contains seven conserved helicase motifs (Q, I, Ia, GG, Ib,

II, and III) and the C-terminal domain four (IV, Q*XX*R, V, and VI). The crystal structure of BAT1 indicates that the overall fold of amino-and carboxy-terminal domains is highly similar (by 35%) to that of the prototypic DExD/H-box protein eIF4A; the latter is involved in translation initiation (Caruthers 2002; Benz *et al.* 1999). The overall fold of each RecA-like domain is similar to that of eIF4A, with differences at the loops and termini. Taken together, the structural and sequential similarity might also suggest functional similarity between BAT1 and eIF4A.

Table 1.6.5 Crystal structure of the human ATP-dependent splicing and export factor UAP56 - Summary of crystallographic analysis (adopted by Shi *et al*. 2004)

Data sets	UAP56	UAP56-ADP	UAP56 (C198A)
Spacegroup	P1	$P2_1$	P1
Cell dimensions, Å	37.16 × 49.91 × 62.27	37.00 × 78.09 × 63.19	37.43 × 49.76 × 62.81
Cell angles, °	95.7 × 101.6 × 111.1	90 × 103.4 × 90	95.6 × 101.9 × 110.9
No. of proteins per asu,* V_m	1 (2.33 Å^3/Da)	1 (1.98 Å^3/Da)	1 (2.36 Å^3/Da)
Resolution, Å	1.95	2.7	2.4
Measured reflections	107,519	27,997	55,301
Unique reflections	28,304	9,164	14,805
Average I/σ	19.7	15.0	20.9
Completeness, % ($I/\sigma \geq 0$)	96.1 (87.3)	94.6 (89.1)	92.9 (75.6)
R_{merge},† %	4.0 (18.0)	8.5 (14.1)	6.0 (10.1)
Refinement			
Resolution range, Å	50.0-1.95	50.0-2.8	50.0-2.4
R factor/R_{free},‡ %	21.8/25.8	21/30	24.3/29.8
No. of protein atoms	3,089	3,017 (1 MgADP)	3,095
No. of solvent molecules	4 isopropanol, 245 water	1 acetate, 85 water	1 BME, 59 water
rms deviations			
Bond lengths, Å	0.008	0.007	0.008
Bond angles, °	1.22	1.35	1.31

- ↵ * asu, asymmetric unit; V_m, Matthews coefficient.
- ↵ † $R_{merge} = \Sigma|I - \langle I \rangle|/\Sigma\langle I \rangle$, where I and $\langle I \rangle$ are the measured and averaged intensities of multiple measurements of the same reflection. The sum is over all the observed reflections.
- ↵ ‡ R factor = $\Sigma||F_o| - |F_o||\Sigma|F_o|$, where F_o denotes the observed structure factor amplitude and F_C denotes the structure factor calculated from the model.

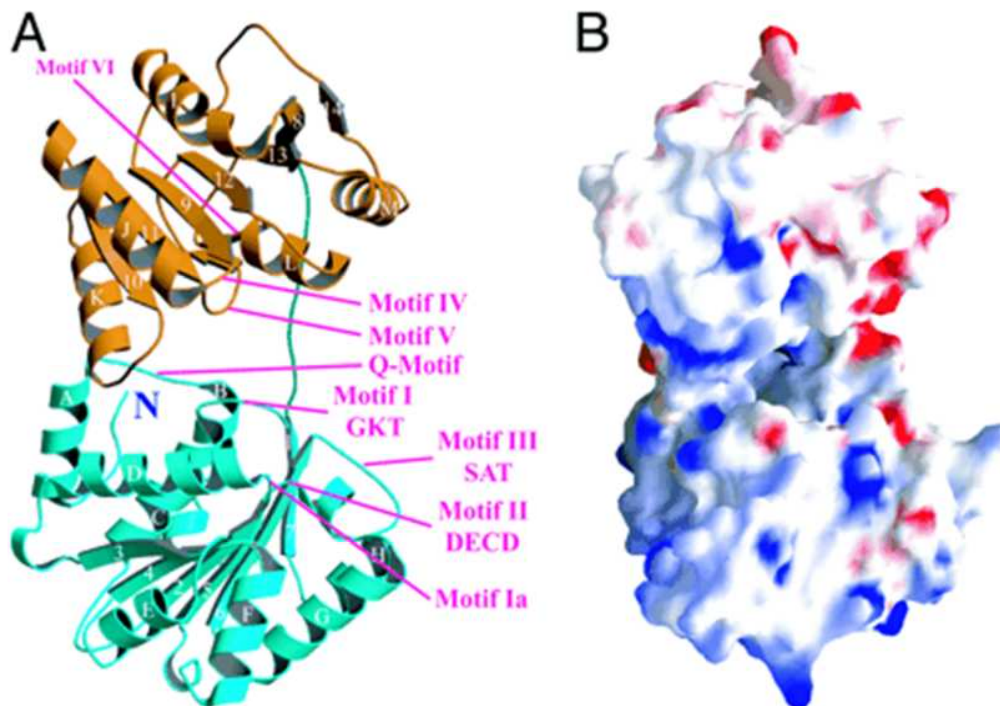


Fig 1.6.5 BAT1 Structure. The structure of UAP56 is shown as a ribbon model (*A*) and in a surface representation (*B*), viewed from a similar direction. (*A*) Cyan, N-terminal domain; brown, C-terminal domain; green, interdomain linker. Locations of conserved helicase sequence motifs are labeled. (*B*) Blue, positively charged electrostatic potential; white, neutral electrostatic potential; red, negatively charged electrostatic potential. A large ATP-binding cleft is formed between the N- and C-terminal helicase domains (adopted by Shi *et al*, 2004)

1.6.6 BAT1 and pre-mRNA splicing

As a member of the DEAD-box RNA helicase family, purified human BAT1 is an active RNA-stimulated ATPase that can hydrolyze ATP (Shen *et al*. 2007; Zhao *et al*. 2004; Benz *et al*. 1999) but also an ATP-dependent RNA helicase that gives BAT1 the ability to unwind 5' or 3' overhangs or blunt end RNA duplexes in vitro. It was shown that BAT1 attains an important role in pre mRNA splicing process and it is essential for

the first ATP-dependent spliceosome assembly step (the binding of U2 snRNP to the branch point sequence) (Fleckner *et al*. 1997); in this process, the human UAP56 (hUAP56) hydrolyzes ATP to facilitate the U2 snRNP-branch point interaction (Fleckner *et al*. 1997). More details regarding role of hUAP56 in pre-mRNA splicing were recently highlighted (Shen *et al*. 2008). It was shown that human BAT1 is actively involved in several pre-mRNA splicing steps. Thus, the ATP-binding and ATPase properties of BAT1 are required for the interaction of complex E with U2 snRNA for the formation of pre-spliceosome (complex A). For the following steps of mature spliceosome formation process and pre-mRNA remodeling, the ATP-binding, ATPase activity and dsRNA unwindase/helicase properties of BAT1 are required; it has been shown that human BAT1contacts U4 and U6 small nuclear ribonucleoproteins to promote the unwinding of the U4/U6 duplex and facilitate stepwise assembly of mature spliceosome (Shen *et al*. 2008). Remarkably, mutations in the N-terminal domain conserved helicase motifs I, II, and III result in loss of the ATPase or helicase activity of BAT1 (Zhao *et al*. 2004).

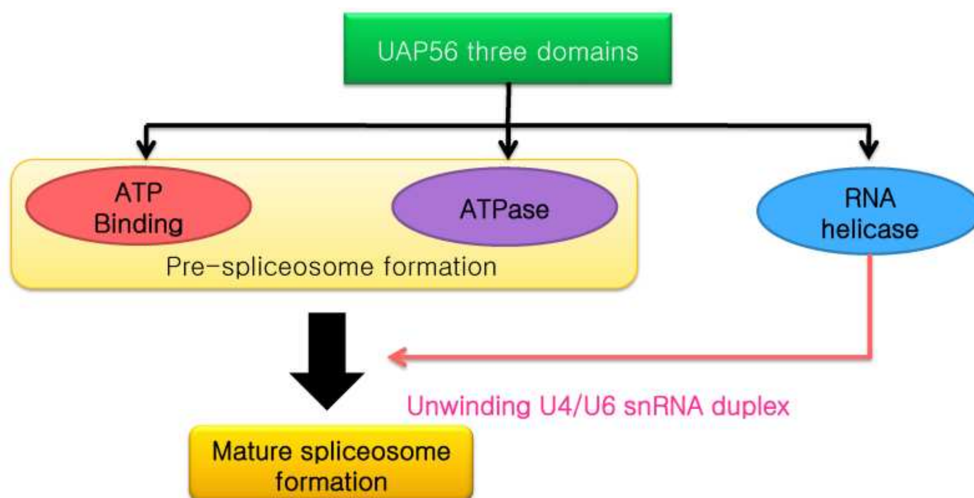


Fig 1.6.6 Function of UAP56 in pre-mRNA splicing (adopted by Shen *et al*. 2009)

1.6.7 BAT1 and nuclear export

The splicing and export processes are coupled. After pre-mRNA splicing and mature mRNA formation, it is exported through nuclear pore complexes to the cytoplasm for translation (figure 1.5) (Mandel *et al*. 2008; Cheng *et al*. 2006; Chan *et al*. 2004; Maniatis and Tasic 2002; Blencowe *et al*. 1998). Experiments in different organisms support an essential role of BAT1 for the mature mRNA nucleocytoplasmic export (Macmorris *et al*. 2003; Herold *et al*. 2003; Jensen *et al*. 2001). In *Drosophila* for example, the mature mRNA export is mediated by a heterodimeric transport receptor (NXF1-p15). The latter, binds the RNA directly or indirectly and transports it from the nucleus to the cytoplasm. Studies in NXF1-p15 and BAT1 knockdowns revealed striking similarities of the mRNA expression profiles (significant reduction of the exported mRNAs, not as a consequence of higher mRNA turnover rates) indicating that these proteins act in the same pathway plus that BAT1 is critical for the export of the majority of mRNAs (Herold *et al*. 2003); remarkable is the existence of a feedback loop by which blocking to mRNA export triggers the upregulation of BAT1. Similar were the findings in BAT1 knockdown studies in Caenorhabditis elegans which demonstrated accumulation of mRNA in the nucleus in the absence of BAT1; these findings support a key role of UAP56 in mRNA export (MacMorris *et al*. 2003). The latter was also supported by studies in higher eukaryotes in which BAT1 associates with spliced mRNAs with exon junction complex thus might provide a functional link between splicing and nuclear export (Reichert *et al*. 2002). It was shown in higher eukaryotes that BAT1 recruits an adaptor protein (REF1) to nascent mRNA which is in turn associated with NXF1-p15 that eventually translocates the mRNA across the nuclear pore complex. It

should be noted though that although essential, mRNA exporting properties have been attributed to BAT1 in mammalian cells, although it is still not clear whether BAT1's ATPase or helicase activity is required for its function to export mRNA (Gatfield and Izaurralde 2002; Strässer *et al*. 2002). It has been shown that, in contrast to REF1, which is recruited co-transcriptionally only to regions of the transcript in which introns have been removed, BAT1 is bound along the mRNA independently of the presence of introns (Kiesler *et al*. 2002). This means that BAT1 binding to nascent transcripts does not necessarily lead to the recruitment of REF1. In addition, it was also shown that BAT1 is essential for the export of bulk mRNA, while REF1 is not (Gatfield D *et al*. 2002 and 2003). Putting together, in higher eukaryotes, BAT1 may act as a critical recruitment factor that recruits not only REF1but also other/others unidentified protein, which in turn promotes binding of NXF1:p15 heterodimers or its role in mRNA export may be that of an helicase which means triggering ATP-dependent mRNA rearrangements that may facilitate binding of proteins that act as adaptors for NXF1.

Finally, in addition to the role of UAP56 as component of splicing and nuclear export complexes, it is also supported its role participation in transcription complexes. The latter comes from findings in yeast where Sub2p (essential messenger RNA export factor in yeast – its conserved counterpart in metazoans is BAT1) is associated with the heterotetrameric THO complex that functions in transcription in yeast (Strässer *et al*. 2002). Putting together, BAT1 provides a bridge between transcription, processing, and export (Reichert *et al*. 2002).

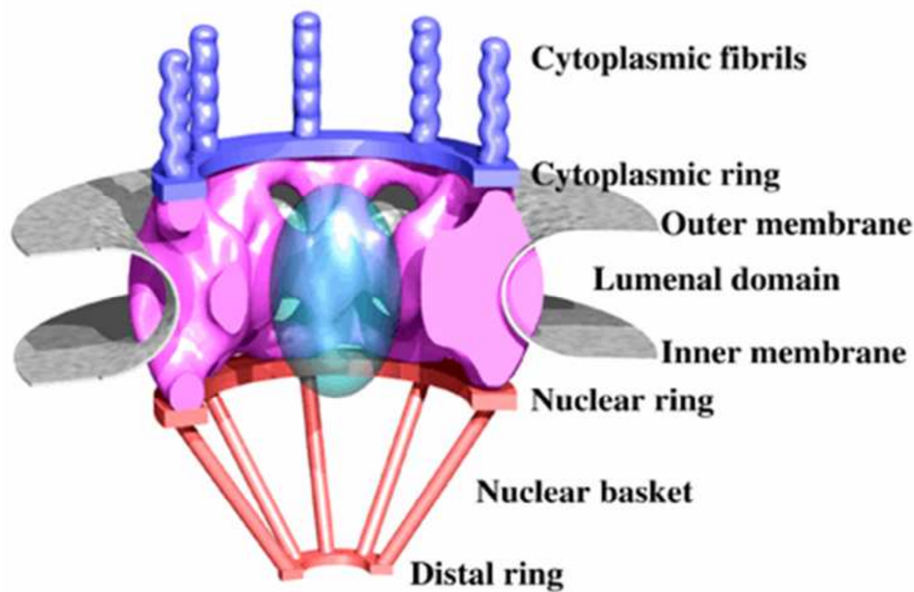


Fig 1.6.7 Nucleocytoplasmic mRNA Export Through the Nuclear Pore Complex
(adopted by Daniel Stoffler web page, Scripps Research Institute)

1.6.8 Clinical significance of BAT1

UAP56 gene is located in the central part of class III MHC genomic locus which affects susceptibility to immunopathologic disorders (Cheong *et al*. 2001; Ota *et al*. 2001; Price *et al*. 1999). It is ubiquitously present in organisms from yeasts to humans and widely expressed in multiple cell types (for example macrophages and hepatocytes) which implies important role for survival (Allcock *et al*. 1999). Findings from several studies have linked reduced BAT1 production with susceptibility to various autoimmune disorders including myasthenia gravis (Degli-Esposti *et al*. 1992), T1DM (Price *et al*. 2004), rheumatoid arthritis (Quiñones-Lombraña *et al*. 2008) amongst other diseases. These disorders are characterized by dysregulation of inflammatory cytokines; hence they

may be influenced by single gene. However, antisense studies have also shown that BAT1 protein can down-regulate inflammatory cytokines such as TNF-α, IL-1 and IL-6 and may therefore influence immunological processes including the development of immunopathological diseases (Allcock *et al*. 2001). Taken together, BAT1 may be directly responsible for the genetic association with the above mentioned pathologies.

1.7 Aim of thesis

Obesity and obesity associated disorders such as insulin resistance, T2DM and cardiovascular disease (CVD) is a pandemic problem. Currently, experimental, epidemiological and clinical studies casually link inflammation to obesity and T2DM, characterized by abnormal cytokine production and activation of inflammatory signaling pathways (Wellen and Hotamisligil 2005). The inflammatory response that emerges in obesity appears triggered by and resides predominantly in adipose tissue, although other metabolically active sites, particularly liver, may also be involved during the course of the disease (Shoelson *et al*. 2006; Fantuzzi 2005).

The function of BAT1 in human adipose tissue has never been investigated. We hypothesize that BAT 1 plays an important role in the regulation of the obesity-associated pro-inflammatory state within the adipose tissue which is considered fundamental in the development of whole body inflammatory state and subsequent metabolic disorders such as insulin resistance, T2DM, endothelial dysfunction and CVD. Initially studies will investigate whether BAT1 is expressed in human adipocytes as well as pre-adipocytes. If BAT1 expression is detected in human adipose tissue studies will investigate its expression in the adipose tissue of lean and obese subjects with or without T2DM, as well

as in different adipose tissue depots (abdominal subcutaneous and visceral depots). Furthermore studies will address whether different adipose tissue depots alters distribution of BAT1 examining the effect of body fat distribution on BAT1 expression ('Effect of Body Fat Distribution' hypothesis). If according to our hypothesis, BAT1 is suppressed in the obese individuals ('Effect of Obesity' hypothesis) and in those with T2DM, as well as in the central (visceral) fat tissue compared to peripheral (subcutaneous) adipose tissue further analysis will examine whether BAT1 repression directly contributes to an increase in inflammation by increasing the production of inflammatory cytokines TNF-α and IL-6, manipulating BAT1 expression in human adipocytes (cell line; repression as well as over-expression). In addition, *in vitro* studies will examine regulation of BAT1 in human adipose tissue investigating the effect of hormonal (leptin, adiponectin, resistin), inflammatory (LPS, TNF-α) and nutritional factors (high glucose concentration, high saturated fatty acid concentration) factors known to pathogenesis of the obesity-associated metabolic defects. As such these combined studies will elucidate the molecular link between BAT1, obesity and inflammation which may lead to a new potential pathway to manipulate in the development of new treatments for insulin resistance, T2DM and CVD.

Chapter 2

General Methods and Materials

2.1 General Methods and Materials

This chapter provides a detailed description of the experimental methods and materials used in this thesis. Furthermore, details of the concentrations and composition of reagents are provided in the appendices.

2.2. Human Subcutaneous and Omental Adipose Tissue Collection and Processing

Human subcutaneous (Sc) and omental (Om) adipose tissue samples were isolated from patients undergoing biopsies or elective surgery/liposuction for cosmetic reasons. The approval of the local ethics committee was issued for all the studies and informed consent was obtained from all the patients prior to enrolment. Subjects with a history of malignancy were excluded from the study, while patients with type 2 diabetes on treatment with anti-diabetic medications or insulin were included and randomized according to their body mass index (BMI) to the appropriate study subgroup. The fat samples were utilized in a category II laboratory.

2.2.1 Extracted Adipose Tissue Freezing

Immediately after the collection, 40mL of adipose tissue was aliquoted into a sterile 50mL falcon (centrifuge tube). The tube was then immediately frozen in liquid nitrogen (N_2) and subsequently transferred into a -80°C freezer, so that any level of protein degradation or damage to be minimized. The samples were stored in -80°C for future use.

2.2.2 Isolation of Mature Adipocytes from the Extracted Subcutaneous and Omental Adipose Tissue - Collagenase Digestion process

For the isolation of the mature adipocytes from the whole adipose tissue, 25-30mL of whole fat tissue from patients undergoing biopsies or elective surgery/liposuction was transferred to a sterile 50 mL falcon. 10 mL of pre-warmed collagenase (2 mg/mL in HBSS, Worthington Biochemical Corporation, Lakewood, New Jersey, USA) was added to this and the tissue/collagenase sample was vigorously shaken by hand so that the collagenase to be well distributed through the whole sample. The mixture was then incubated in a continuously shaking water bath of 37°C (100 cycles/minute) for 40 minutes, with four vigorous shaking at 10 minute intervals. This step was considered completed when a smooth homogenous consistency of the sample was reached meaning complete collagenase digestion. The falcons were then removed from the water bath, wiped down with 70°C ethanol and put in a sterile primary cell culture hood, where their contents were filtered through a layer of sterile autoclaved cotton mesh (Medistore, UK) into a fresh sterile 50 mL falcon. To separate the mature adipocytes, the mixture was centrifuged at 360 x *g* for 5 minutes and the falcon content was separated into three layers-phases; the upper phase containing the adipocytes; the middle phase containing the collagenase and the lower phase at the bottom, containing the stromal-vascular fraction (formed by various cell types, including pre-adipocytes, leukocytes and macrophages).

Any lipid sample was carefully removed from the upper phase of the densely packed adipocytes; the latter was gently slided into a fresh, sterile 50 mL falcon by tilting the centrifuge tube. Any contamination of the adipocyte layer from blood or collagenase

was removed by washing the layer with 30 mL of warmed culture medium (37°C, DMEM/F-12 Ham's phenol red free medium, containing penicillin (100 units(U)/mL) and streptomycin (100 µg/mL); Sigma, UK) and then by gently inverting the tube to suspend the adipocytes and centrifuging at 190 x g for 30 seconds; this process was repeated and each wash was followed by removal and discard of the liquid beneath the adipocyte layer with a sterile 10 mL pipette till the adipocytes appeared free from blood. When the presence of any macrophage-monocyte/blood contamination in the so isolated mature adipocyte sample was excluded using immunohistochemistry (McTernan *et al*. 2002), 1 mL aliquots of adipocytes (approximately 500,000 cells as determined by haemocytometer analysis) were transferred into 1.5 mL microcentrifuge tubes containing 1 ml 10% Dimethyl sulfoxide (DMSO) in 15% Foetal Calf serum and then immediately frozen in liquid nitrogen (N2) and subsequently transferred into a -80°C freezer, so that any level of protein degradation or damage to be minimized; the samples were stored for future use.

2.2.3 Assessment of Adipocyte Cell Viability by Trypan Blue Staining

A dye-exclusion method was used to assess the viability of mature adipocytes. An aliquot of compact adipocytes was re-suspended in trypan blue dye (Sigma, UK; Appendix I) containing phosphate buffer saline (PBS, 120 mM, pH 7.6) and distilled water (dH2O), followed by vortexing and incubation of the sample mixture for 5 minutes at room temperature (RT). A homogenous aliquot of the mixture was measured with a haemocytometer and both viable and non-viable adipocytes were analyzed under a light

microscope and counted using a cell counter; the non-viable cells stain blue as they take up the dye.

2.3 Protein Isolation and Quantification

2.3.1 Protein Isolation from human Abdominal Subcutaneous and Omental Adipose Tissue using Radio-Immunoprecipitation Assay (RIPA) Buffer

For extraction of protein from whole abdominal subcutaneous or omental adipose tissue, the 50 ml falcons with the tissue sample were transferred from -80oC freezer to a 2 liter Dewar flask containing liquid nitrogen so that the sample did not defrost. Approximately 100 mg of frozen tissue was excised with a sterile scalpel and the excised sample was placed in a 5 mL bijou tube with 600 µL Radio-Immunoprecipitation Assay (RIPA) buffer. A Rotor-Stator Homogenizer (PowerGen 125, Fisher Scientific, UK) was used at 1100-4200 x g for 30-40 seconds to homogenize the sample, which was then flash frozen in liquid nitrogen and then removed and allowed to thaw. After thawing, the mixture was spun at 18,000 x g at 4oC for 30 minutes. Using a 5ml sterile syringe and hypodermic needle, the infranatant was carefully removed from beneath the lipid layer, transferred to a fresh, labeled 1.5 mL microcentrifuge tube, and stored in a -80oC for future use.

2.3.2 Quantification of Protein Concentration

The BioRad DC (detergent compatible) protein assay kit (BioRad, Hercules, California, USA) (Peterson 1979; Lowry *et al*. 1951) containing reagent A (alkaline Copper Tartrate solution), Reagent B (dilute Folin Reagent) and Reagent S (Surfactant

solution) was used to quantify protein concentration of RIPA extracted samples. The used protein assay allows the concentration of protein extracted from adipocytes and adipose tissue to be quantified, thus enabling equal loading of protein samples when carrying out western blot analysis.

To quantify the protein concentration, 1.5 mL labeled microcentrifuge tubes containing RIPA extracted protein samples were removed from -80oC storage and allowed to thaw; then the samples was vortexed to ensure a homogenous sample. 980 µL of reagent A and 20 µL of reagent S of the BioRad DC (detergent compatible) protein assay kit were mixed in a glass test-tube, creating a working reagent, A'. 3 µL of protein extracted from adipose tissue was added to each polypropylene cuvette (Starstedt, Germany), in which 125 µL of the previously described reagent A' and then 1 mL of reagent B were added. Finally, parafilm was placed over each cuvette which was then mixed by inversion and incubated for 15 minutes at RT.

2.3.3 Standard Curve for BioRad DC Protein Assay

A standard curve of absorbance against protein concentration was concurrently created using a series of known dilutions of bovine serum albumin (BSA, fraction V, Sigma, UK) (2 µg/µL) in the range 0-100 µg/µL, using the method outlined above. The absorbance of both samples and standards was measured at a wavelength of 655 nm, using a 6505 UV/VIS spectrophotometer (Jenway, UK), allowing the protein concentration of samples (µg/µL) to be determined.

2.4 Western Blot Analysis of Protein Isolated from Adipocytes and Whole Adipose Tissue

2.4.1. Western Blot Analysis of Protein

The western blot (alternatively, protein immunoblot) is an analytical technique used in the sizing, detection and quantification of specific proteins from a given sample of tissue homogenate or extract. In short, it uses agarose gel electrophoresis, pre-loaded with the protein samples, to separate proteins by their size. A molecular weight marker is loaded with the sample for size comparison. The proteins are then electrophoretically transferred from the gel to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) with an antibody raised against the targeted protein. The latter is then detected using a chemiluminescence detection method as shown in **Table 2.1**

Table 2.4.1: Composition of Sample Loading Buffer for Western Blot Analysis.

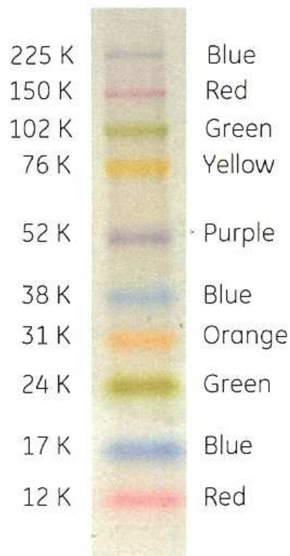
Sample Loading Buffer		
Reagent Used	**Volume Added**	**Final Concentration**
Tris HCl (pH 6.8) (BioRad, Hercules, CA, USA)	625 μL	125 mM
SDS (10%) (Sigma, UK)	500 μL	4%
Glycerol (Sigma, UK)	1000 μL	20% (w/v)
β 2-Mercaptoethanol (BioRad, Hercules, CA, USA)	200 μL	6.5 x 10-3%M (w/v)
Bromophenol Blue	125 μL	2.5 x 10-3%M (w/v)
dH2O	250 μL	N/A

2.4.2 Preparation of Protein Samples Isolated from Adipocytes and Whole Adipose Tissue for Western Blot Analysis

Quantified proteins were removed from -80°C, defrosted on ice and vortexed to ensure a homogenous sample. 12-25 µg from each protein sample was pipetted into a pre-labeled 1.5 mL centrifuge tube containing 10µl loading buffer (**Table 2.4.2**). Sample volumes were equalized with varying quantities of distilled water and the mixture (50 µl) was heated to 95oC for 5 minutes to denature and linearise the protein. After heating, the microcentrifuge tubes containing the protein samples, loading buffer and distilled water at a total volume of 50 µl were centrifuged briefly, kept in 4-5°C for 10 minutes so that any evaporated water to be re-precipitated and then loaded onto the gel .

A rainbow molecular weight marker / ladder (Amersham Biosciences, product code 2892534 – RPN800E, batch 9; UK) with a range of 12 – 225 kDa (**Fig 2.1**) was diluted 1:5 with loading buffer, also heated to 95°C for 5 minutes and then loaded onto the gel with each set of samples (one lane per gel). The ladder provides a method of identifying a protein of a specific molecular weight- size by measuring it against a selection of proteins of a known size (detailed in appendix III).

Fig 2.4.2 Rainbow molecular weight marker (Amersham Biosciences, product code 2892534 – RPN800E, batch 9; UK)



2.4.3 Protein Separation using Gel Electrophoresis

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out as a method of separating out the proteins by the molecular weight-length of the polypeptide (4% stacking gel, pH 6.8; 10% resolving gel, pH 8.8); the percentage of resolving gel was chosen according to the size of the protein of interest. The gels casings were prepared following manufacturer's instructions; 1.5 millimeter (mm) glass plates were arranged and clamped in place; distilled water was poured between the plates to ensure they were watertight and free from any leaks.

The resolving gel was prepared in a 50 mL falcon; the mixture was vortexed for 30 seconds and gently poured into the gel casings, leaving a gap of approximately 15 mm

from the top of the glass plates for the stacking buffer to be added. A small volume of methanol was poured onto the top of the resolving gel to initiate polymerization. The gel was left for about 30 minutes at room temperature to set. Once completely set, methanol was removed and the gel surface washed gently with distilled water.

The stacking gel was then prepared in a 50 ml falcon; the mixture was vortexed for 30 seconds and gently poured onto the pre-set resolving gel. Taking care not to introduce bubbles wells were created into the stacking buffer by inserting 1.5 mm thick, 5 mm wide, 10 well-combs. The stacking buffer was then left to set for 45-60 minutes at room temperature.

Once completely set, the combs were carefully removed vertically and the wells rinsed twice with distilled water, using a syringe and hypodermic needle to remove any traces of excess acrylamide. The glass encased set gels were then securely fixed to electrophoretic apparatus, placed inside an electrophoresis tank and subsequently filled with 1 L of 1 x electrode buffer.

The 1.5ml microcentrifuge tubes containing the protein samples, loading buffer and distilled water that were kept in 5°C for about 10 minutes so that any evaporated water to be re-precipitated, were removed from the fridge, vortexed well, centrifuged briefly and loaded onto the gels, using duck-billed tips (Fisher, UK). Using a voltage of approximately 120 volts (V), proteins were resolved by electrophoresis until the loading buffer had completely run through the gel and the proteins had separated sufficiently, as determined by the position of bands visible on the molecular weight marker. Details of the gels composition are given in appendix III.

2.4.4 Transfer of Proteins – Electroblotting

After the completion of electrophoresis determined by complete run of the loading buffer through the gel, the glass plates containing the gels were removed from the electrophoretic apparatus, and carefully prised open, exposing the gel. The stacking gel was removed and discarded, and the gels submerged in cold transfer buffer (appendix I) for approximately 10 minutes at room temperature. Immobilon-P™ PVDF membranes (0.45 µm) (Millipore, Bedford, Massachusetts, USA) of the size of the gel were immersed in methanol (100%) for 3 seconds and subsequently washed for 60 seconds with distilled water. The permeated membranes were then left in transfer buffer for approximately 10 minutes at RT. Fibre pads and filter paper (2 sets for each gel) at least of the size of the Immobilon-P™ PVDF membranes were also soaked in transfer buffer and left for 10 minutes to equilibrate. For the transfer process, a section of filter paper was firstly laid over a fibre pad and a gel laid onto the filter paper (orientated to be identical to the loading sequence). The permeabilized Immobilon-P™ membrane was then carefully placed on top of the gel, taking care throughout to remove any air bubbles. This arrangement was further sandwiched with a top layer of filter paper and a fibre pad. The stacks were then placed in transfer casings, in an orientation that would allow migration of proteins from the gel onto the Immobilon-P™ membrane and submerged into a tank, containing 1 L transfer buffer (appendix I). A magnetic ‘flea’ was deposited in the bottom of the tank and an ice pack inserted into the tank to keep the transfer buffer cool. The transfer apparatus was then placed onto a magnetic stirrer to enhance ion circulation in the buffer for the generation of a current. Proteins were electrophoretically transferred at a constant voltage of 100 V for 1 hour in 5°C.

2.4.5 Primary Antibody Application

After the completion of electrophoresis and the protein transfer, the membranes were gently removed from the transfer casings and the top right corner was marked, so that the orientation of the proteins to be distinguished. They were then incubated in 10% non-fat milk solution (Marvel Milk Powder, Premier Brands, Merseyside, UK), dissolved and diluted in 0.5% PBS-polyoxyethylene sorbitan monolaurate (tween 20 (0.1% (v/v)); Sigma, UK) on an orbital shaker for 1 hour at room temperature. To block and minimize any non-specific binding of primary antibodies to the Immobilon-PTM membrane, the membranes were incubated with milk. After this step, the membranes were removed from the blocking agent and rinsed three times with PBS, followed by three 10 minute washes in 0.1% PBS-tween 20 (PBS-T).

The primary antibody was prepared at a concentration of 0.05% PBS-T, to a total volume of 5 ml so that the membrane to be completely covered and incubated in a 50 mL centrifuge tube, on an orbital shaker, for either 2 hours at room temperature. The exact antibody dilutions can be found in chapter 3 where details of the experiments are sited. Following this, the membranes were transferred to individual trays and rinsed three times in PBS (120 mM, pH 7.6). Trays were then placed on an orbital shaker and membranes washed 4 x 10-15 minutes in excess of 0.1% PBS-T to remove any excess primary antibody.

2.4.6 Secondary Antibody Application

After the completion of the previous step, the membranes were incubated in the secondary antibody that was previously prepared in 0.05% PBS-T; this process took

place at room temperature for 1 hour on an orbital shaker (80-100 cycles/min). The membranes were then rinsed thrice consecutively with PBS, and then underwent a further 4 x 10-15 minute washes in excess of 0.1% PBS-T to remove any excess secondary antibody.

2.5 Immunodetection of Antibody-Labeled Proteins

2.5.1 Principles of ECL/ECL+ Detection

The horseradish peroxidase (HRP) is an enzyme extensively used in molecular biology applications primarily for its ability to conjugate to a labeled molecule and produce a color, fluorimetric or luminescent derivative of the molecule of interest; thus HRP amplifies a weak signal and allows the targeted molecule to be detected and quantified. However, the HRP enzyme alone is of little value and its presence must be made visible. Therefore, the use of a substrate which when oxidized by HRP using hydrogen peroxide as the oxidizing agent, yields a characteristic change that is detectable by spectrophotometric methods is necessary.

There are several substrates for the HRP including the light transmitting chemical luminol. The HRP catalyses the oxidation of luminol; the reaction is accompanied by emission of low intensity light. However, in the presence of certain chemicals, the light emitted may be enhanced up to 1000-fold making the light easier to detect, increasing thus the sensitivity of the reaction. Such a light transmitting enhancer is phenol and the method that makes use of the enhancement of light emission is called enhanced chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

The HRP enzyme is bound to the molecule of interest and acts as the catalyst for the luminol. Thousands of acridinium ester intermediates per minute are generated by the oxidation of the lumigen PS-3 acridan substrate, which is catalyzed by the combined HRP and peroxide. The so produced intermediate products react with peroxide in PH slightly above 7, producing a sustained, high intensity chemiluminescence. The application of photographic X-ray film or real-time digital photography can exactly visualize the light exuding from this reaction.

After the completion of secondary antibody incubation, the three times rinse with PBS and the further 4 x 10-15 minute washes in excess of 0.1% PBS-T to remove any excess secondary antibody the membranes were ready for chemiluminescent detection. For protein immunodetection, the membranes were placed protein side up on an even sheet of Saran Wrap (Appleton Woods, Birmingham, UK), making sure that any air bubbles were removed. Depending on the protein being studied, either ECL or ECL+ was utilized and reagents were mixed accordingly in the ratios given in **Table 2.2.**

In order to allow even and total coverage of the membranes, the reagents were mixed in sufficient quantities (approximately 1 mL/membrane). The membranes were then incubated with the reagent mixture at room temperature for 1 minute (ECL) or 5 minutes (ECL+). To remove any excess ECL/ECL+ the corner of the membrane was blotted onto tissue paper following the 1 minute (ECL) or 5 minutes (ECL+) incubation. The membranes were then immediately transferred to a clear plastic wallet, not allowed to dry. Using tissue paper and by gentle wiping over the surface of the plastic any air bubbles were removed. The wallet was then placed inside a film cassette and the membranes exposed to photographic X-ray film (Kodak, UK) for varying time points

(from 1minute to 24 hours) depending on the antibody used, until the targeted protein became suitably visible for detection and quantification.

Table 2.5.1 Quantities, ratios, and incubation times for chemiluminescence detection systems used for western blot analysis.

Chemiluminescence Detection System Used	***Reagent Ratio Used (1 : 2)***	***Membrane Incubation Time***
ECL	*1 mL : 1 mL*	*1 min*
ECL+	*50 µL : 2 mL*	*5 mins*

2.5.2 Quantification of Western Blot Protein Bands

The quantification of western blot protein bands was done using Chemigenius (Syngene, UK). For this purpose, 12-24 µg of the extracted protein was loaded onto a 10% polyacrylamide gel (Geneflow Ltd., Fradley, UK), under reducing conditions. Then a mouse BAT1 monoclonal antibody (primary antibody) was utilized (product code: ab50986, UAP56 antibody [2060C10a], 1:1000; abcam Ltd., UK) and the blots were developed using a secondary anti-mouse antibody (product code: ab6729, mouse IgG secondary antibody – H&L, abcam Ltd., UK). Equal protein loading and transfer was confirmed by examining β-actin (Cell Signaling, UK) expression (Primary antibody, 1:1000; Secondary antibody, 1:10,000). The ratio of BAT1 to β-actin was used to minimize the potential effect of any insignificant differences in protein loading in the results. Visualization of the produced western blot protein blots was developed by the

usage of a chemiluminescent detection system ECL/ECL+ (GE Healthcare, Amersham Biosciences, Little Chalfont, UK) following exposure to X-ray film and then intensity was determined using densitometry (GeneTool software, Syngene, UK). The autographs' quantification was followed by appropriate statistical analysis

2.5.3 Statistical Analysis

An unpaired Students' t-test was used to compare the protein expression data between control and treatments. Data were presented as mean±SD. Analyses were carried out using the SPSS (SPSS Inc. 14.0, Woking, UK) software package. The threshold for significance was $p<0.05$. Further details of the statistical tests used are given in each chapter.

2.6 Isolation and Purification of RNA from adipose tissue

Total RNA was extracted from whole AT, using a column-based isolation method (RNeasy Lipid Mini Tissue Kit; Qiagen, UK), according to manufacturer's instructions. After the completion of this process, a DN*ase* digestion step followed so that any possible genomic DNA (gDNA) contamination, to be removed (appendix II). For the DN*ase* digestion step, 7 μL (1000 U/mL) of DN*ase* I digestion enzyme (DN*ase* I Kit, Sigma, UK) was added to the eluted RNA, along with 7 μL reaction buffer (DN*ase* I Kit, Sigma, UK); the reaction was incubated at room temperature for 15 minutes. After the completion of 15 minutes the reaction was stopped with the addition of 7 μL stop solution (50 mM EDTA) (DN*ase* I Kit, Sigma, UK). Following this, the samples were vortexed, centrifuged briefly and heated to 70°C for 10 minutes for DN*ase* (the process id described below).

2.6.1 RNA Quantification

As nucleic acids only absorb light at 260 nm, RNA was quantified using a spectrophotometer (Nanodrop ND-1000, Labtech, UK) measuring at this wavelength. The ratios between absorbances 260/280 nm and 260/230 nm were measured to give an estimate of RNA purity, with respect to contaminants (e.g. proteins, solvents and salts), which can alter the absorbance ratios as they absorb ultraviolet (UV) light at or around 280 nm (e.g. proteins) or 230 nm (e.g. phenols). Values of ~1.8 for both ratios between absorbances represent an accepted RNA purity for use.

2.6.2 Reverse Transcription of RNA

1 µL of random hexamers (Invitrogen, UK) and 1 µL 10 mM dNTP mix (dATP, dGTP, dCTP, dTTP at neutral pH, Invitrogen, UK) was mixed with an aliquot containing 200 ng RNA (diluted with distilled water to reach the desired RNA concentration) into a 1.5ml sterile microcentrifuge tube to a total volume of 13 µL. The mixture was then vortexed, spun for 15 seconds and heated for 5 minutes to 65°C. After the completion of this process, the samples were vortexed thoroughly and spun for 15 seconds and reverse transcription mastermix was prepared to a sufficient volume as to allow for all reactions (**Table 2.6.2**). 7 µL of the mastermix were added to each reaction, to a final volume of 20 µL. Each sample was subsequently vortexed thoroughly, spun briefly, incubated at room temperature for15 minutes and then transferred to a thermocycler (Biorad, UK); there, the samples were initially heated to 50°C for an hour, and subsequently heated to 70°C for 15 minutes, so that the reaction to be inactivated. The product of this reaction which is complimentary DNA (cDNA) was then removed from the thermocycler, mixed, spun and

stored at -20°C for use in the future (further information regarding reverse transcription, in appendix II).

Table 2.6.2 Contents of Reverse Transcription master mix. *Invitrogen, UK; **Promega, UK.

Reverse Transcription Master Mix Components	**Volume Added**	**Concentration in Final 20 µL Reaction Volume**
5 x Reaction Buffer*	4 µL	1 x (v/v)
SuperScript III Reverse Transcriptase* (200 U/µL)	1 µL	10 U/µL (v/v)
RNasin (40 U/µL)**	1 µL	2 U/µL (v/v)
0.1M DTT*	1 µL	5 mM

2.6.3 Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR/qRT-PCR)

The quantitative real time polymerase chain reaction is a laboratory technique used in molecular biology that enables both detection and quantification, as absolute number of copies of a targeted DNA sequence. It is based on the polymerase chain reaction, which amplifies and simultaneously quantifies a specific DNA sample. The reactions were prepared to 25 µL volumes in a 96 well plate, in a reaction buffer containing Taqman universal PCR mastermix (Applera, UK), 1 µL cDNA and a specific commercially available TaqMan™ Gene Expression Assay (Applera, UK); the qRT-PCR reaction was carried out using an ABI 7500 Sequence Detection System (Applera, UK). All reactions were multiplexed with the housekeeping gene 18S (ribosomal RNA), provided as a pre-optimized endogenous control assay (Applera, UK). The usage of an

endogenous control enabled data to be expressed in relation to an internal reference, allowing for differences in 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 with the manufacturer's guidelines, ΔCt values were determined (ΔCt is equal to the Ct of the target gene minus the Ct of the housekeeping gene), and the ΔΔCt method used for comparison of gene expression between groups (Applera, UK). To exclude any potential bias due to averaging data which had been transformed through the equation 2-ΔΔCt, all statistics were performed at the ΔCt stage; each measurements was carried out in triplicate. The fluorescent label FAM was used to label all target gene probes while the housekeeping gene was labeled with the fluorescent label VIC. Reactions were as follows: 50oC for 2 minutes, 95oC for 10 minutes, 44 cycles of 95oC for 15 seconds, and finally 60oC for 60seconds. Statistical analysis was performed using student's t-test. Measurements were carried out on at least three occasions for each sample.

2.7 Culture and Differentiation of Primary Human Pre-adipocytes

Primary human pre-adipocytes were isolated from human abdominal subcutaneous and omental adipose tissue as described in 2.2b. The adipocytes were maintained into 1.5 mL microcentrifuge tubes containing 1 ml 10% Dimethyl Sulfoxide (DMSO) in 15% Foetal Calf serum in a -80°C freezer, so that any cellular nuclear function to be ceased. For primary cell culture to be done, the pre-adipocytes were removed from -80°C and quickly thawed in water bath (temperature 37°C). When the content of the microcentrifuge tubes was thaw, they were removed from the water bath

and put in a sterile primary cell culture hood, where their contents (human abdominal subcutaneous or omental pre-adipocyte cells (n = 5-10) were cultured in an incubator (37oC, 95% O2, 5% CO2 mix) and grown in 6-well plates to confluence in DMEM/Ham's F-12 medium containing 15% foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml), and transferrin (5 µg/ml). At confluence, pre-adipocytes were differentiated in differentiation media containing DMEM/Ham's F-12 phenol red free medium containing glucose (1000 mg/liter), insulin (500 ng/ml), transferrin (5 µg/ml), biotin (8 µg/ml), Dexamethasone (400 ng/ml), IBMX (44 µg/ml), Ciglitazone (3µg/ml) and L-Thyroxine (9ng/ml) for 96 hours. The differentiation media were refreshed every 48 hours. After 96 hours the differentiated cells were grown in nutrition media containing DMEM/Ham's F-12 phenol red free medium with d-biotin (8 µg/ml), 3% FCS, Dexamethasone (400 ng/ml) and insulin (500 ng/ml) until the cells were fully differentiated, as indicated by the accumulation of lipid droplets shown under x12,000 microscopic observation of the adipocytes (14-20 days). The nutrition media were also refreshed every 48 hours.

Before any treatments, the growth media were removed and the fully differentiated adipocytes were grown in normal DMEM/Ham's F-12 phenol red free medium containing only 2% serum for 24 hours, so that any effects of growth factors and other components used in nutrition media to be removed. After 24 hours, the detoxification media were refreshed and any treatments were then placed in the refreshed detoxification media for the required time points, depending upon the type of treatment.

The isolation, purification and estimation of RNA and protein extracted from cultured primary cells are the same with those used in primary cells.

2.8 Enzyme Linked Immunosorbent Assay (ELISA) / Principles of ELISA Analysis

The ELISA is an immunological technique used to detect the presence and quantify specific proteins in a sample (in our case cell culture media). An antibody, specific to the protein of interest, is coated onto individual wells of a microtiter plate where the samples with the protein of interest (antigen) are pipetted. With this process, an unknown amount of the antigen is captured by the immobilized antibody on the base of the well. Then a specific detection antibody is washed over the surface, forming a complex with the antigen or the antigen antibody. It is important between each step the plate to be washed with a mild detergent solution so that any proteins or antibodies that are not specifically bound to be removed. After removal of excess antibody, a HRP-labeled 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 a detectable signal for *e.g.* color. The color intensity is directly proportional to the concentration of the protein of interest in the original sample. The intensity of the color is then read on a plate reader (Tecan, UK), alongside a standard curve that is set up concurrently with the samples of interest.

In this thesis, ready prepared, commercially available assay kits were used for all ELISA assays performed and the analysis was carried out in accordance with the manufacturer's instructions contained within the individual kits. The particular kit sources and all the corresponding CVs, intra- and inter-assay values for each individual assay are outlined in the relevant chapters. Appropriate statistical analysis for ELISA

was carried out using a statistical software package (SPSS for Windows, Version 16.0, Woking, UK).

Chapter 3

Characterisation of BAT1 Expression

in

Human Adipose Tissue

3.1 Introduction

More than 45 morbidities from all body systems have been linked to obesity. Among them, the increasing prevalence of T2DM has been strongly linked to increasing adiposity; the term "diabesity" has been suggested as providing the point that T2DM and obesity often go hand in hand. Data from large epidemiologic studies reveal the parallel escalation of obesity and diabetes epidemics. It is estimated that 50-60% of patients with T2DM are obese (NHANES and SHIELD, respectively). Several theories linking different pathogenic mechanisms that make obese individuals prone to develop diabetes have been suggested, including the Randle's cycle', the 'Ectopic Fat Storage Hypothesis' and the role of adipose tissue as endocrine organ. Only in the last few years scientists have come to consider obesity as a low-grade inflammatory state in the view of increased plasma circulating mononuclear cells and lymphocytes (Perfetto *et al*. 2002) in obese individuals, as well as in the view of increased plasma concentration of pro-inflammatory cytokines (TNF-α, IL-1, IL-6) and acute phase proteins (CRP) in this group of the population (Yudkin *et al*. 2002; Van Dielen *et al*. 2001; Visser *et al*. 1999; Dandona *et al*. 1998). It has been proposed that source of the pro-inflammatory molecules is the adipose tissue (Xu *et al*. 2003) and in particular the enlarged/hypertrophic adipocytes and the locally accumulated/chemo-attracted macrophages (Curat *et al*. 2004; Weisberg *et al*. 2003; Xu *et al*. 2003). The close relationship between metabolic and inflammatory signaling pathways in insulin-sensitive peripheral tissues has been demonstrated, providing the pathway for the inflammatory-induced insulin resistance and T2DM.

Although significant progress has been made regarding the understanding of the changes in the adipose tissue hormonal, metabolic and biochemical milieu with

increasing adiposity, the crucial fundamental factor that drives the inflammatory state development in the adipose tissue of obese individuals still remains unknown. The HLA-B associated transcript 1 (BAT1), is an RNA helicase, member of the DEAD-box protein family that participates in RNA metabolism in particular pre-mRNA splicing and mRNA nuclear export (Luo *et al*. 2001) and down-regulates inflammatory cytokines (Allcock *et al*. 2001). This anti-inflammatory protein could be of great importance in the inflammatory state generation within the adipose tissue as its suppression could influence the local immunological processes and precipitate the inflammatory state generation.

BAT1 also called D6S81E or UAP56 is a human gene encoding a DEAD-box family RNA helicase. It lies in the central human major histocompatibility complex class (MHC) III region on chromosome 6, a region of great importance for the immune function. The HLA-B associated transcript 1 encodes a protein with important role in the mRNA splicing and mature mRNA transport from the nucleus to the cytoplasm for translation. Mutations of BAT1 have been linked to several autoimmune disorders including insulin-dependent diabetes mellitus, rheumatoid arthritis, systemic lupus erythematous, myasthenia gravis, coeliac disease, dermatitis herpetiformis (Allcock *et al*. 1999). Little is known about the role and function of the protein encoded by BAT1 gene. Its ubiquitous presence however, in organisms from yeasts to humans implies a potential crucial role of this protein in organisms' survival and immune response. Furthermore, screening of human cells and tissues for BAT1 revealed its expression in multiple cell types, notably in macrophages and hepatocytes (Quinones-Lombrana *et al*. 2008; Price *et al*. 2004; Allcock *et al*. 2001 and 1999).

The aim of this study was to investigate the expression of BAT1 in human adipose tissue, the effect of adiposity as well as diabetic status. Further the influence of obesity with and without diabetes on BAT1 expression to elucidate the importance of the metabolic state in expression of BAT1.

3.2 Methods & Materials

3.2.1 Subjects

Human AT was collected from patients (age: 44.7 (mean ± SD) ±9.3yrs; BMI: 27.9 (mean ± SD) ±7.3kg/m^2; male: female ratio 1:4, fasted for 8hr) undergoing elective surgery with informed consent, obtained in accordance with LREC guidelines and approval. All tissue samples were flash frozen and/or utilized for *in vitro* studies as detailed below. In total, 43 human non-diabetic AT samples were analyzed, which were sub-divided into: AbdSc (n=25), and Om (n=18). Subjects providing fat samples were not on endocrine therapy, (*e.g*. steroids, HRT, thyroxine) or receiving any anti-hypertensive therapy. Abdominal subcutaneous adipose tissue was also utilized from T2DM patients (age: 59.1±8.2 yr; BMI: 35.2±9.2kg/m^2, n=8 male: female ratio 2:1).

3.2.2 Extraction of AT RNA for Quantitative PCR

RNA was extracted from AT from AbdSc (n=23), Om (n=18), using RNeasy Lipid Tissue Mini Kit (Quiagen, Crawley, UK), which included a DNase digestion step to remove any contaminating genomic DNA. 1μg of RNA from each sample was reverse transcribed (RT) using AMV reverse transcriptase (Promega, Southampton, UK) according to manufacturers' instructions (McTernan *et al*. 2002, Diabetes).

3.2.3 Microarray

Paired abdominal sc and abdominal omental (n = 10) adipose tissue biopsies were divided into two cohorts according to BMI (lean, n = 5; BMI, 23.0 ± 1.2 kg/m^2; obese, n = 5; BMI, 33.2 ± 3.1 kg/m^2). Gene expression in paired abdominal sc and omental adipose tissue samples were analyzed using the Human Genome U133 plus 2.0 DNA microarrays (Affymetrix). Preparation of cDNA and hybridization to DNA microarray was performed according to standard Affymetrix protocols. The hybridization and analysis were performed according to the Minimum Information about a Microarray Experiment guideline and as previously assessed for other genes (Saiki *et al*. 2009; Brazma *et al*. 2001). Gene expression profiles from the adipose tissue samples were analyzed using the RMA software (Affymetrix).

3.2.4 Protein determination & Western blot analysis

Protein concentrations were determined using the Bio-Rad Detergent Compatible (DC) protein assay kit (Biorad UK). Homogenized human AT were extracted using a 10% RIPA buffer method (McTernan *et al*. 2002). Western blot analysis was performed using a method previously described (McTernan PG *et al*. 2002) and relative expression was standardized by using a densitometry quantification software (GeneTools, Geneflow, Fradley, UK). In brief, 5-20µg of protein was loaded onto an 8% polyacrylamide gel (Geneflow, Fradley, UK), a mouse BAT1 monoclonal antibody (primary antibody; product code: ab50986, UAP56 antibody [2060C10a], 1:1000; Abcam Ltd., UK) was used to assess BAT1 expression (Biosource UK, Nivelles, Belgium). Equal protein loading was confirmed by densitometry using the β–actin antibody (2.04µg/mL, Abcam, Cambridge,

UK). A chemiluminescent detection system ECL/ECL+ (GE Healthcare, Little Chalfont, UK) enabled visualization after exposure to X-ray film.

3.2.5 Statistical Analyses

Descriptive statistics for numeric variables (mean, standard deviation, median) along with 95% confidence limits for mean. In order to explore correlations between numeric variables Pearson coefficient and additionally non parametric Spearman coefficient were used. Shapiro-Francia Normality test was used to test the normality of the data. To compare mean values of BAT1 2 independent samples t-test (the threshold for significance was $p<0.05$), or one-way ANOVA (in case of 3 or more categories) were used. In addition the non parametric tests Mann-Whitney and Kruskal-Wallis were used. For the multiple comparisons after ANOVA, the Scheffe's multiple test was used. Finally the linear multiple regression model was used to assess the overall effect of the predictors to our primary end-point variable (value BAT1). For the graphical presentation bar charts and box-plots were used.

3.3 Results

3.3.1 BAT-1 microarray expressed in human Sc and Om abdominal adipose tissue

The micro-array analysis met all quality standard criteria with 5'/3' ratios of 78% for GAPDH and 60% for β-actin, where it should be noted that values greater than 33% are considered acceptable. Furthermore transcript integrity had been maintained at all stages of RNA extraction and target preparation. Across the arrays there was an average

present call, as determined by GCOS software, of 38% of total probe sets indicating that target integrity was with normal boundaries and that low abundance signals were detectable (expected range of 31.9% for liver to 40.5% for brain; Affymetrix technical notes). Overall 10,895 probe sets were called as present on all 20 of the arrays with a further 22,965 being called as present on at least one array.

Initial microarray expression data examined whether BAT1 expression mRNA expression levels were detectable in human adipose tissue (AT) depots. This data identified BAT1 mRNA expression in all adipose tissue depots. There was also a noted change in expression between AT from lean and obese subjects [AbdSc AT lean: 1567 (±SEM) 73 optical density units (ODU), AbdSc AT obese: 1211±53 ODU; Om AT Lean: 1598±34 ODU, OM AT obese: 1387±68 ODU].

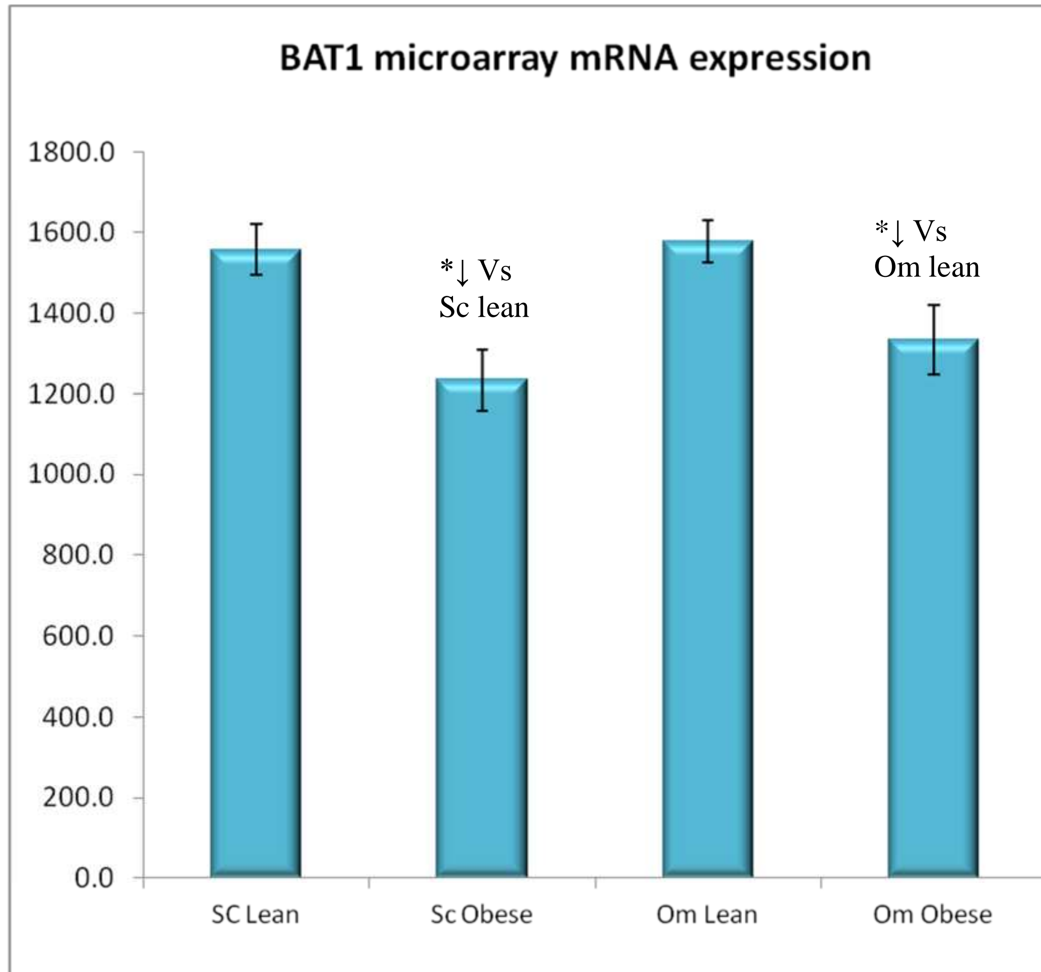
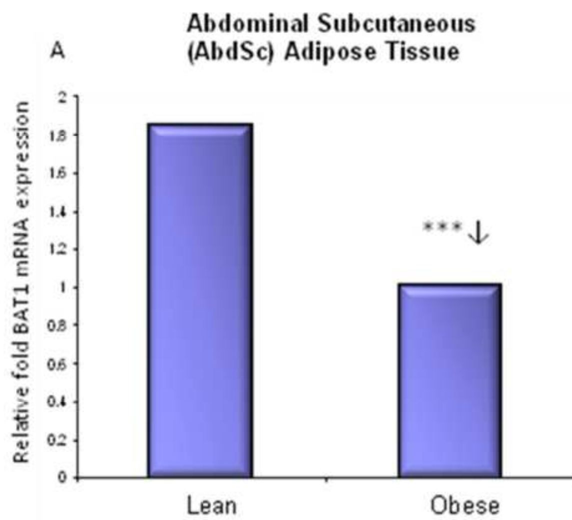


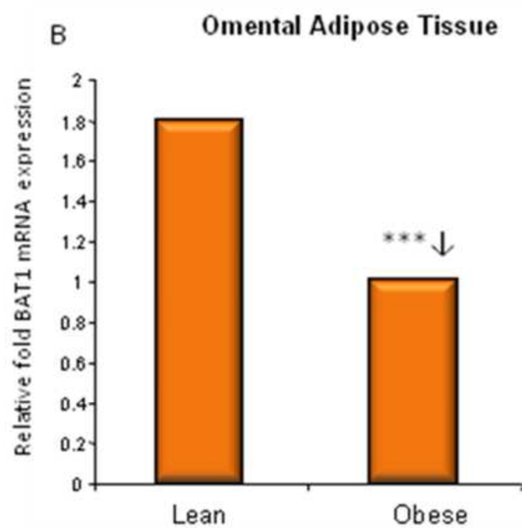
Figure legend 3.3.1. **Microarray mRNA expression of BAT1** in Abdominal Subcutaneous (Sc) and Omental (Om) adipose tissue from lean and obese subjects. Actual optical intensities are plotted (P-values: *p<0.05).

3.3.2 mRNA expression of BAT1 in human adipose tissue using real-time data analysis

Real time PCR analysis data confirmed the initial microarray BAT1 mRNA expression levels in human AT depots however the RT-PCR identified more pronounced changes with adiposity and depot specific expression [AbdSc AT lean: 8.86 (±SEM) 0.19

ΔCT, AbdSc AT obese: 12.37±0.28ΔCT; Om AT Lean: 9.76±0.16ΔCT, OM AT obese: 11.64±0.17ΔCT; **Figure 3.3.2; Figure 3.3.2.1**].





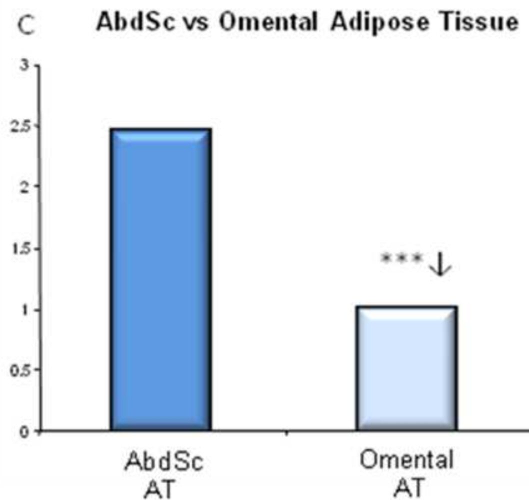


Figure 3.3.2: Relative mRNA expression levels of BAT1 quantified by Real-Time RT-PCR (n=20) (A) abdominal Subcutaneous (AbdSc) Adipose Tissue (AT): Lean vs Obese (B) abdominal Omental (Om) AT: Lean vs Obese, and (C) AbdSc Vs Om AT (P-Value: ***$p<0.001$).

The final figure below also details the relative fold changes in expression compared between the AT depots as well as examining the effect of how AT taken form T2DM subjects changes BAT1 mRNA expression [T2DM AbdSc AT: 12.5±0.40ΔCT Vs T2DM Om AT 10.81±0.24ΔCT; Figure 3.3.2.1].

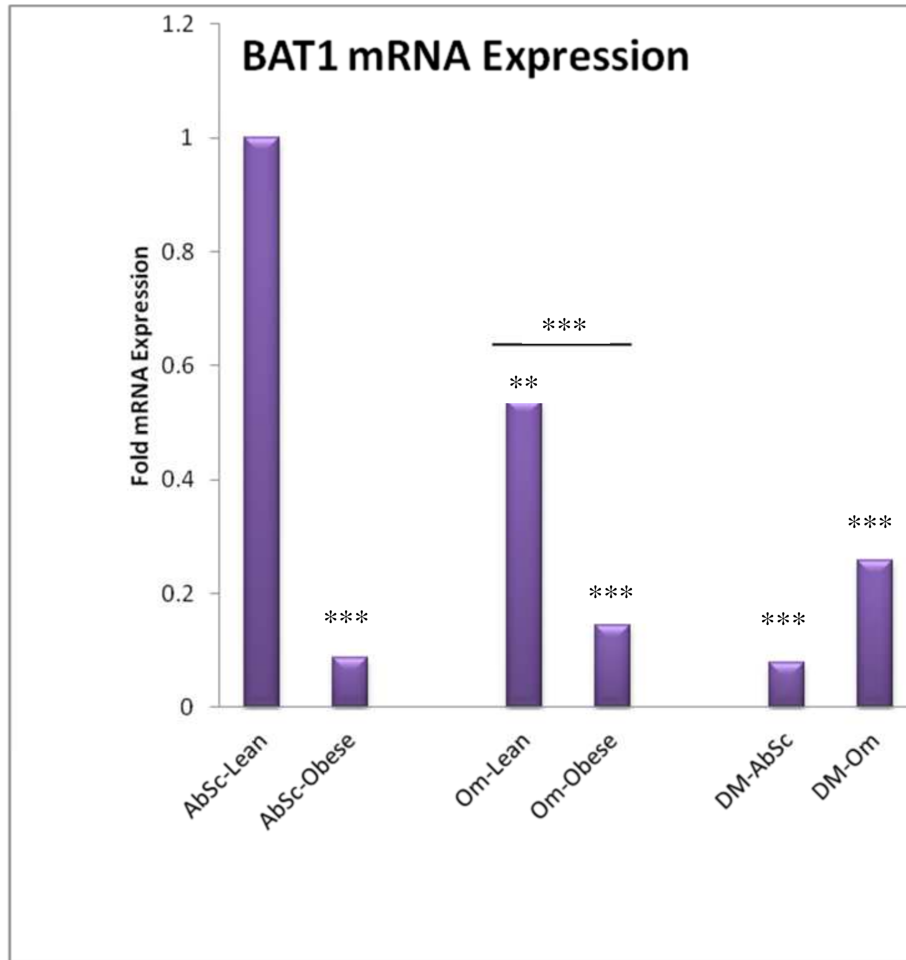


Figure legend 3.3.2.1. Relative fold mRNA expression of BAT1 in Abdominal Subcutaneous (AbdSc) and Omental (Om) adipose tissue from lean and obese subjects, as well as subjects with type 2 diabetes mellitus (DM). AbdSc lean adipose tissue has been given an arbitrary figure of 1 to compare with other tissues (P-values: *p<0.05, **p<0.01, ***p<0.001).

3.3.3 The effect of adiposity and gender on mRNA BAT1 expression

Although the data highlighted a change with adiposity further analysis addressed whether there was a statistically significant correlation between BAT1 expression and BMI using Pearson and Spearman coefficient. The results showed that using the Pearson

Coefficient -0.609 with a p-value of p<0.001, which was also affirmed using the Spearman coefficient -0.603 and a p-value of p<0.001, highlighting that as BMI increased BAT1 mRNA expression reduced. With analysis of the mRNA data women tended to have a higher BAT1 mRNA level compared with men (R-value 0.266; p<0.001).

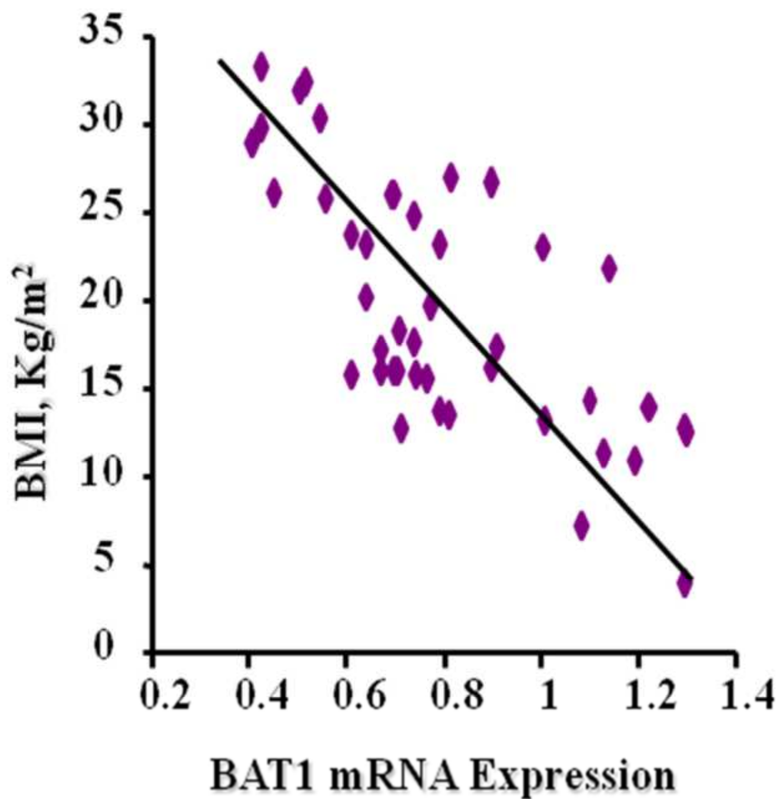


Figure 3.3.3: Scatter plot of BAT1 mRNA expression versus BMI (Kg/m2, R value*****; P value, p <0.001)

3.3.4 Protein expression of BAT1 in human adipose tissue

The effect of AT depot and adiposity for BAT1 protein expression was also analyzed in both depots (Lean: BMI: AbdSc and Om (mean±SD) 22.26±2.62kg/m^2 and Obese: BMI: AbdSc and Om 31.2±6.2kg/m^2). BAT1 expression was significantly increased in the AbdSc depot compared with Om AT group (**Figure: 3.3.4**; AbdSc: 6.46±(SEM)1.10 ODU Vs Om AT: 2.97±1.34 ODU; $P < 0.05$). Furthermore BAT 1 expression altered significantly with adiposity in AbdSc AT (6.46±1.10 ODU Vs Om AT: 2.97±1.34 ODU; $P<0.05$; **Figure 3.3.4.1**).

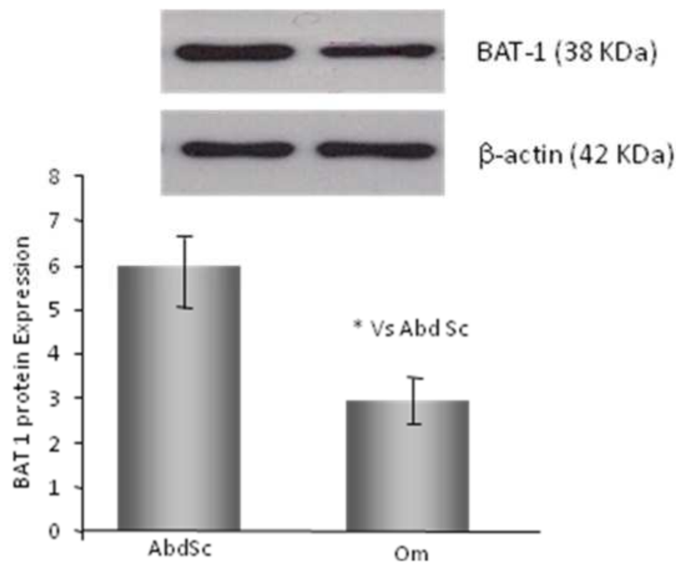


Figure 3.3.4: Protein expression of BAT1 (38 KDa) between AbdSc and Om AT. A representative Western blot is shown for BAT1 and equal protein loading was confirmed by using β-actin (P-Value: *$P<0.05$).

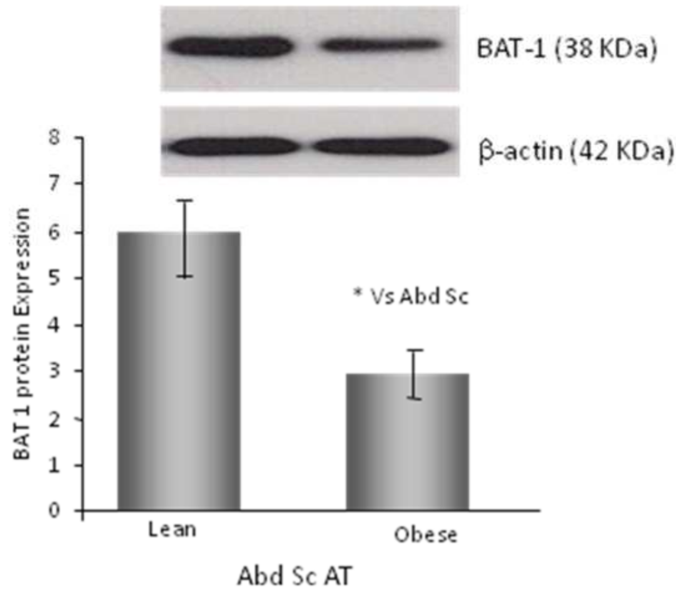


Figure 3.3.4.1: Protein expression of BAT1 (38 KDa) between AbdSc lean and obese subjects is shown. A representative Western blot is shown for BAT1 and equal protein loading was confirmed by using β-actin (P-Value: *P<0.05).

Finally, utilizing the human pre-adipocyte cells line Chub-S7 protein expression of BAT1 was assessed across differentiation, to determine the level of expression in a pure fat cell population. The figure below highlights clear expression of BAT1 in isolated human adipocyte cell line (**Figure 3.3.4.2**).

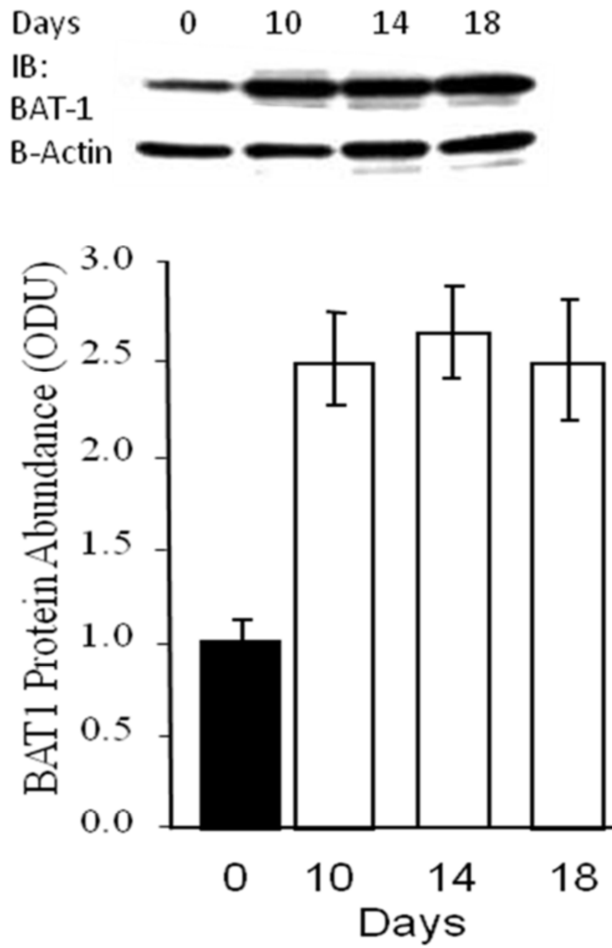


Figure 3.3.4.2: BAT1 Protein expression (38KDa) across differentiation in human Chub-7 cells (n=3).

3.4 Discussion

The premise of this chapter was to understand the relevance of BAT1 in human adipose tissue and understand whether its perceived roles in other tissues and cell line studies may be influential in human adipocytes. To examine this, a series of molecular techniques were utilized. Initially studies investigated the presence of BAT1 in lean and obese Abdominal Sc and Om AT through microarray analysis. These studies revealed that BAT1 was expressed in human Abd Sc and Om AT altered by adiposity and T2DM status.

Although no functional studies explored the role of BAT1 in this chapter following microarray analysis further studies addressed whether increasing adiposity and/or diabetic status may reduce the expression of BAT1 within the AT. Interestingly initial microarray studies identified that BAT1 expression was reduced with obesity in either Abd Sc or Abd Om AT. Subsequent analysis by RT-PCR confirmed this observation with BAT1 mRNA expression reduced with increasing adiposity in both fat depots and a further reduction in subjects with T2DM. However it should be noted that microarray data has to be interpreted cautiously as although an extremely sensitive technique, it can lead to false positives and therefore as in this case the data should always been supported with specifically designed probe sequences that can ensure the data is accurate. To affirm the findings from the real time data subsequent studies examined protein expression of BAT1 and confirmed the real time PCR data. Additionally, whilst it was established that BAT1 was expressed at the gene and protein level in human adipose tissue further studies determine the expression of BAT1 in isolated human adipocytes, as assessed by undifferentiated and differentiating human pre-

adipocyte Chub 7 cells. These findings established that pure cultured human adipocytes express BAT1.

Although obesity reduced expression of BAT1 in either Abd Sc or Abd Om AT it was interesting to note that BAT1 expression was observed to be much higher in lean AbdSc AT than in either omental AT groups (lean or obese) or in either Abd Sc or Om depot once subjects became diabetic. Such an observation may be due to the knowledge that Abd Sc appears to represent the least insulin resistant tissue compared with Abd Om AT or an epicardial AT. However Abd Sc AT importance should not be underestimated in the pathogenesis of T2DM and may represent the last adipose tissue which tries to buffer ectopic fat from other tissues reducing inflammatory responses (Carobbio *et al*. 2011). With BAT1 mRNA expression reduced in obese and T2DM states this may incapacitate BAT1 functionality to reprise the production of inflammatory cytokines and co-current insulin resistance. The BAT 1 expression would also tend to fit with the subjects at most risk of insulin resistance, with men tending to show a lower BAT 1 expression compared with women.

Taken these finding together, indicate that if BAT1 expression is suppressed with increasing adiposity and remain suppressed, through to the development of T2DM and thereafter this may reduce the capacity to response to the inflammatory insults. Such BAT 1 suppression in the adipose tissue of obese subjects could be associated with unhindered local release of inflammatory cytokines e.g. IL-6, TNF-α that generate a local pro-inflammatory state initially with later systemic effects. Interestingly, within the subgroup of the obese subjects (with or without diabetes) BAT1 was significantly more suppressed in the Abd Sc adipose tissue compared with Om AT. This may not be what

many studies would anticipate as omental AT is often viewed as the critical site in the generation of the metabolic disturbances (e.g. Isomaa *et al*. 2001; Kahn, Flier 2000; Kissebah & Krakower 1994; Larsson *et al*. 1984). This finding however, could imply a more important role of peripheral Abd Sc AT in the generation of inflammation in obesity than other studies might consider currently (Carobbio *et al*. 2011). Although often noted in Abd Sc AT is to have comparable or significant adipokine protein expression and release compared with Om AT (McGee *et al*. 2011; Harte *et al*. 2005; Fisher *et al*. 2005; McTernan *et al*. 2003; McTernan *et al*. 2002). Besides AT depot being important for BAT 1 expression these current studies also examined predictors of BAT1 expression such as BMI, age, gender and T2DM status. The statistic analysis revealed that increased BMI, male gender and presence of diabetes were all associated with suppressed BAT1 expression.

In conclusion this chapter has identified that BAT1 is clearly expressed in human adipose tissue and isolated human adipocytes, further that fat depot, adiposity, diabetic status and gender can impact on the expression of BAT 1. However, as the human adipose tissue contains several cell types except of adipocytes, including fibroblasts, macrophages, lymphocytes and endothelial cells, and some particular cell types increase with increasing adiposity for *e.g.* macrophages (Weisberg *et al*. 2003) and lymphocytes (Kintscher *et al*. 2008; Wu *et al*. 2007) it will be important to establish if BAT1 expression is regulated in human adipocytes. Further to understand whether local release of inflammatory cytokines influence BAT 1 expression and as such suggest how obesity and diabetic status may affect BAT1 activity.

Chapter 4

BAT1 Expression in the Immortalized Human Subcutaneous Pre-adipocyte Cell Line Chub-S7

4.1 Introduction

In the previous chapter BAT1 expression at mRNA and protein level was established in human adipose tissue. However, since adipose tissue is composed of several cell types including macrophages, lymphocytes, endothelial cells, amongst other cell type, the identification of BAT1 expression in the whole adipose tissue is obviously not synonymous with BAT1 expression in the isolated adipocytes. Therefore, this chapter will focus on the expression of BAT1 in isolated human adipocyte cell line and how expression changes through differentiation using the human subcutaneous pre-adipocyte cell line Chub-S7, also called CNCM I-2663. The human pre-adipocyte Chub-S7 (CNCM I-2663) cell line is a well-characterized human preadipose cell line (Jha, Banga *et al.* 1998). Chub-S7 represents a transformed human pre-adipocytes immortalized by co-expression of human telomerase reverse transcriptase (hTERT) and papillomavirus E7 oncoprotein (HPV-E7) genes. The pre-adipocytes are derived from the subcutaneous abdominal white adipose tissue of a 33-year-old severely obese female subject.

Cell lines are widely used in molecular and cellular biology, for the study of intracellular activities, but also for the elucidation of the effects of extracellular molecules and cell-cell interactions. They are produced by extraction from tissue containing a heterogeneous cell population including the cell type of interest, which is separated from several other cell types allowing for subsequent isolation of the cell clone of interest. As soon as the cell clone is isolated, it is then cultured so that the total cell number increases over several generations and the population is uniform in its lineage.

However, the isolated and grown homogenous cellular population will survive only a limited number of passages in an *in vitro* culture and then they will start to

senesce. Therefore, the cells need to be immortalized. This is usually achieved by infecting the cells with a recombinant vector, which could be a recombinant plasmid, a recombinant virus or a retrovirus that harbors in its genome an oncoprotein gene and the telomerase reverse transcriptase gene (hTERT). It was demonstrated (Kiyono, Foster *et al*. 1998) that if the hTERT genes are derived from the same species as the cells being immortalized, this could prevent senesce of cells caused by the shortening of the chromosome's telomeres as it happens with allogenic TERTs. The immortalized cell line derived still attains the karyotype and exhibit metabolic patterns that are essentially identical to those of the non-immortalized original cells that are not tumorigenic. These special properties make cell lines useful human cellular models for research studies.

The immortalized Chub-S7 pre-adipocytes can be maintained in a serum-free chemically defined medium to achieve differentiation into mature white adipose cells. These Chub-S7 cells have been used in a limited fashion in comparison with either human pre-adipocytes or 3T3-LI studies; as such these present studies will also examine how the Chub-S7 cells differentiate over time (Marshak, Greenwalt *et al.* 2002). The aim of the current chapter therefore was to use the immortalized Chub-S7 pre-adipocytes and examine the cells through differentiation, as a model cell line for subsequent experiments. For these studies it was important to investigate lipid metabolism as noted by a glycerol release assay, lipid accumulation by an oil red O staining. Measuring lipid accumulation is important as adipocytes can rapidly grow in size and number during differentiation with these processes being regulated genetically and hormonally, involving multiple genes, through lipogenesis (triglyceride formation), and lipolysis (triglyceride breakdown (Gregoire and Smas 1998; Dicker *et al.* 2007). Gene expression levels are

also an important genetic marker of differentiation to be analysed during Chub-S7 differentiation assessing adiponectin, PPARγ, CCAAT enhancer-binding protein alpha (CEBPα) and perilipin. The ability of Chub-S7 cells to either proliferate or differentiate is under close regulation from various adipogenic factors, such as peroxisome proliferator activated receptor (PPAR)-γ (MacDougald and Mandrup 2002) and free fatty acids (FFAs) as well as adipogenic inhibitory factors, that inhibit hyperplastic growth, such as inflammatory cytokines, that include interleukin-6 (IL-6) (Skurker *et al*. 2005; Maury and Brichard 2010) growth factors (MacDougald and Mandrup 2002), and certain androgens (Dieudonne *et al.* 2000, Allan and McLachlan 2010). Finally, analysis of differentiation will examine protein release of adiponectin and leptin into the media from differentiating adipocytes, as established protein markers combined with the other data to determine that the Chub-S7 have reached full differentiation.

4.2 Methods

4.2.1 Differentiation of Chub-S7 Cells

Chub-S7 were cultured into tissue culture flasks until confluent and then trypsinized to obtain sufficient number of cells to carry out the study. The preadipocytes from the same passage were grown in 6-well plates (10^4 cells/well in 2 ml media) to confluence in DMEM/Ham's F-12 phenol-free medium (Invitrogen, UK) containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and transferrin (5 μg/ ml). At confluence, preadipocytes were differentiated in differentiation media (Promocell, Germany) containing biotin (8 μg/ml), insulin (500 ng/ml), Dexamethasone (400 ng/ml), IBMX (44 μg/ml), L-Thyroxin (9 ng/ml) and Ciglitazone (3 μg/ml) for 72 h. After this

period, the differentiating cells were grown in nutrition media containing (NM) DMEM/Ham's F-12, 3% FCS, D-biotin (8 μg/ml), insulin (500 ng/ml) and Dexamethasone (400 ng/ml) until fully differentiated (14–25 days). The viability of adipocytes was assessed using the trypan blue dye exclusion method. Total RNA and protein were isolated from these cells during differentiation.

4.2.2 Real time PCR adipogenic gene methodology

To determine the metabolic pattern of the novel human adipocytes cell line Chub-S7, the expression of adipocytes-specific markers was assessed using qRT-PCR, at different time points during cellular maturation. qRT-PCR was used to assess the expression of adipocytes-specific markers in Chub-S7 at different time points during cellular maturation including adipogenic transcriptional factors like PPARγ and C/EBPα, mRNA Expression for adiponectin, PPARγ, perilipin and CEBPα, genes were analyzed using commercially available gene expression assays (PE Applied Biosystems, Warrington, UK). BAT1 mRNA expression through differentiation was also examined using commercially available gene expression assays. All reactions were multiplexed with the housekeeping gene 18S, provided as a preoptimized control probe (PE Applied Biosystems, Warrington, UK), enabling data to be expressed in relation to an internal reference to allow for differences in RT efficiency. Data were obtained as Ct values according to the manufacturer's guidelines and were used to determine ΔCt values (ΔCt = Ct of the target gene - Ct of the housekeeping gene). Measurements were carried out on at least three occasions for each sample. To exclude potential bias due to averaging

data that had been transformed through the equation $2^{-\Delta\Delta Ct}$, all statistics were performed at the ΔCt stage as part of the standard operating procedures.

4.2.3 Measurement of adipokine release in differentiating Chub-S7 cells.

During differentiation conditioned media was collected and assayed for both leptin and adiponectin secretion, using commercially available solid phase enzyme-linked immunosorbent assay (ELISA) kits (leptin, Millipore; adiponectin Millipore, UK). Conditioned media were analyzed using a ELISA, the leptin ELISA had an assay limit of 0.5 ng/ml, CV intra-assay 2.5-4.7% and inter-assay variability was 1.2-4.6%; adiponectin's assay limit was 0.75ng/ml, CV intra-assay 7.2% and inter-assay variability was 2.2-8.2%.

4.2.4 Lipolysis studies in differentiating Chub-S7 cells.

To undertake the lipolysis analysis conditioned media was collected during differentiation of the Chub-S7 cells and examined for glycerol release as a measure of lipolysis (µM/mL) using a commercial colorimetric kit (Randox Laboratories, Co. Antrim, UK). Conditioned media was analyzed for free glycerol content using the glycerol free assay reagent and glycerol standards according to manufacturer's protocol. 25µl of conditioned media was taken and added to 200µl of free glycerol reagent this was incubated for 15mins at RT and absorbance read at 540nm. Glycerol release in the samples was calculated from the standard curve using known standards of glycerol.

4.2.5 Lipid staining of differentiating Chub-S7 cells

Oil red O is a fat soluble dye and stains lipids within the adipose cell, therefore it represents a well established reagent to use to assess lipid accumulation in adipocytes. Removal of oil red O following staining of the adipocytes acts as an indirect measure of lipid accumulation. Lipid staining using Oil Red O was undertaken through a modified method by Mcvean *et al,* (Mcvean *et al,* 1965). To assess lipid accumulation at set time points during differentiation cells were washed with hanks' balanced salt solution (HBSS) and stained with 2.5% Oil Red O (wt./vol in isopropanol) (Gurr Ltd., London, UK) for 15 min at room temperature (RT). Cells were quickly washed with distilled water and viewed with a light microscope. Subsequently 100% isopropanol (Fisher Scientific Ltd., Loughborough, UK) was added to elute Oil red O staining and amount of lipid determined by measuring absorbance 520nM. Photographs were also taken to examine lipid accumulation during differentiation.

4.2.6 Protein Expression of BAT1 in Chub-S7 cells

As described in chapter 3 western blot analysis was performed using a method previously described (McTernan PG, McTernan CL, *et al*. 2002) and relative expression was standardized by using a densitometry quantification software (GeneTools, Geneflow, Fradley, UK). In brief, 5-20μg of protein was loaded onto an 8% polyacrylamide gel (Geneflow, Fradley, UK), a mouse anti-human BAT1 monoclonal antibody (primary antibody; product code: ab50986, UAP56 antibody [2060C10a], 1:1000; Abcam Ltd., UK) was used to assess BAT1 expression (Biosource UK, Nivelles, Belgium). Equal protein loading was confirmed by densitometry using the β−actin antibody (2.04μg/mL, Abcam,

Cambridge, UK) for loading control. A chemiluminescent detection system ECL/ECL+ (GE Healthcare, Little Chalfont, UK) enabled visualization after exposure to X-ray film.

4.2.7 Statistical analysis

For analysis of protein expression and gene expression data, statistical analysis was undertaken using unpaired t tests unless otherwise stated, where data were analyzed using nonparametric tests as previously stated in Chapter 3 with full details given in Chapter 2 and appendices. The threshold for significance was $P < 0.05$. The data in the text and figures are presented as the mean ± SEM unless otherwise stated.

4.3 Results

4.3.1 Analysis of adipogenic gene in CHUB-S7 cells during differentiation

For the preadipocyte differentiation, the induction of PPARγ and C/EBPα is very critical. The analysis of the RT-PCR data showed that during the process of differentiation the Chub-S7 PPARγ expression increased about 4.2-fold at day 3 compared to day 0; the level of PPARγ expression peaked at day 6 to 7.5 fold and then gradually decreased but always above the control levels **(Figure: 4.3.1.1)**. Upon differentiation induction expression of C/EBPα increased by almost 20 fold after day 03 in DM and then gradually decreased after day 06 but was always significantly elevated at all time points. Even after day 25, the expression was still 6 fold high. After day 03 IBMX and PPAR-agonist, Ciglitazone were removed from the treatments. Even in the

absence of ciglitazone and IBMX the PPAR-γ and C/EBPα induction was maintained. [276].

The mRNA expression was examined across differentiation also for perilipin, Hexose-6-Phosphate Dehydrogenase (H6PD) and adiponectin. **Table 4.3.1** shows all the actual ΔCt values for the genes examined during Chub-S7 cell differentiation (n=4). This data is then presented in a graphic form showing the mRNA expression for each individual gene as a relative fold expression with day 0 given an arbitrary value of 1. Expression of all these genes significantly increased during adipogenesis (**Figures: 4.3.1.1; 4.3.1.2; 4.3.1.3; 4.3.1.4 & 4.3.1.5**).

Table 4.3.1 The gene expression profile for PPARγ, Perilipin, CEBPα Hexose-6-Phosphate Dehydrogenase (H6PD) and adiponectin across differentiation. The gene expression data show the ΔCt±(SEM) changes at several interval points duriong diffferentation (Day 0-25).

Day	PPARγ SEM ΔCt	Perilipin SEM ΔCt	CERBα SEM ΔCt	Hexose 6PD SEM ΔCt	Adiponectin SEM ΔCt
0	18.27±0.19	29.13±0.12	27.19±0.54	22.31±0.53	25.56±0.05
3	17.12±0.38	17.08±0.34	21.94±0.61	18.76±0.12	22.06±0.54
6	15.63±0.31	16.74±0.27	22.17±0.32	17.77±0.09	22.75±0.66
9	16.08±0.51	19.63±0.41	26.16±0.27	21.29±0.23	23.59±0.17
12	17.78±0.09	17.68±0.16	25.05±0.38	20.15±0.28	24.11±0.25
15	17.69±0.13	20.92±0.47	24.9±0.19	20.56±0.47	23.13±0.21
18	18.17±0.18	20.02±0.4	24.48±0.46	18.57±0.32	23.22±0.82
25	18.11±0.32	24.24±0.43	25.73±0.34	26.89±0.08	24.15±0.16

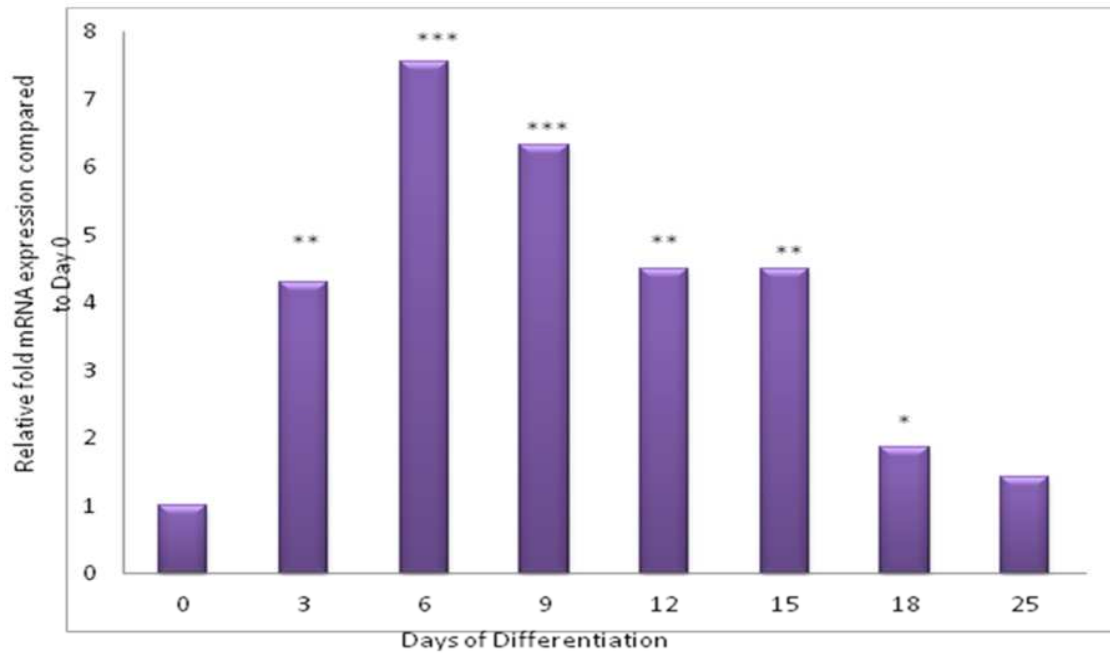


Figure 4.3.1.1 PPARγ mRNA expression in Chub-S7 cells. This figure shows the relative fold difference in PPARγ mRNA expression, with Day 0 taken as 1. All ΔCt±(SEM) data are shown in Table 4.3.1; P-value: *p<0.05).

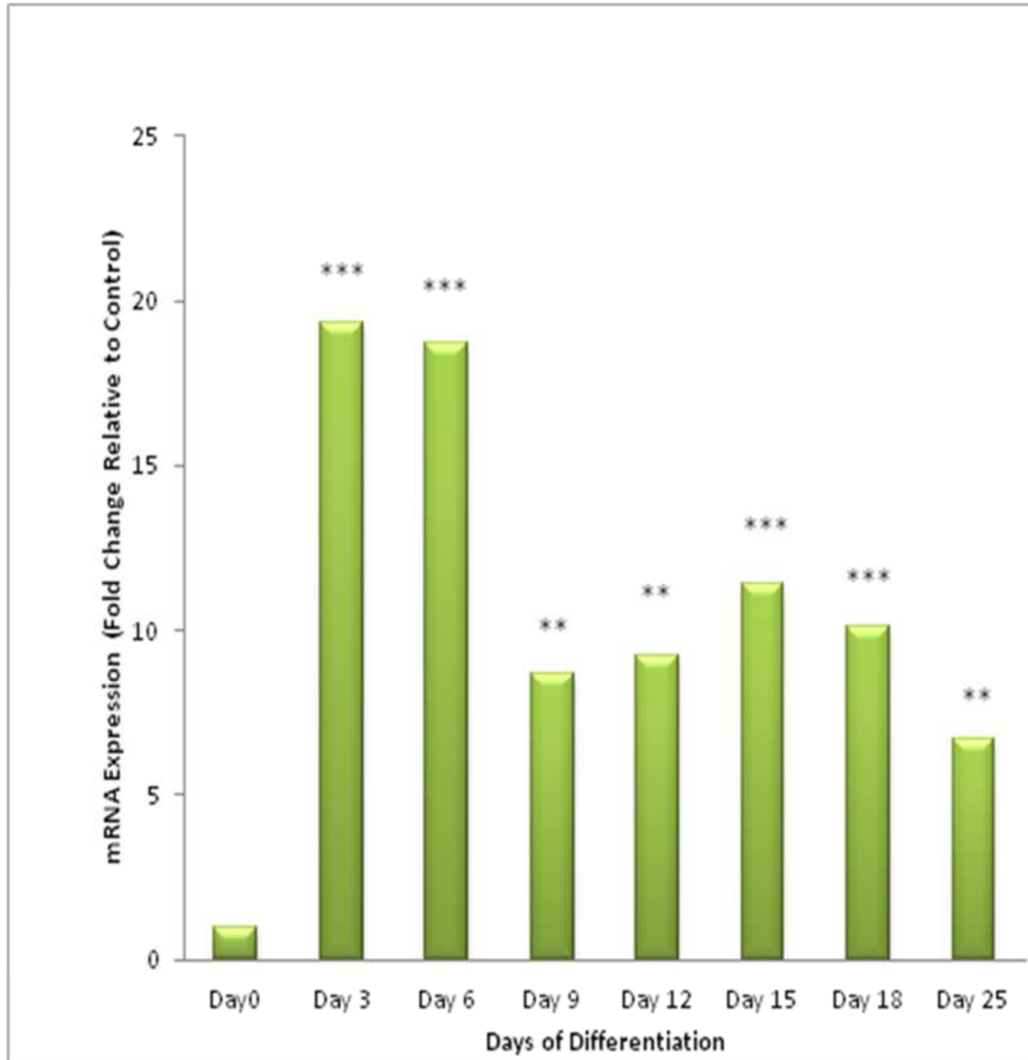


Figure 4.3.1.2 CERBα mRNA expression in Chub-S7 cells. This figure shows the relative fold difference in CERBα mRNA expression, with Day 0 taken as 1. All ΔCt±(SEM) data are shown in Table 4.3.1; P-value: *p<0.05).

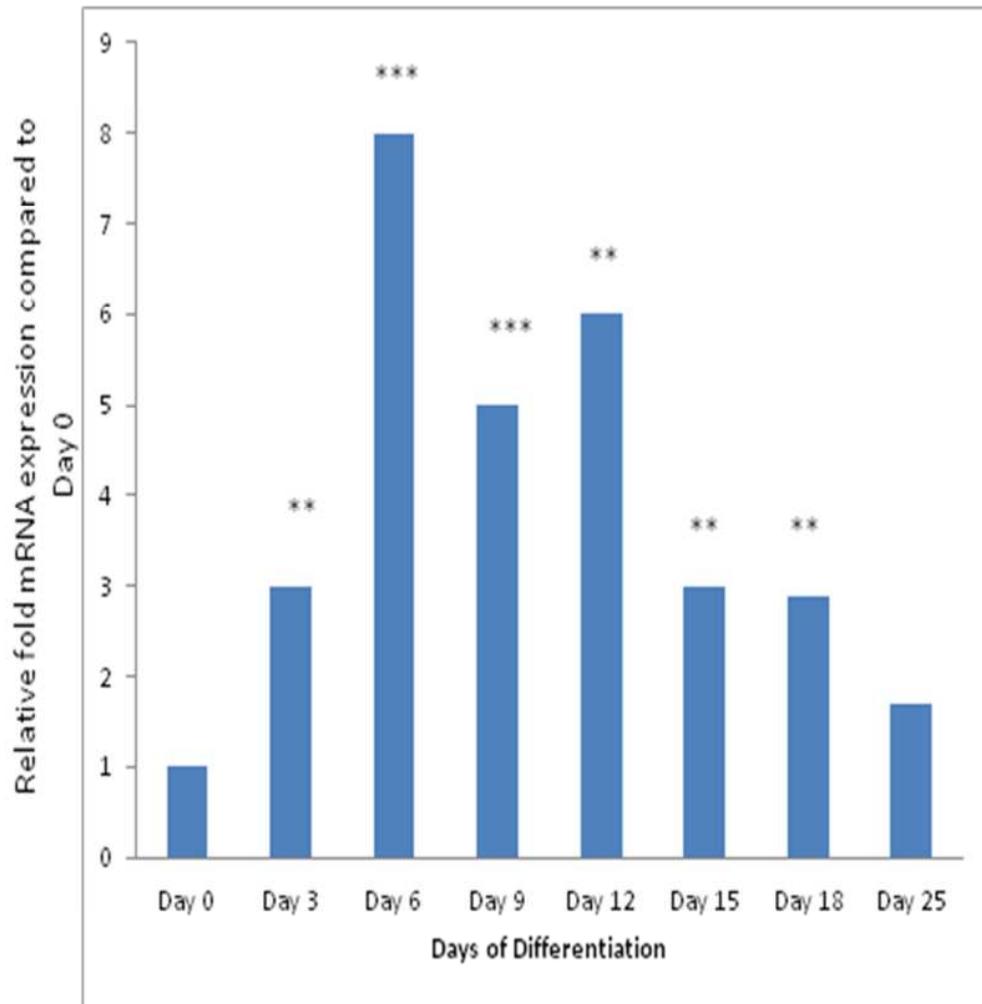


Figure 4.3.1.3 Perilipin mRNA expression in Chub-S7 cells. This figure shows the relative fold difference in Perilipin mRNA expression, with Day 0 taken as 1. All ΔCt±(SEM) data are shown in Table 4.3.1; P-value: *p<0.05).

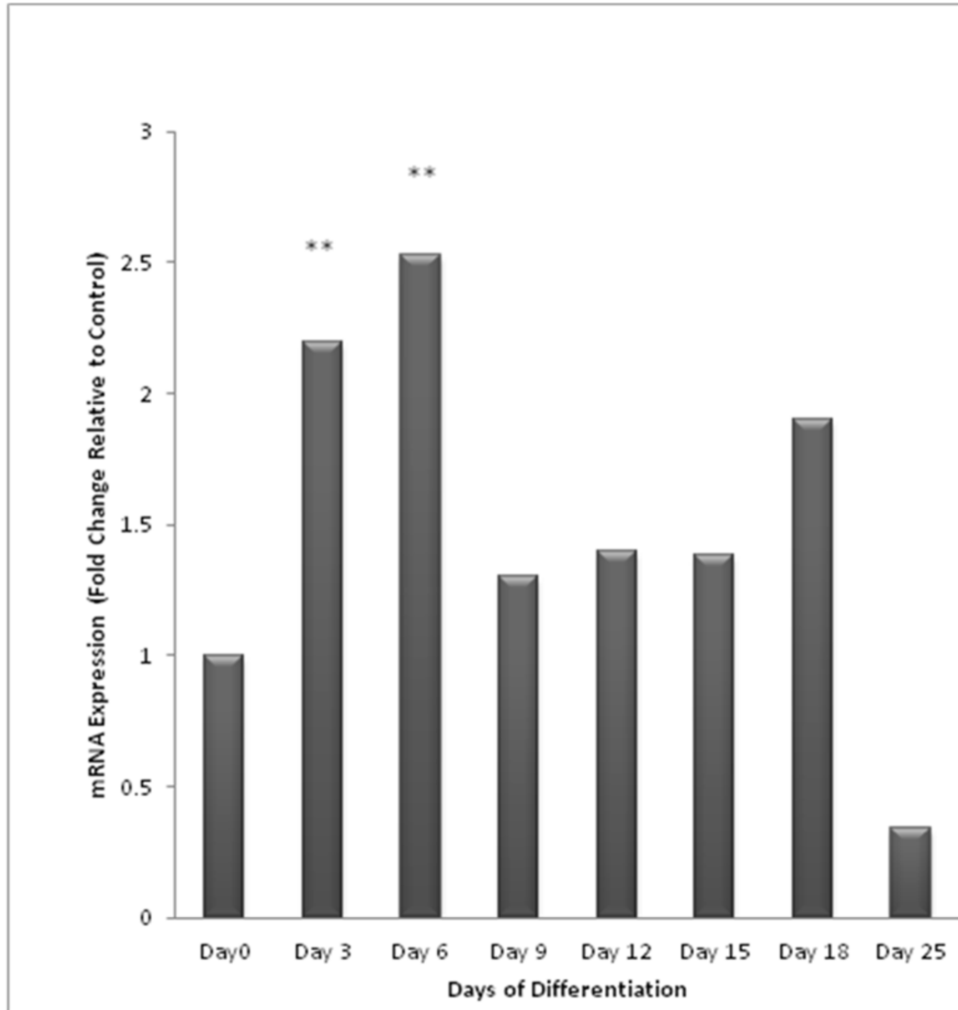


Figure 4.3.1.4 Hexose 6PD mRNA expression in Chub-S7 cells. This figure shows the relative fold difference in Hexose 6PD mRNA expression, with Day 0 taken as 1. All ΔCt±(SEM) data are shown in Table 4.3.1; P-value: *p<0.05).

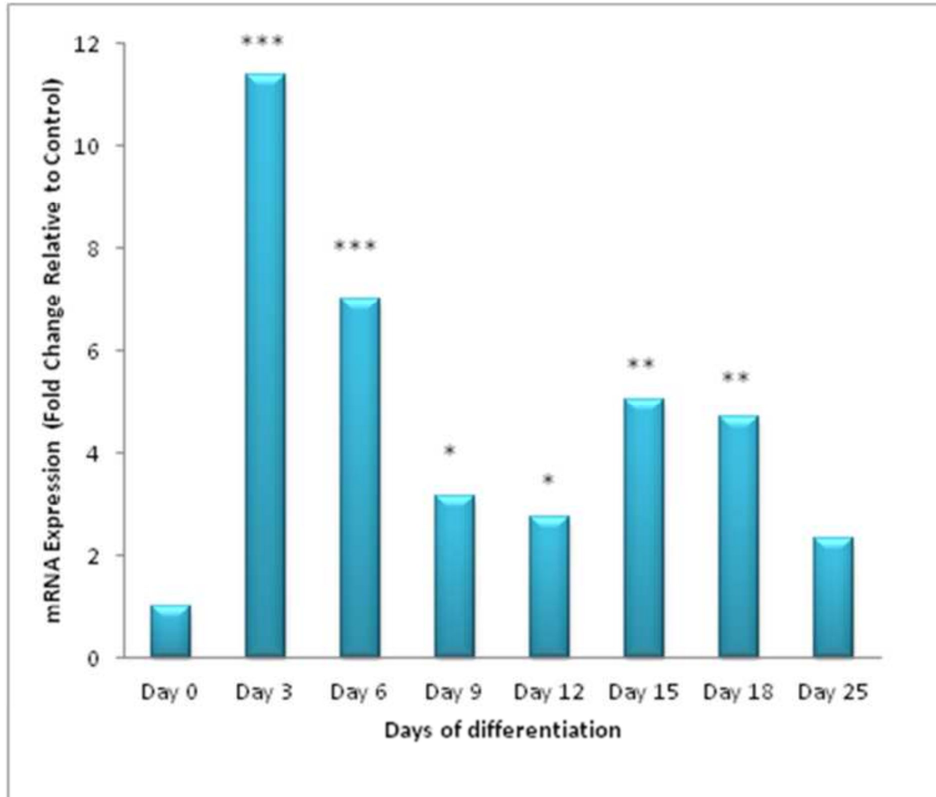


Figure 4.3.1.5 Adiponectin mRNA expression in Chub-S7 cells. This figure shows the relative fold difference in Adiponectin mRNA expression, with Day 0 taken as 1. All ΔCt±(SEM) data are shown in Table 4.3.1; P-value: *p<0.05).

4.3.2 BAT1 mRNA expression in Chub-S7 during differentiation

BAT 1 mRNA expression was measured during the process of differentiation. There was a significant increase in BAT mRNA expression over time up until day 12 (Day 0: 11.41±0.10 ΔCt; Day 3: 10.65±0.03ΔCt*; Day 6: 10.31±0.03ΔCt**; Day 9: 10.07±0.03 ΔCt**; Day 12: 9.37±0.06 ΔCT***; *p<0.05, **p<0.01; ***p<0.001). After day 12 BAT1 mRNA expression appeared to reduce, however after day 12 the BAT1

expression significantly decreased below control levels (Day 15: 11.65±0.04 ΔCt; Day 18: 12.87±0.01 ΔCt**; p-values: **p<0.01, n=4; **Figure 4.3.2.1**).

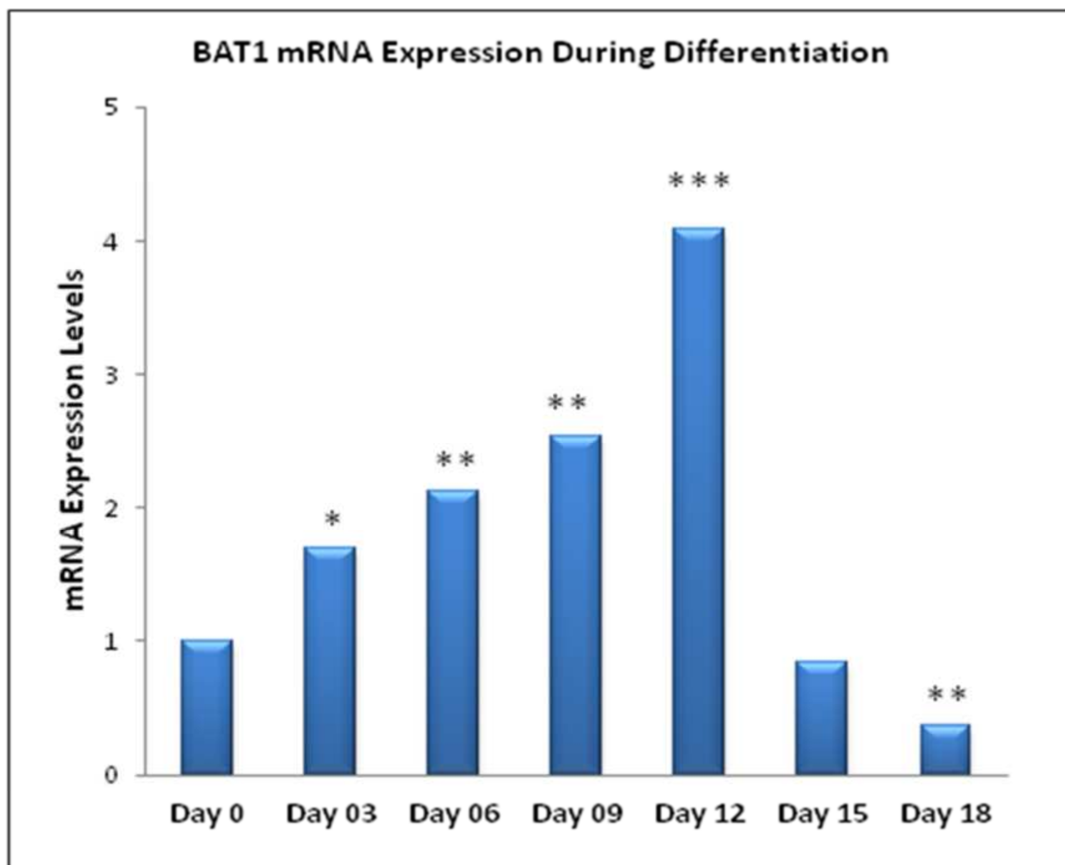


Figure 4.3.2.1 BAT 1 mRNA expression in Chub-S7 cells. This figure shows the relative fold difference in BAT 1 mRNA expression, with Day 0 taken as 1. All ΔCt±(SEM) data are shown in text above; P-value: *p<0.05; **p<0.01).

4.3.3 Measurement of adipokine release from differentiating Chub-S7 cells.

Secreted leptin and adiponectin were measured in conditioned media from differentiating Chub-S7 by ELISA. Secretion of leptin increased over differentiation from day 0 to a maximum level at Day 18 and Day 21 (Day 0: 1.02±0.039ng/ml; Day 3: 1.12±0.33ng/ml; Day 6: 4±0.40ng/ml*; Day 9: 4.7±0.35ng/ml*; Day 12: 5.6±0.64ng/ml*; Day 15: 7.75±0.7ng/ml**; Day 21: 9.7±1.1ng/ml**; Day 25: 8.8±0.34ng/ml** p-values: $p<0.05$; **$p<0.01$, n=4; **Figure 4.3.3.1**).

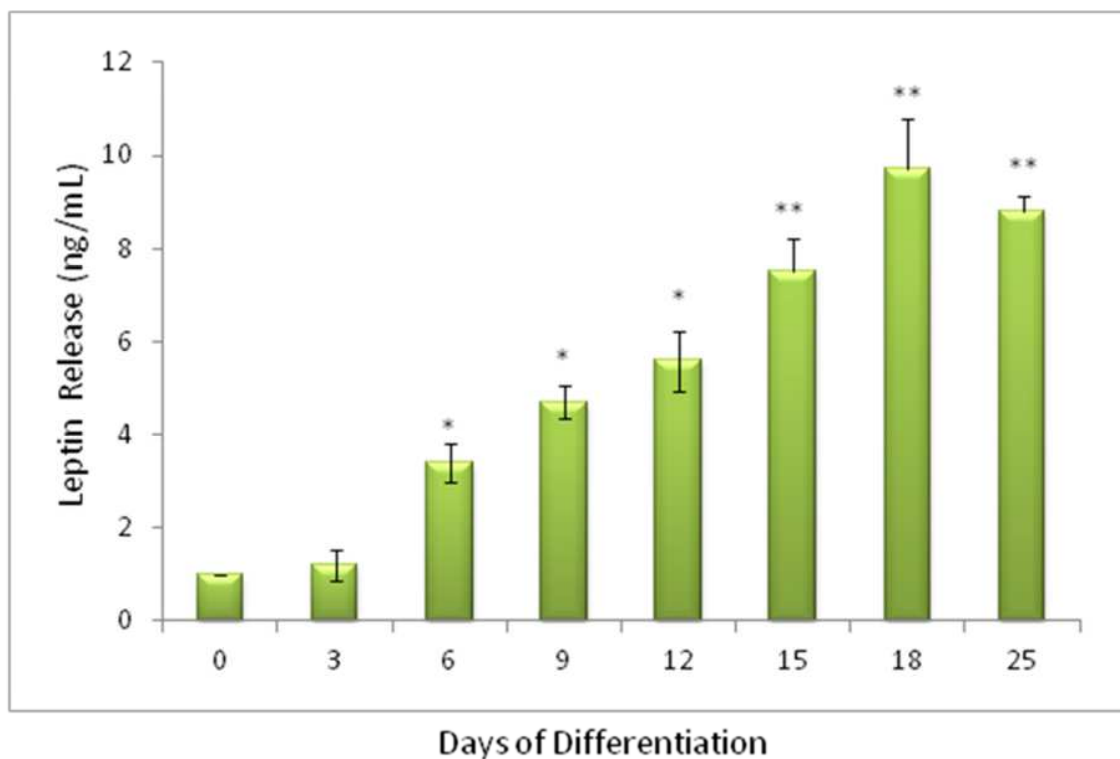


Figure 4.3.3.1: Leptin release from differentiating Chub-S7 cells over time. This figure shows leptin secretion over time, with significant changes noted comparing day 0 versus days 3-25 (p-values: *$p<0.05$; **$p<0.01$).

Additionally adiponectin release was also measured. Adiponectin release data showed that secretion increased over time with a maximal increase measured at Day 9 with a subsequent reduction. It should be stressed that adiponectin secretion was very low from these human adipocytes in comparison to other adipokines. (Day 0: 0.3±0.01ng/ml; Day 3: 0.42±0.03ng/ml; Day 6: 0.5±0.01ng/ml; Day 9: 0.7±0.05ng/ml*; Day 12: 0.4±0.02ng/ml; Day 15: 0.45±0.06ng/ml; Day 21: 0.45±0.07ng/ml; Day 25: 5±0.04ng/ml; p-value: *p<0.05; n=4; **Figure 4.3.3.2**).

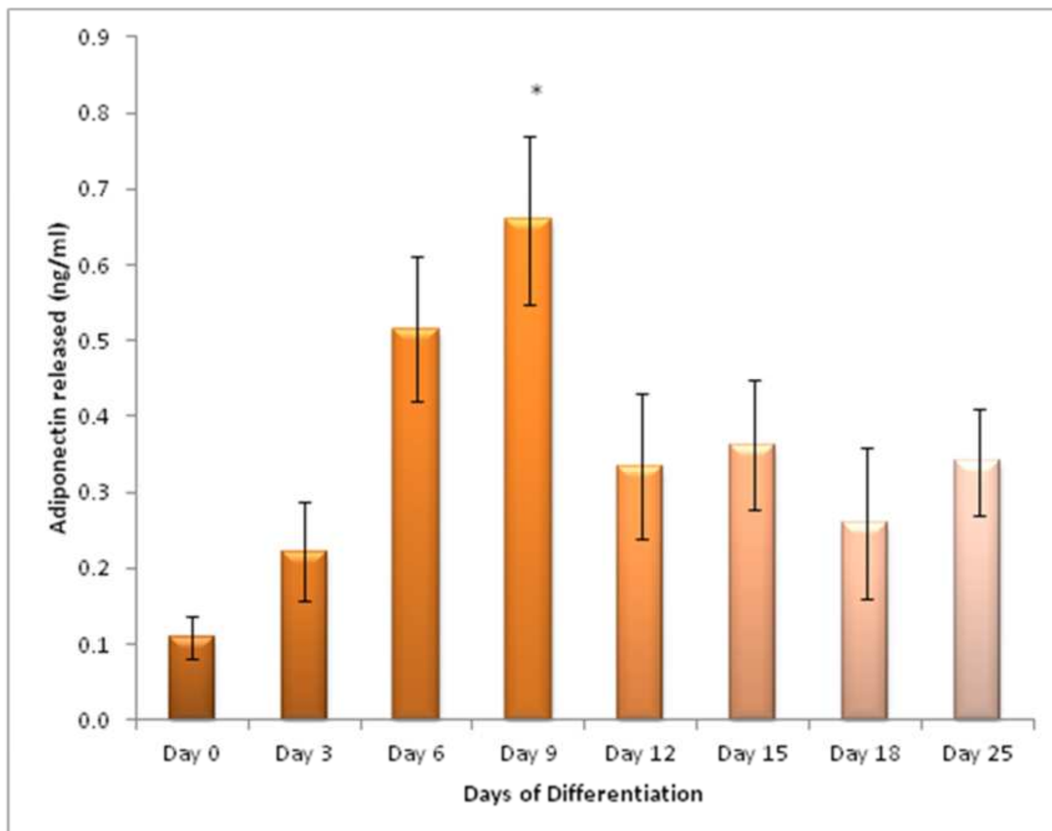


Figure 4.3.3.2: **Adiponectin release from differentiating Chub-S7 cells over time**. This figure shows adioponectin secretion over time, with significant changes noted comparing day 0 versus days 3-25 (p-values: *p<0.05; **p<0.01).

4.3.4 Lipid accumulation in differentiating Chub-S7 cells.

To undertake the lipolysis analysis these studies examined glycerol release as a measure of lipolysis. Glycerol release data showed that lipolysis increased over time as lipid content within the differentiating Chub-S7 cells increased, showing a clear turnover of glycerol. (Day 0: 4.9±0.61μg/ml; Day 3: 5.3±0.75μg/ml; Day 6: 8.5±0.91μg/ml; Day 9: 13.4±0.56μg/ml*; Day 12: 14.64±1.62μg/ml*; Day 15: 15.5±1.56μg/ml**; Day 21: 16.7±1.97μg/ml**; Day 25: 16.75±2.04μg/ml**; p-value: *p<0.05; **p<0.01; n=4; **Figure 4.3.4.1**).

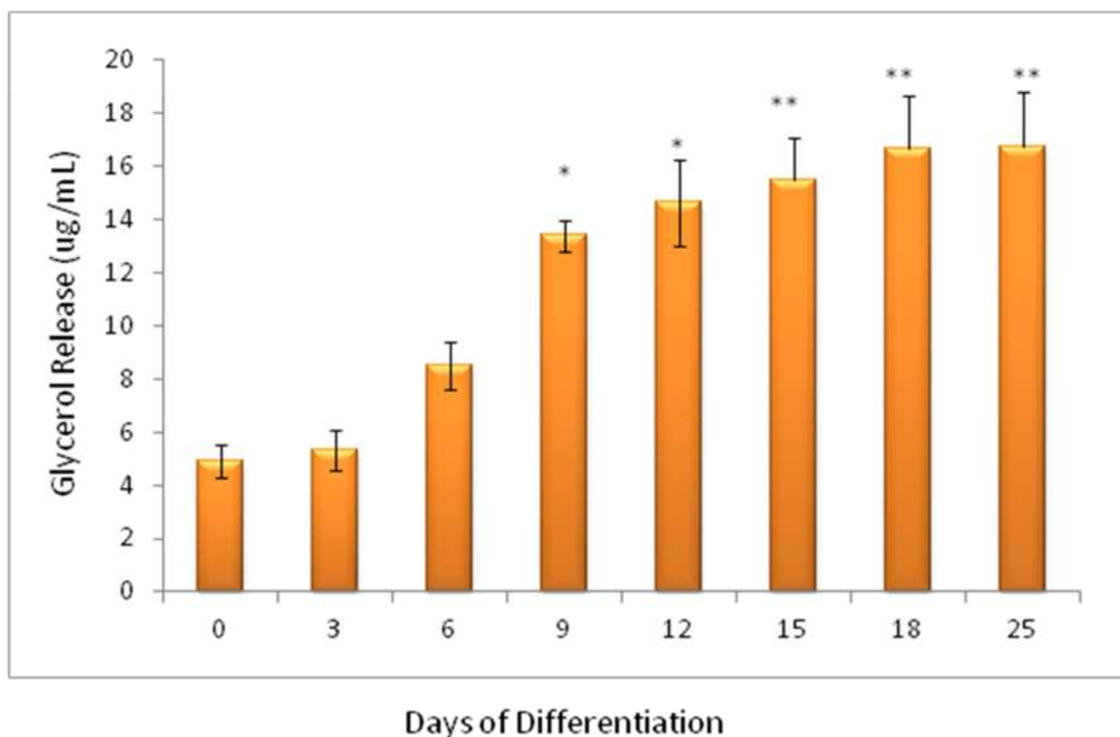


Figure 4.3.4.1: Glycerol release from differentiating Chub-S7 cells over time This figure highlights the change in glycerol release over time, Day 0-25 with significant changes noted comparing day 0 versus days 3-25 (p-values: *p<0.05; **p<0.01).

4.3.5 Measurement of lipid accumulation using Oil Red O in differentiating Chub-S7 cells.

Oil red O release from lipid stained Chub-S7 cell was measured across differentiation with lipid accumulation quantified with spectrophotometric analysis noted at a wavelength 520nM with absorbance defined as optical density units ODU. There was a significant increase in lipid accumulation over time compared with baseline ((Day 0: 0.1±0.01 ODU; Day 3: 0.12±0.01 ODU; Day 6: 0.14±0.01 ODU Day 9: 0.16±0.01 ODU; Day 12: 0.22±0.01 ODU; Day 15: 0.24±0.01 ODU; Day 18: 0.26±0.02 ODU**; Day 25:0.26±0.02 ODU**; p-values: **p<0.01, n=4; **Figure 4.3.5.1.**).

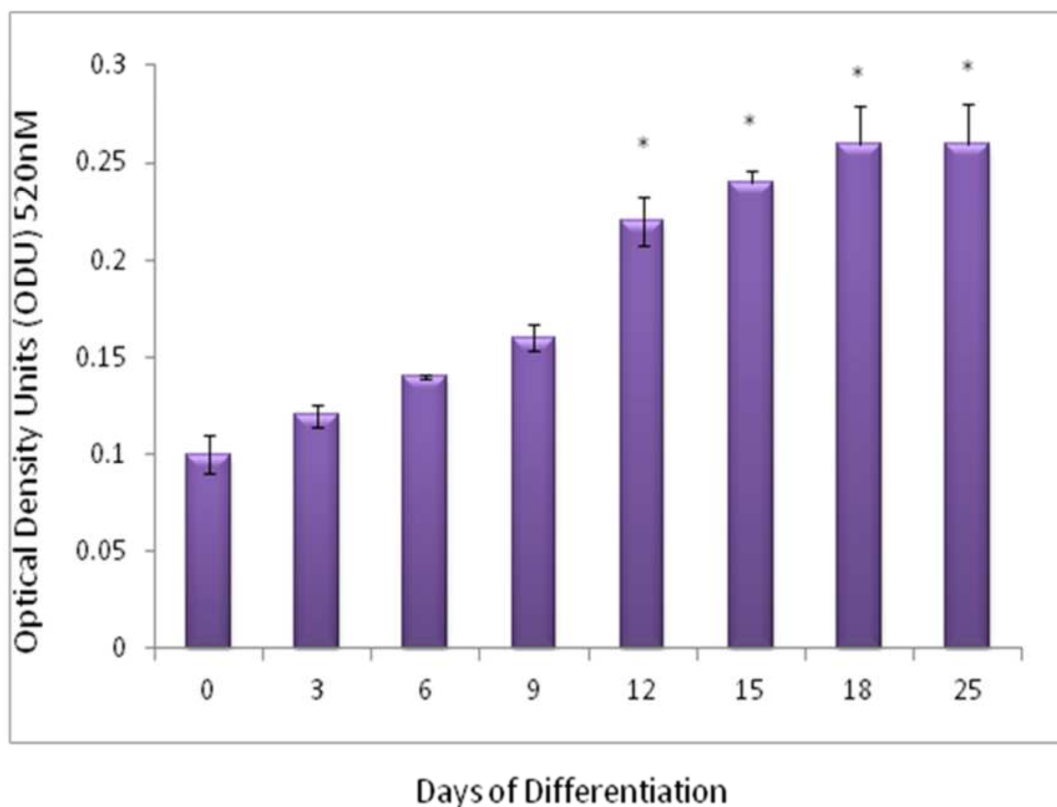


Figure 4.3.5.1: Oil Red O staining from differentiating Chub-S7 cells over time. This figure highlights the change in Oil Red O staining over time, Day 0-25 with significant changes noted comparing day 0 versus days 3-25 (p-value: *p<0.05).

Furthermore, oil red O stained pictures of Chub-S7 at different stages of differentiation clearly highlighted lipid droplets accumulation in the cytoplasm which is compatible with maturation of the primary human abdominal subcutaneous adipocytes and full expression of adipocyte-specific metabolic profile (**Figure 4.3.5.2**). It should be noticed that there was no significant changes in lipid droplet accumulation after day 12. As such this data taken together suggest that Chub-S7 are differentiated to adipocytes (expression of specific adipogenic markers) soon after adding differentiation media (day 3) and become fully maturated (lipid droplet accumulation) by day 12.

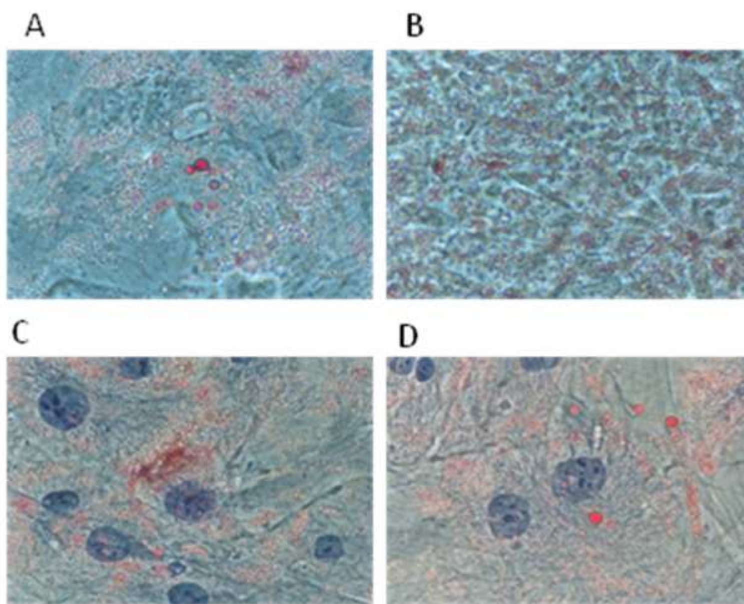


Fig 4.3.5.2 Cytoplasmic lipid accumulation in Chub-S7 during differentiation. This figure shows Oil Red O staining in differentiating Chub-S7 cells over time, Day 0-25. **The photographs show the oil red O staining at A) day 3; B) Day 6; C Day 9; Day 12 (magnification x40).**

4.3.6 BAT1 protein expression in differentiating Chub-S7 cells.

BAT 1 protein expression was measured during differentiation. There was a significant increase in BAT protein expression over time which was comparable to mRNA data up until day 12 (Day 0: 0.808 ± 0.0157 ODU Day 6: 1.0963 ±0.0883 ODU; Day 12: 1.4037±0.0699 ODU; Day 18: 1.655 ±0.0569 ODU; *p<0.05, **p<0.01, Figure 4.3.6.1). BAT1 protein was expressed in the pre-adipocyte and increased significantly with progressing maturation.

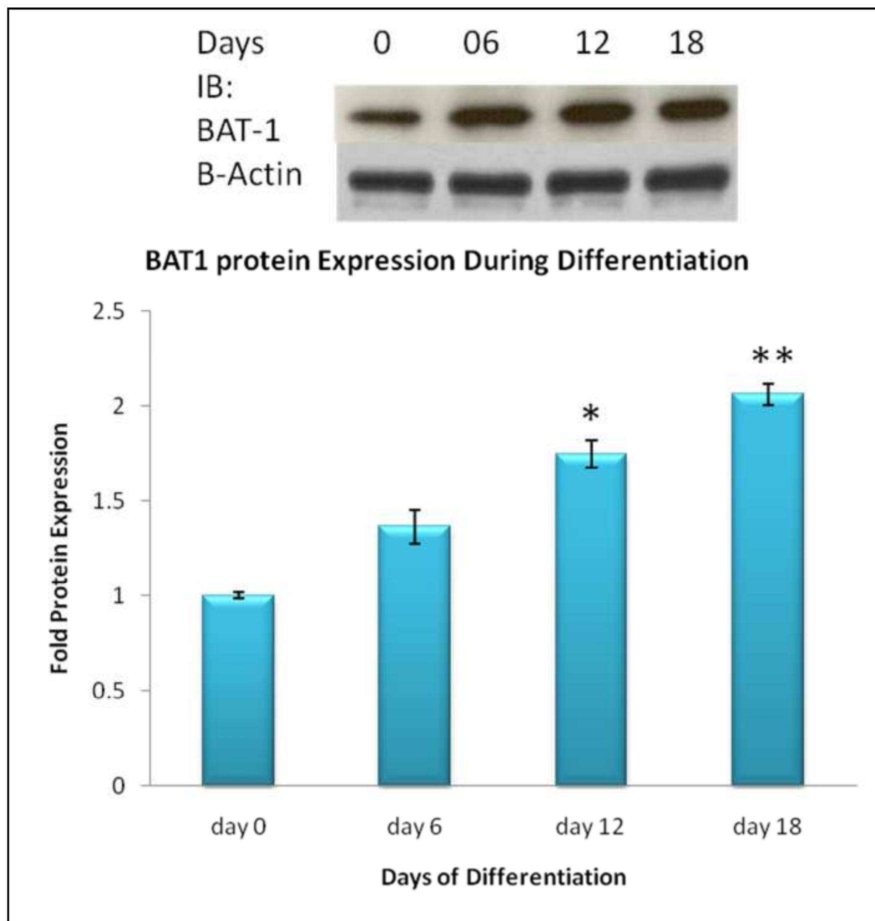


Figure 4.3.6.1 BAT 1 protein expression in Chub-S7 cells. This figure shows the changes in BAT 1 protein expression, over, day 0-18, with significant changes noted comparing day 0 versus days 3-25

4.4 Discussion

Whilst we understand the role of the human pre-adipocyte differentiation and factors that can affect lipid metabolism, mRNA and protein expression and secretion of adipokines in human subcutaneous pre-adipocytes (Tian *et al*. 2010; Dicker. *et al*, 2007; Payne *et al.* 2007; Marshak *et al.* 2007; Deng *et al.* 2006; Kappes *et al.* 2000), it is less documented in the human preadipocyte cell line Chub-S7. Previous studies have utilised Chub-S7 cells as a useful model to characterise human metabolism but less have focussed on factors which change as a result of differentiation (Leyvraz *et al.* 2009; Bujalska *et al.* 2008; Garruti *et al.* 2007; Gathercole *et al.* 2007; Darimont *et al.* 2005; Qiao *et al.* 2005).

Interestingly there are many parallels to be drawn between the previous papers examining differentiation in human pre-adipocytes to the data presented here on the Chub-S7 cells. Many examining differentiation investigated lipolytic action and the role of lipolysis as a predictor in the state of preadipocyte differentiation. Dicker *et al.* (2007) noted that mRNA expression of lipolysis related metabolism reached maximal effect much earlier during differentiation than measuring glycerol release which mirrors the findings for the glycerol and oil-red O data noted form the Chub-S7 cells. Many mRNA gene patterns examined over differentiation appeared to reach their maximum by day 8 (Dicker *et al.* 2007). The data presented in this chapter examining perilipin and PPARγ mRNA expression, identified a similar pattern with a significant increase in mRNA expression from day 0 up until day 6 and a clear reduction after that day. During the process of differentiation the cells were given rosiglitazone to drive differentiation substantially. The mRNA PPARγ data showed the Chub-S7 cells clearly responded to this stimulus – thus affirming PPARγ‘s role in lipid metabolism, during differentiation of

pre-adipocytes (Deng *et al.* 2006). All mRNA data confirmed that the Chub-S7 behave in a similar fashion to abdominal subcutaneous differentiating pre-adipocytes (Dicker *et al.* 2007).

These current studies also examined mRNA expression of CAAT/enhancer-binding protein alpha (C/EBP alpha) which, along with C/EBP beta, is documented to regulate diacylglycerol acyltransferase 2 (DGAT2) expression during adipogenesis. DGAT2 is an important enzyme to catalyze the final step of triglyceride (TG) synthesis for lipid accumulation. Previous studies have shown that in primary murine pre-adipocytes and 3T3-L1 cells DGAT2 expression closely mimics expression of C/EBP genes: C/EBPα and β expression known to change during differentiation. These current studies showed that Chub-S7 cells increased prior to day 6 and then led to a significant reduction in CEBPα mRNA expression which mirrors previous analysis in other murine cells (Payne *et al.* 2007; Dicker *et al.* 2007). Hexose-6-phosphate dehydrogenase (H6PD) catalyzes the first two principal steps in the reactions of the pentose phosphate pathway in the endoplasmic reticulum (ER), thereby generating reduced NAD (NADPH) within the luminal compartment. It has an important role in adipogenesis which has been documented to be attributed to H6PD (Atanasov *et al*. 2004; Hewitt *et al*. 2004; Odermatt *et al*. 2006). The current data on H6PD in this chapter again affirms previous studies from other human cultured pre-adipocytes undergoing differentiation (Senesi *et al.* 2008).

Secretion of adipokines was also examined as a marker of differentiation. These current finding of leptin release from Chub-S7 cells align with previous studies examining changes in leptin secretion during differentiation (Mutch *et al.* 2009; McTernan *et al.* 2003). Additionally the measurement of adiponectin release was also in

agreement with previous studies noting a significant up regulation of adiponectin mRNA and protein secretion post 3 days (Kappes *et al.* 2000; Tian *et al.* 2010 Mutch *et al.* 2009).

As the current findings of the Chub-S7 cells were similar to those noted in primary human adipocytes, the expression of BAT1 mRNA and protein during differentiation in this cell system was investigated. BAT1 expression increased with progressing maturation up to a stage of lipid accumulation after which BAT1 remains unchanged; further (extreme) increase in adiposity and abnormally high fat storage may result in suppressed BAT1 expression as described in chapter 3. This will be further investigated in the later chapters. BAT1 is a cellular DExD/H-box RNA-helicases which performs an essential role for cellular mRNA export by recruiting the adaptor proteins to spliced and unspliced mRNAs (Thomas *et al*. 2011). BAT1 therefore may have a role in the translational control of some of the genes required for differentiation or adipogenesis and inflammation which needs to be further investigated.

In summary, this chapter has examined differentiation markers including lipolysis, adipogenesis, gene and protein expression data, to highlight that Chub-S7 cells are comparable with primary human pre-adipocytes and that BAT1 is expressed at mRNA and protein levels in adipocytes at concentrations that increase with increasing differentiation. In the following chapters we utilise primary human adipocytes to test how hormonal, nutritional and inflammatory molecules influence BAT1 expression and modulation of gene and protein levels.

Chapter 5

The influence of nutritional and inflammatory factors on BAT1 expression in primary adipocytes

5.1 Introduction

Obesity has been considered a state of chronic low-grade systemic inflammation, as it is suggested by the increased levels of C-reactive protein (CRP) (Visser *et al*; 1999), tumor necrosis factor-α (TNF-α) (Dandona *et al*. 1998), interleukin-1 (IL-1), IL-6 (Van Dielen *et al*. 2001; Yudkin *et al*. 2000; Visser *et al*. 1999), mononuclear cells and lymphocytes (Perfetto *et al*. 2002) in the blood of otherwise healthy obese individuals. The low-grade inflammation has been implicated in the development of insulin resistance (IR), (Laaksonen *et al*. 2004; Pradhan, Ridker 2002) and β-cell failure (Wogensen *et al*. 1990). The adipose tissue participates actively in this pro-inflammatory state through generation of bioactive molecules (Cachofeiro *et al*. 2006) that promote inflammation but also by recruiting inflammatory cells (lymphocytes and macrophages) (Brake *et al*. 2006; Weisberg *et al*. 2006) which in turn secrete inflammatory cytokines (e.g. IL-6 and TNF-α) fueling whole body inflammation (Lumeng *et al*. 2007). Whilst inflammation is known to occur the insults that mediate these chronic low-grade effects are less clear, although nutritional status appears important.

Most population-based studies evaluating the relationship between diet and risk of T2DM demonstrate that high fat intake, especially the saturated fat, has adverse effect on insulin effectiveness, increase risk of diabetes (Hunnicutt *et al*. 1994; Marshall *et al*. 1994) plus enhance systemic inflammation in the whole body (Cani *et al*. 2008 and 2007; Nappo *et al*. 2002) but also in adipocytes (Schäffler *et al*. 2008)**.** Similarly to inflammatory factors (e.g. TNF-α), elevated levels of free fatty acids (FFAs) stimulate the inflammatory serine/threonine kinases JNK, IKK and PKC (Schmitz-Peiffer and Biden 2008; Arkan *et al* 2005; Hirosumi *et al*. 2002) causing serine phosphorylation of

IRS-1 thus blockage of insulin action. These kinases also stimulate the AP-1 complexes and the NF-κB (Hotamisligil 2006) thus enhance the production of inflammatory mediators including TNF-α and IL-6 (Shoelson *et al*. 2003; Gao *et al*. 2002) (Figure 5.1). Other epidemiologic studies have also revealed that diets with high glycemic index increase the risk of T2DM (Ludwig 2007; Lindstrom *et al*. 2006) while postprandial hyperglycemia *per se* has been directly linked to inflammation, IR and β cell dysfunction (Dandona *et al*. 2005; Ludwig 2002). The enhanced fuel oxidation and excessive mitochondrial ROS production provide the pathogenic mechanisms by which increased carbohydrate intake exerts pro-inflammatory effect (Ceriello, Motz 2004).

Furthermore, potential insults may also arise directly from the gut besides glucose and lipids. In recent years the connection between gut microbiota and obesity-related disorders have been increasingly recognized and explored (Cani, *et al*, 2007, 2008; Brun *et al*, 2007; Al-Attas *et al*. 2009; Baker *et al*. 2009; Miller *et al*. 2009; Shoelson and Goldfine 2009; Creely *et a*l. 2007). The hypothesis of 'metabolic endotoxinaemia' suggests that toxins produced in the gut may disrupt the energy balance equation (Turnbaugh *et al*. 2009; Martin *et al*. 2008; Turnbaugh *et al*. 2008; Bäckhed *et al*. 2007; Dumas *et al*. 2006), alter fatty acid metabolism and composition in adipose tissue and liver (Cani *et al*. 2007, 2008), modulate gut-derived peptides (PYY and GLP-1) (Samuel *et al*. 2008; Cani *et al*. 2009; Zhou *et al*. 2008; Cani *et al*. 2006) and activate the TLR-4 axis (Ghanim *et al*. 2009; Cani *et al*. 2008; Anderson *et al*. 2007; Cani *et al*. 2007), leading to obesity, IR and diabetes in the host. Lipopolysaccharide (LPS) is such a gut bacteria-derived toxin implicated in inflammation and IR seen in obesity; it suggests the outer cell wall membrane of gram-negative bacteria transverses the gut

mucosa attached to damaging lipoproteins to lead to systemic inflammation (Creely *et al*, 2007; Baker *et al*, 2009; Al-Attas *et al*, 2009; Brun *et al*, 2007; Miller *et al*, 2009; Harte *et al*, 2010; Dixon *et al*, 2008). More specifically previous work has shown that LPS has an immediate impact on the innate immune pathway in human AT, acting via TLRs which recognize antigens, such as LPS, to initiate an acute phase response to infection (Kaisho & Akira 2002). Stimulation of the TLRs leads to intracellular activation of NFκB, a key transcription factor in the inflammatory cascade that regulates the transcription of numerous pro-inflammatory adipokines (including IL-6, IL-11, PAI-1, ANG II, resistin and TNF-α). As such, LPS may act as a mediator of inflammation through activation of NFκB leading to a rapid response within AT, primarily increasing inflammation and subsequent metabolic risk (Muzio, *et al*, 2000; Lin *et al*, 2000; Kopp *et al*, 2009; Song *et al*, 2006; Shoelso & Goldfine 2009; Alhusani *et al*, 2010; Gregor & Hotamisligil 2007; Wellen & Hostamisligil, 2005; Hotamisligil 2005; Xu *et al*, 2009; Doroudgar *et al*, 2009; Nishimura *et al*, 2009).

The aim of these studies were therefore to look both at the direct effects of nutritional factors (saturated fats and glucose) and inflammatory insults (LPS) on changes in BAT1 expression in human differentiated Abd Sc adipocytes, as well as to examine the role of particular inflammatory cellular pathways such as TLR-4/NFκB and JNK in BAT1 modulation.

5.2 Research Design and Methods

5.2.1 Subjects

Human AbSc AT was collected from overweight non smoking female patients (age: 54.0 (mean±SD)±2.65yr; BMI: 28.43(mean±SD)±1.0 kg/m^2, undergoing elective or liposuction surgery with informed consent obtained in accordance with LREC guidelines and with ethics committee approval. The selected samples were from subjects of the same gender (females), postmenopausal so that any effect of sex hormones to be elucidated. In addition, the subjects were not smoking, had no other medical issues and were not on any medication. In total, 9 human non-diabetic primary AT samples were analyzed.

5.2.2 Cell Culture

In brief, human abdominal subcutaneous (Abd Sc) pre-adipocytes were differentiated as previously described in chapter 4. On day 12, the fully differentiated adipocytes were grown in normal DMEM/Ham's F-12 phenol-free medium containing only 2% serum (detoxification media) for 24 hr to remove effects of growth factors and other components in nutrition media. The selected treatments (NEFA, glucose, LPS) were then placed in the fresh detoxification media for several different time points. LPS and glucose were purchased from Sigma-Aldrich Corp. (Poole, UK), NEFA (Stearic-Palmitic acid Mixture) from Fluka Chemicals Ltd. (Gillingham, UK).

5.2.3 Protein determination & Western blot analysis

Protein concentrations were determined using the Bio-Rad Detergent Compatible (DC) protein assay kit (Biorad UK). Homogenized human AT were extracted using a 10% RIPA buffer method (McTernan PG *et al*. 2002). Western blot analysis was performed

using a method previously described (McTernan CL *et al*. 2002) and relative expression was standardized by using a densitometry quantification software (GeneTools, Geneflow, Fradley, UK). In brief, 5-20μg of protein was loaded onto an 8% polyacrylamide gel (Geneflow, Fradley, UK), a mouse BAT1 monoclonal antibody (primary antibody; product code: ab50986, UAP56 antibody [2060C10a], 1:1000; Abcam Ltd., UK) was used to assess BAT1 expression (Biosource UK, Nivelles, Belgium). For inhibition studies, Abd Sc adipocytes were incubated with NFκB inhibitor (NF-κB: SN50, 50 μg/mL; Calbiochem, UK) or c-Jun N-terminal Kinase (JNK) inhibitor (SP600125, 10 μM; A.G. Scientific, Inc., San Diego, CA). Equal protein loading was confirmed by densitometry using the β−actin antibody (2.04μg/mL, Abcam, Cambridge, UK). A chemiluminescent detection system ECL/ECL+ (GE Healthcare, Little Chalfont, UK) enabled visualization after exposure to X-ray film.

5.2.4 Statistical methods

For analysis of protein expression and gene expression data, statistical analysis was undertaken using unpaired t tests unless otherwise stated, where data were analyzed using nonparametric tests as previously stated in Chapter 3 with full details given in Chapter 2 and appendices. Treatment effect was defined relatively to control value as $\frac{(TRT - Control)}{Control}\%$. In every case, at least three independent experiments performed in triplicates to ensure reproducibility. The threshold for significance was $P < 0.05$. The data in the text and figures are presented as the mean ± SEM unless otherwise stated.

5.3 Results

5.3.1 Effect of NEFAs on BAT1 expression in human primary AbdSc adipocytes; the role of NFκB and JNK pathways

Previous studies have shown the effectiveness of the selected NEFA concentrations and the particular time points in human differentiated adipocytes (Gao *et al*. 2010; Richard *et al*. 2008; Wen *et al*. 2006; Bueno *et al*. 2008). Dose and time course studies were performed to assess BAT1 protein at 24, 48 and 72 h with control and NEFA-treated adipocytes (0.5 and 2mM). BAT1 was significantly suppressed by the effect of NEFA on all selected different times and treatment concentrations in comparison with control, baseline time zero [NEFA 72hr, 0.5 mM -11.4% (mean±SEM) ± 11.7%***; 24hr 2 mM -43.3% ± 11.0%***; 48hr 2 mM -8.9% ± 9.8%**; 72hr 2 mM -87.8% ± 3.2%***; Total 0.5 mM -11.4% ± 11.7%***; Total 2 mM -52.7% ± 7.1%***, p-value: **$p<0.01$, ***$p<0.001$, **Figure 5.3.1]**

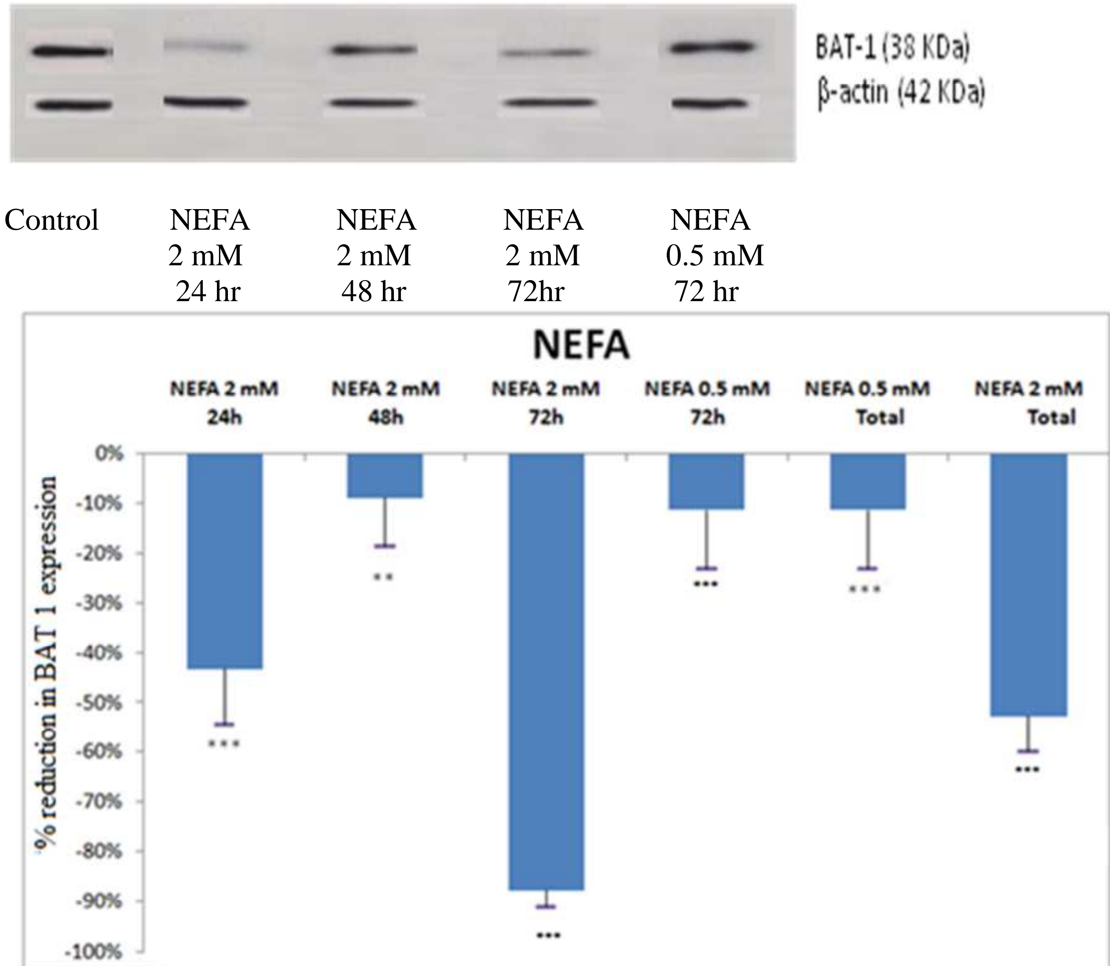
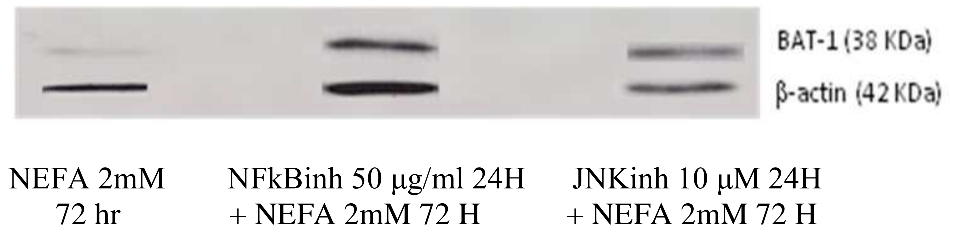


Figure 5.3.1 BAT1 protein expression in NEFA-treated AbdSc adipocytes (n=3-7), on different times and treatment concentrations. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared with control [(TRT-Control)%/Control], (p-values: **p<0.01, ***p<0.001).

In order to investigate whether NEFA modulate BAT1 expression via TLR-4/NFκB and JNK inflammatory pathway, these pathways were blocked by treating the adipocytes with NFκB inhibitor (50 µg/ml) (SN50, CalBiochem, Nottingham, UK) or JNK inhibitor (10 µM/ml) (SP600125, A.G. Scientific, Inc., San Diego, CA), respectively for 24 hours; the adipocytes were then treated with NEFA 2mM for 72 hours. At the end

of the study, BAT1 protein was examined and compared with control (baseline time zero) and NEFA-only treated adipocytes (2mM for 72 hr, without previous treatment with NFκB inhibitor or JNK inhibitor); dose and time course studies were performed to assess BAT1 protein at 14, 24, and 48 hr. The used treatment doses and conditions were based on previous studies in adipocytes (Takahashi *et al*. 2008; Kusminski *et al*. 2007; Baan *et al*. 2006). BAT1 expression in NEFA-treated adipocytes in which JNK pathway was previously blocked didn't differ compared with control, baseline time zero ($p>0.05$). NFkB inhibitor, failed to prevent the inhibitory effect of NEFA on BAT1 [NEFA 72h 2 mM -87.8% (mean±SEM) ± 3.2%***; NFkB inh 24h 50μg/ml + NEFA 72h 2 mM -45.2% ± 5.2%***; JNK 24h 50 μg/ml +NEFA 72h 2mM -5.3% ± 0%, p-value: ***$p<0.001$, **Figure 5.3.1.1**]. The ANOVA F-test (for means comparison) and Kruskal-Wallis rank test confirmed the previous findings (**Figure 5.3.1.2 and 5.3.1.3**).



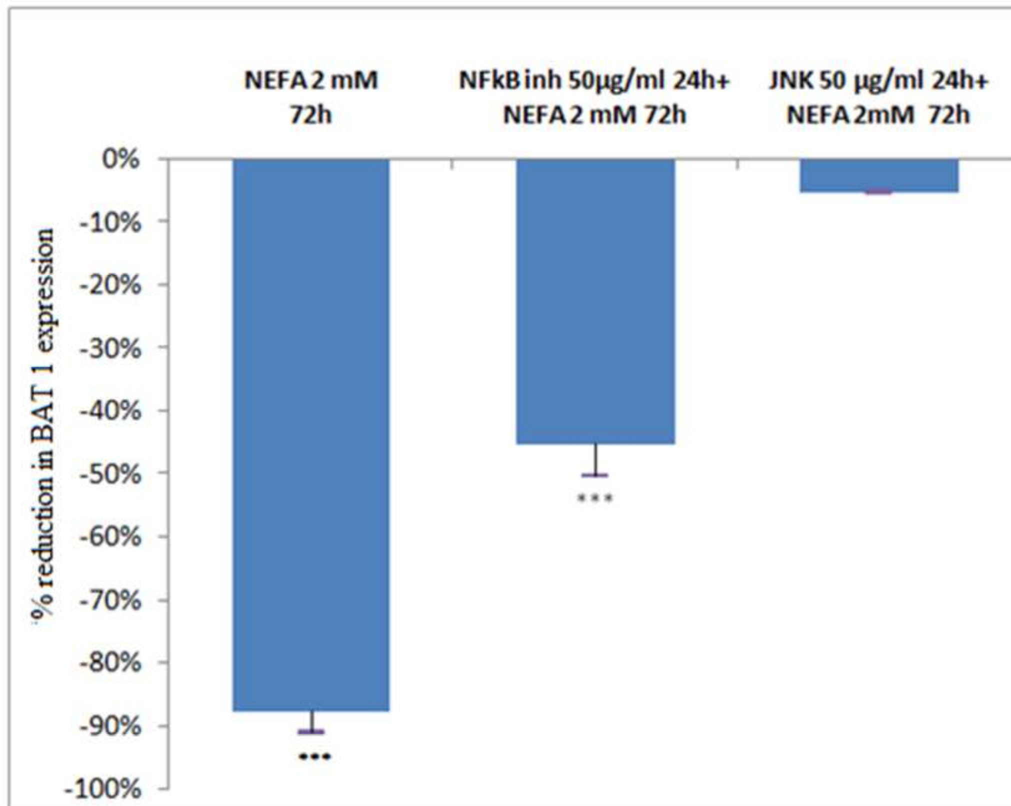
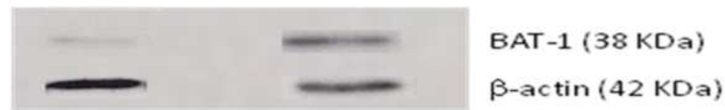


Figure 5.3.1.1 BAT1 protein expression in AbdSc adipocytes (n=3-7) treated only with NEFA 2mM for 72 hr or with NFkB inhibitor 50µg/ml for 24 hr and then with NEFA 2mM for 72 hr or with JNK inhibitor 10 µM/ml for 24 hr and then with NEFA 2mM for 72 hr. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared with control [(TRT-Control)%/Control], (p-values: ***p<0.001).



NEFA 2mM 72H   JNKinh 10 μM 24H + NEFA 2mM 72 H

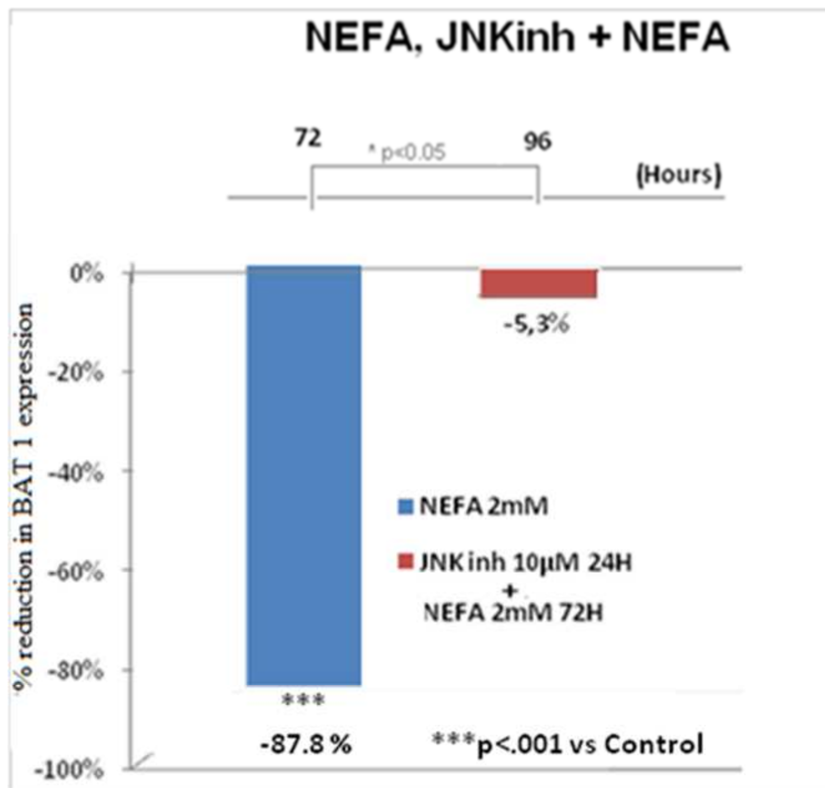


Figure 5.3.1.2 BAT1 protein expression in AbdSc adipocytes (n=3-7) treated with NEFA 2mM for 72 hours or with JNK inhibitor 10 μM/ml for 24 hrs to block JNK pathway and then with NEFA 2mM for 72 hr. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared with control [(TRT-Control)%/Control], (p-values: *p<0.05, ***p<.001).

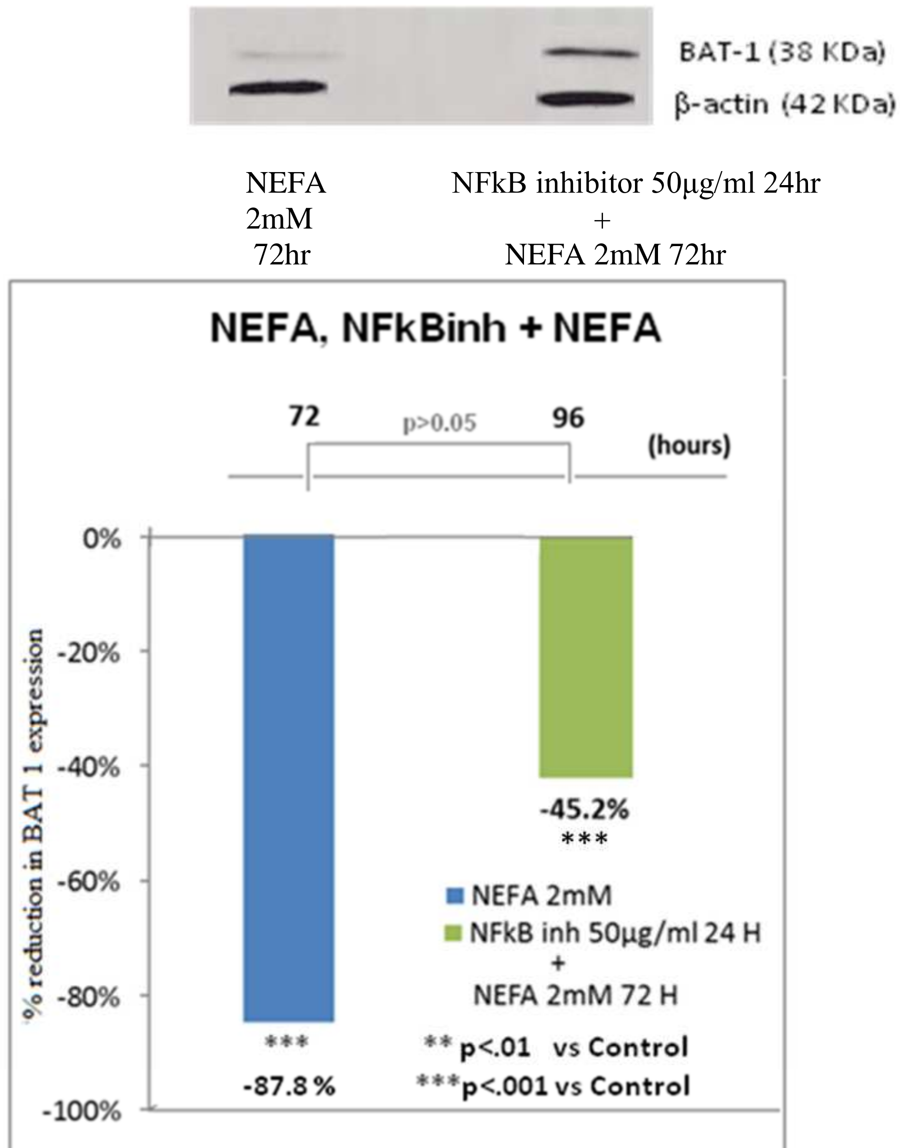


Figure 5.3.1.3 BAT1 protein expression in AbdSc adipocytes (n=3-9) treated with NEFA 2mM for 72 hr or with NFkB inhibitor 50μg/ml for 24 hr to block NFkB pathway and then with NEFA 2mM for 72 hr. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared with control [(TRT-Control)%/Control], (p-values: **p<0.01, ***p<.001)

The following figure (**Figure 5.3.1.4**), summarizes the mean effect of NEFA, regardless of treatment duration. As it is shown NEFA at both high (2mM) and low (0,5mM) concentrations have significant repressive effect on BAT1 protein expression in human adipocytes, while inhibition of JNK pathway ameliorates this effect.

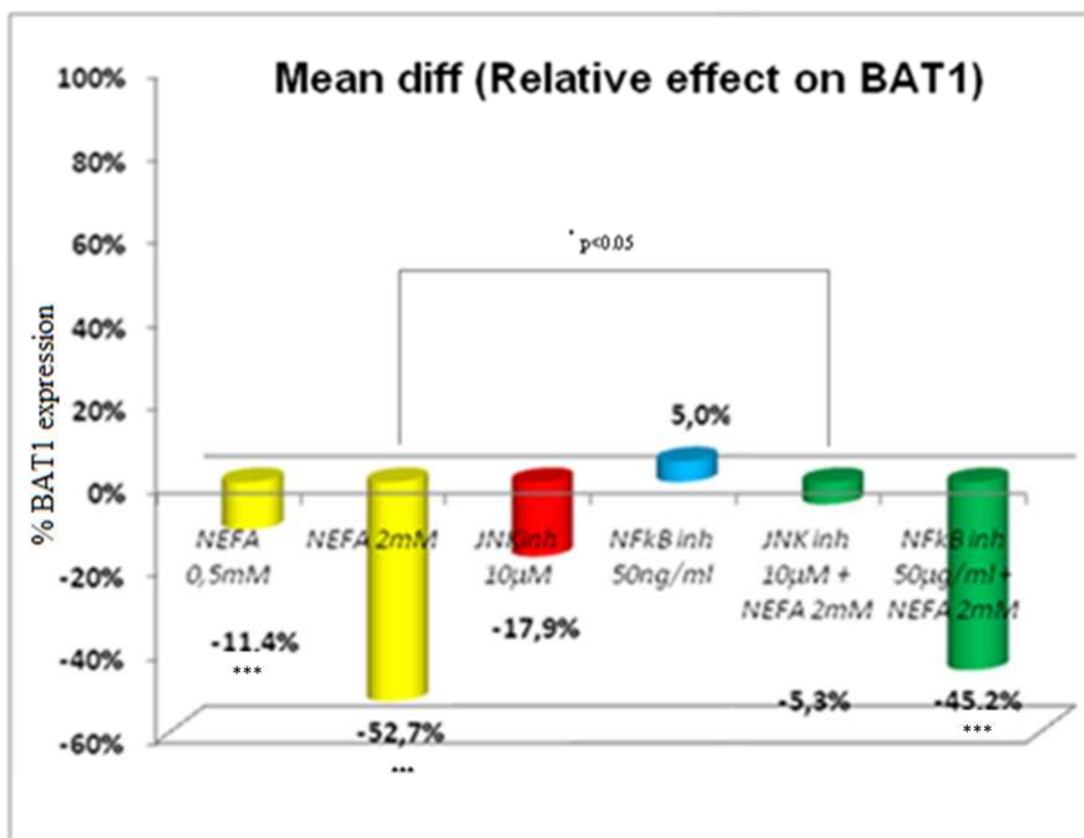


Figure 5.3.1.4 Summary effects of NEFA on BAT1 protein expression in naive or previously treated with JNK or NFkB inhibitor human AbSc AT-derived adipocytes. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared with control [(TRT-Control)%/Control], (p-values: *p<0.05, **p<0.01, ***p<0.001).

5.3.2 Effect of high glucose concentration on BAT1 expression in primary adipocytes

Fully differentiated AbdSc adipocytes (n=3-5) were incubated in serum-free medium and treated with high concentrations of glucose (30mM and 60mM, respectively). The BAT1 protein was estimated at two different time points (24 and 72 hr) in naive (control) and treated cells. The selected doses and treatment duration were based on previous studies with glucose treatments in adipocytes (Gao *et al,* 2010; Zhang *et al,* 2009; Zu *et al,* 2008; Nelson *et al,* 2000). The analysis using t-test showed that BAT1 was significantly repressed by glucose in a dose and time dependent manner in comparison with control, baseline time zero [24h Glucose 30nM -0.1% (mean±SEM) ± 15.7%; 72h 30mM -16.1% ± 17.2%*; 72h 60mM -15.6% ± 4.1%***; total Glucose 30mM -8.1% ± 20.1%**, p-value: *p<0.05, **p<0.01, ***p<0.001, **Figure 5.3.2**].

Control   Glucose 30mM 24 hr   Glucose 60mM 72 hr   Glucose 30mM 72 hr

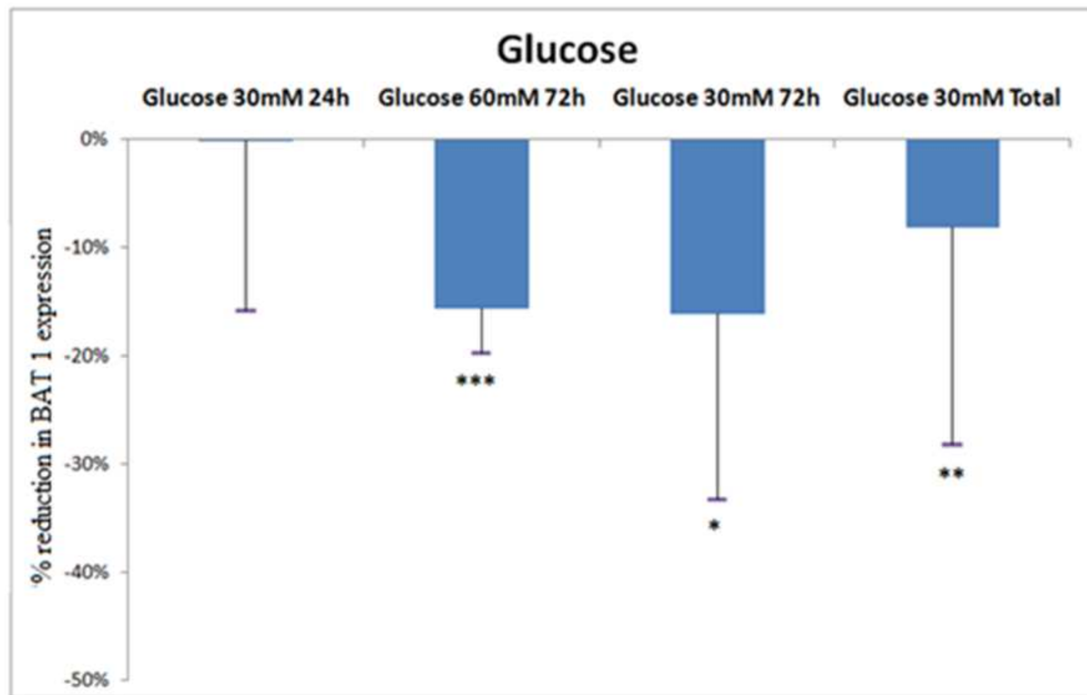


Figure 5.3.2 BAT1 protein expression in AbdSc adipocytes (n=3-5) treated with high concentrations of glucose (30mM and 60mM, respectively) for 24, 72 hr, respectively. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared with control [(TRT-Control)%/Control], (p-values: *$p<0.05$, **$p<0.01$, ***$p<0.001$).

5.3.3 Effect of LPS on BAT1 protein expression in primary adipocytes

For this study, the primary human adipocytes were treated with LPS (5ng/ml and 25ng/ml). Dose and time course studies were performed to assess BAT1 protein at 1, 6, 12, 24 and 72 hr in controls and LPS-treated adipocytes. The used treatment concentrations were based on previous studies with LPS on adipocytes (Lira *et al.* 2011; Penfornis and Marette 2005). Based on the *in vitro* experiments LPS significantly represses BAT1 when compared with control [1 hr LPS 5ng/ml -30.5% (mean±SEM) ± 20.9%**; 6 hr 5ng/ml -43.5% ± 19.3%***; 12 hr 5ng/ml -43.7% ± 17.1%***; 24 hr 5ng/ml -33.2% ± 23.6%**; 1 hr LPS 25ng/ml -34.2% ± 10.2%***; 6 hr 25ng/ml -16.3% ± 10.3%***; 12 hr 25ng/ml -49.5% ± 16.0%***; 24 hr 25ng/ml -45.6% ± 11.0%***; 72 hr LPS 25ng/ml -47.1% ± 16.3%***; Total LPS 5ng/ml -38.3% ± 9.5%***; Total LPS 25ng/ml -38.2% ± 6.1%***, p value: **$p<0.01$, ***$p<0.001$, **Figures: 5.3.3, 5.3.3.1**].

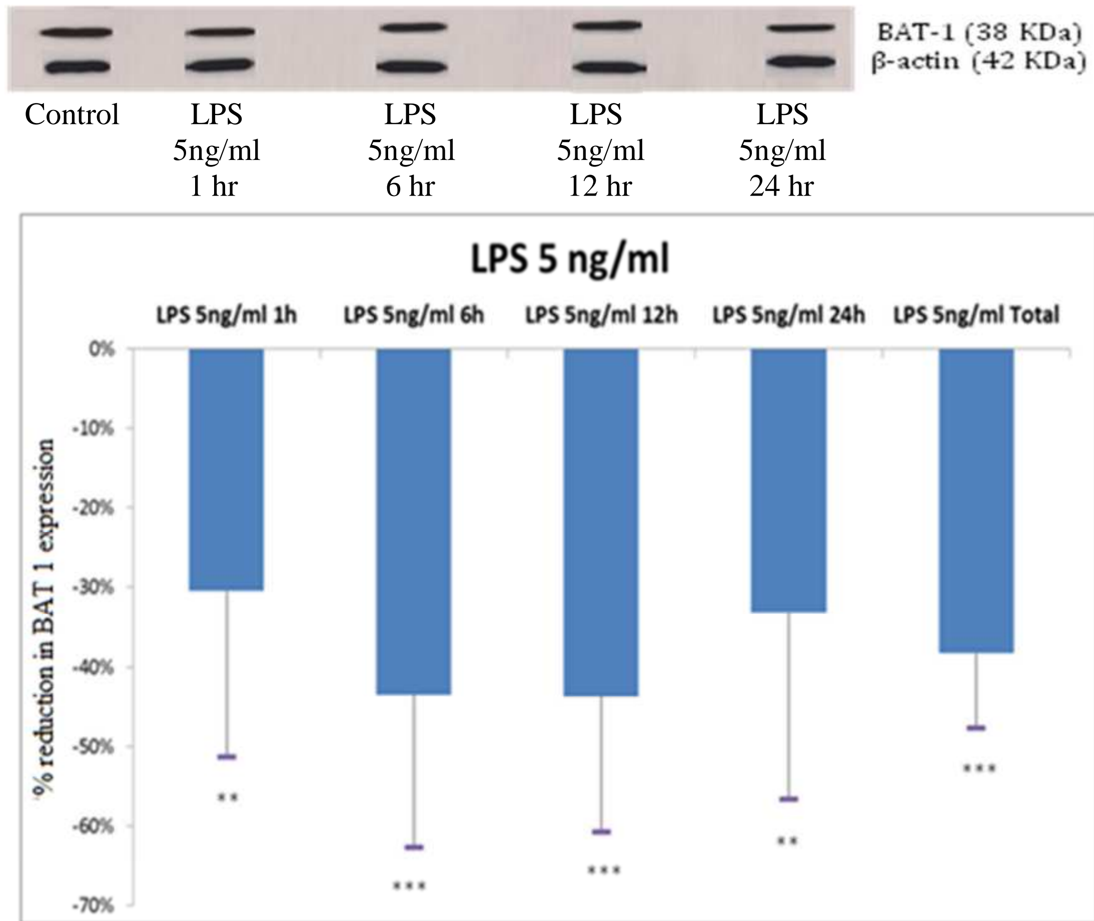


Figure 5.3.3 BAT1 protein expression in AbdSc adipocytes (n=3-5) treated with 5ng/ml LPS over time (1-24 hr). In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared with control [(TRT-Control)%/Control], (p-values: **$p<0.01$, ***$p<0.001$).

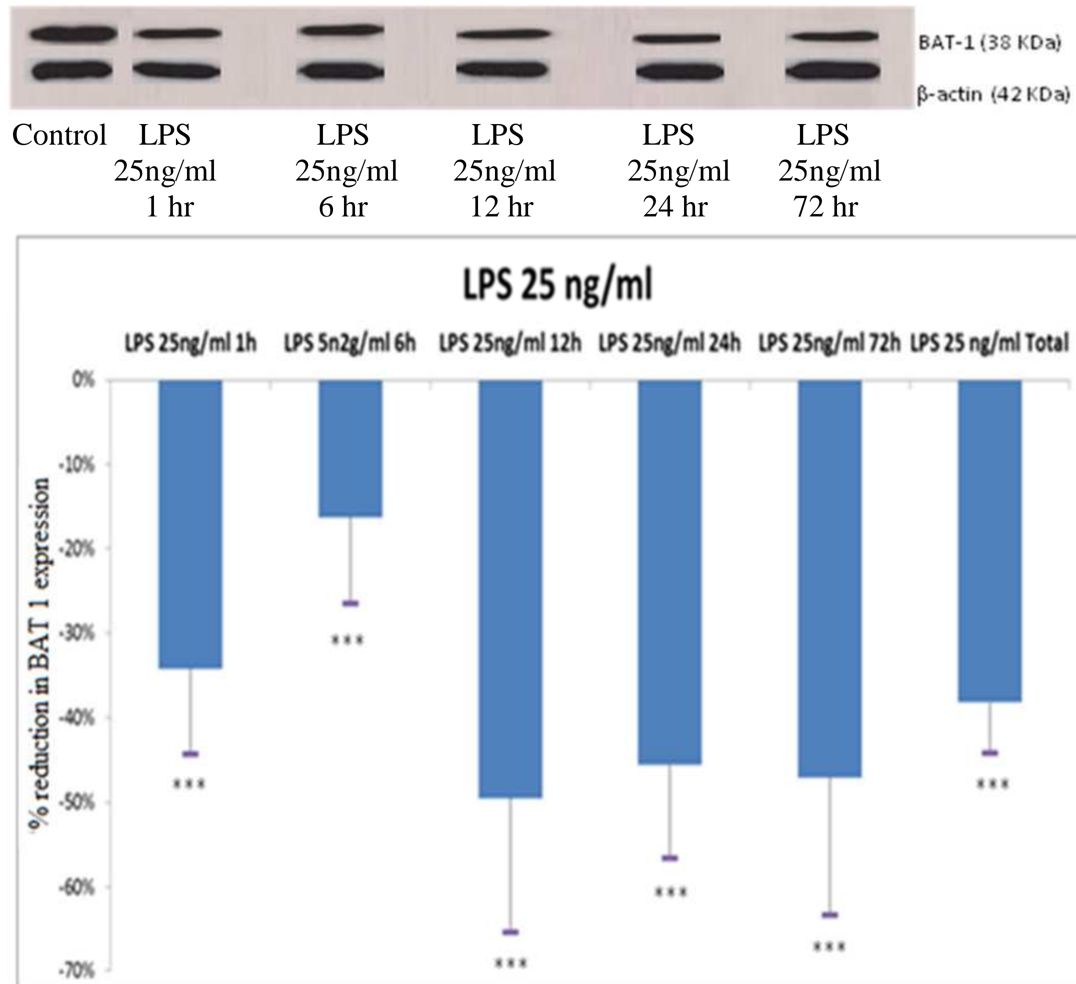


Figure 5.3.3.1 .BAT1 protein expression in AbdSc adipocytes (n=3-5) treated with 25ng/ml LPS over time (1-24 hr) and Total, respectively. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared with control [(TRT-Control)%/Control], (p-values: ***p<0.001).

In summary the selected nutritional factors (glucose and saturated fats) and cytokines involved in the pro-inflammatory state generation in obesity on BAT1 protein expression were compared (analysis with Mann-Whitney test) (**Figure 5.3.3.2**).

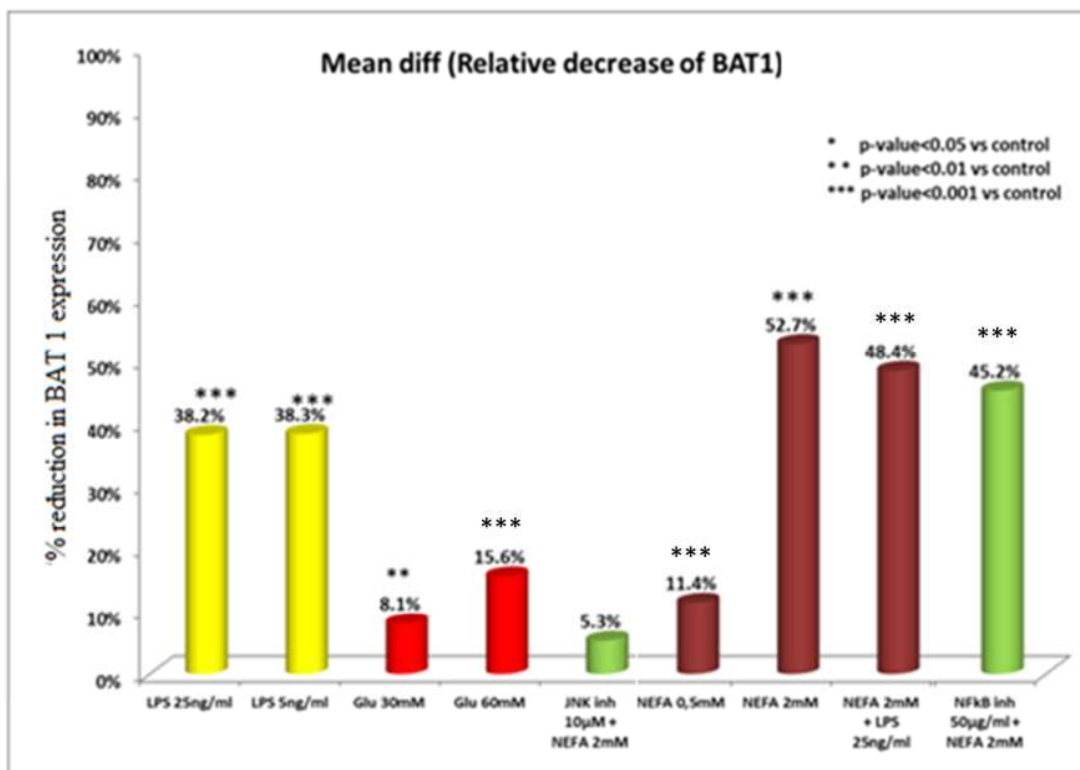


Figure 5.3.3.2 Summary of negative mean effect on BAT1 protein expression in human AbdSc AT adipocytes. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared to baseline time zero controls [(TRT-Control)% / Control], (p-values: *p<0.05, **p<0.01, ***p<0.001)

5.4 Discussion

This study investigated the relationship between nutritional factors and BAT1 specifically those involved in the pre-inflammatory state generation in obesity (glucose and saturated fats). In addition, the effect of pro-inflammatory agents (LPS) on BAT1 expression in human differentiated AbdSc adipocytes was examined, along with potential molecular pathways that mediate BAT1 modulation. AbdSc adipocytes of overweight, non-diabetic, female subjects were used for these studies. The cells were cultured and differentiated as described in chapter 2 and treated with the addition of the agent of interest (glucose, saturated fats or LPS) in the fresh detoxification media for pre-determined time points. At the end of the treatment duration, the cells were collected, and the BAT1 protein expression was evaluated from the isolated cellular protein content.

This study showed that all the selected factors altered BAT1 protein expression (Figure 5.3.4.2). Interestingly despite the insult utilized all achieved negative effects on BAT 1 expression in each case. Thus, it was shown that NEFAs significantly repressed BAT1 expression in human adipocytes. This is in accordance with previous findings linking saturated fats with inflammation within the adipocytes (Schäffler *et al*. 2008). Although findings from *in vitro s*tudies cannot directly translated into the clinical setting, these findings might suggest a central role of NEFA excess in obesity-associated inflammatory process via BAT1 suppression. Furthermore, according to this study, JNK pathway may mediate NEFA-induced BAT1 suppression, highlighting that a main inflammatory pathway has an important influence of BAT1 expression. Furthermore recent studies in human adipocytes has shown that JNK activity appears to influence

NFκB expression in certain circumstances which highlights the interconnectivity between the pathways (McGee, *et al*, 2011).

Previous studies have clearly show a strong link between hyperglycemia and inflammation (Dandona *et al*. 2005; Ludwig 2002), while the primary study in chapter 3 demonstrated BAT1 suppression in the adipose tissue of patients with T2DM compared with non-T2DM subjects. In this direction, these current studies showed that glucose in excess directly represses BAT1 in human adipocytes in a dose- and treatment duration-dependent manner providing another nutritional factor that could exacerbate inflammation.

The study of the effect of LPS on BAT1 expression revealed potent repressive effects of this agent on BAT1 after acute (1 hr) or chronic prolonged (72 hr) exposure of human adipocytes to low (5ng/ml) or high (25ng/ml) dose of LPS. These findings are in accordance with the *metabolic endotoxinaemia* theory according to which toxins produced in the gut may be related to the obesity-associated inflammation and metabolic disorders (Turnbaugh *et al*. 2009; Cani *et al*. 2008; Martin *et al*. 2008; Turnbaugh *et al*. 2008; Bäckhed *et al*. 2007; Cani *et al*. 2007;Dumas *et al*. 2006).

In summary, this study highlighted that nutritional (NEFA and glucose) and inflammatory insults in excess modulate BAT1 expression in adipocytes. All of the examined agents repressed BAT1. This may suggest a protective mechanism by which adipocytes recognize the forthcoming inflammatory storm and get prepared to counteract it. This hypothesis becomes even more interesting considering the downregulating effect of BAT1 on TNF-α in T-cells and monocytes (Allcock *et al*; 2001). The fact that so many different molecules, nutritional or inflammatory, effectively repress BAT1 could

suggest that this protein is sensitive to biochemical/biomolecular changes within the cell and highly regulated, thus predisposes, at least in the case of human adipocytes, to the generation of inflammation, when nutritional factors or inflammatory agents in excess alter the micro-biochemical balance within the adipocyte. As such further studies to evaluate whether local paracrine adipokine factors may also regulate its expression. This would be important to determine to identify the influential nature of primary insults and down-stream pro-inflammatory or anti-inflammatory adipokines.

Chapter 6

The influence of human recombinant pro- and anti-inflammatory adipokines on BAT1 expression in human differentiated adipocytes

6.1 Introduction

Adipose tissue is a complex and highly active organ controlling energy balance, metabolism and the immune system via the expression and secretion of a variety of bioactive peptides, known as adipokines that act locally but also systemically as endocrine hormones (Fruhbeck *et al.* 2001; Ahima, Flier 2000; Figure 1.8). Amongst the myriad of adipokines, some affect insulin signaling and inflammatory pathways and as such may contribute to the pathogenesis of obesity linked T2DM; adipokines of note such as leptin, resistin and adiponectin appear to represent important mediators of metabolism. The following will briefly highlight these adipokines and the rationale for their use in examining their influence on BAT 1 expression which would be considered to form part of an anti-inflammatory intracellular response.

Leptin is secreted by the adipocytes in direct proportion to adipose tissue mass. Insulin, glucocorticoids, IL-6 and TNF-α enhance leptin secretion (Kirchgessner *et al.* 1997; Sarraf *et al.* 1997), while free fatty acids and PPAR-γ agonists have the opposite effect (Margetic *et al.* 2002). Subcutaneous AT accounts for the majority of leptin secretion compared to omental depot (Fain *et al.* 2004). The effects of leptin on energy homeostasis and its diverse endocrine properties in the regulation of neuroendocrine and endocrine systems functions are diverse. Leptin serves as a metabolic signal of energy sufficiency (Friedman, Halaas 1998) and modulates the function of the hypothalamic-pituitary-adrenal/thyroid and -gonadal axes (Margetic *et al.* 2002; Flier *et al.* 2000; Hileman *et al.* 2000). In addition, it increases glucose transport in muscle (Minokoshi, Kahn 2003) via AMPK activation, plus attains immunomodulatory properties (alters the cytokine production by the immune cells) (Lord *et al.* 1998). Within the human adipose

tissue in particular, leptin in excess (as in obesity) exerts pro-inflammatory effects as by inducing the release of TNF-α (Lappas *et al*. 2005) and the endothelial-derived MCP-1 production; the latter facilitates the recruitment of macrophages into the fat tissue (Yamagishi *et al*, 2001).

Within this pro-inflammatory milieu resistin, has also been observed to act in an inflammatory capacity (Mohammed *et al*, 2009) in addition to leading to dysregulation of insulin signaling (Satohet *et al*, 2004; Rajala *et al*.,2004; Rangwala *et al*, 2004; Rajala *et al*, 2003). These previous findings imply a pathogenic role of resistin in the obesity-associated insulin resistance in animal models (Banerjee & Lazar 2003; Steppan *et al*, 2001). However the role of resistin in human has been more controversial. Previous work with the research team has previously documented its expression within both human pre-adipocytes and adipocytes and its expression in higher levels in abdominal fat (Sc and Om fat depots) compared to thigh and breast adipose tissue depots (McTernan *et al*, 2002; Kusminski *et al*, 2007). Other studies demonstrated its synthesis and secretion by the adipose tissue macrophages (ATMs) (Curat *et al*, 2006); the extent of contribution of macrophages to the overall concentration of resistin in adipose tissue though, remains uncertain. Studies in humans have identified a link between resistin concentration and obesity/IR/T2DM (McTernan *et al*, 2002; McTernan *et al*, 2002; Vidal-Puig & O'Rahilly 2001), while others failed to do so (Kielstein *et al*, 2003; Patel *et al*, 2003; Janke *et al*, 2002). Recent data also suggests whoever that there appears to be a positive association between resistin and inflammation in morbidly obese individuals (De Luis *et al*, 2010; Iqbal *et al*, 2005; Kunnari *et al*. 2006) as well as in patients with inflammatory diseases (Qatanani *et al*, 2009; Senolt *et al*, 2007; Lehrke *et al*, 2004); plasma resistin levels in

particular were positively related to inflammatory molecules including TNF-α, IL-6 and CRP. Taken together, resistin seems to attain pro-inflammatory properties. As such resistin remains an interesting pro-inflammatory adipokine to study in human adipose tissue.

Unlike other adipokines, adiponectin an anti-inflammatory adipokine decreases with increasing adiposity (Trujillo *et al*, 2005). Adiponectin is higher in abdominal subcutaneous AT than omental adipose tissue (Fain *et al*, 2004) and a strong and consistent inverse relation with both inflammation and IR has been documented for this adipokine (Chandran *et al*, 2003; Diez & Iglesias 2003); the latter probably via AMPK activation (Yamauchi *et al*, 2003; Diez & Iglesias 2003; Yamauchi *et al*, 2002). Interestingly, its plasma levels decline before the onset of obesity and IR suggesting a potential role of hypoadiponectinemia in the pathogenesis of these disorders (Hotta *et al*, 2000). It has been shown that certain adipokines that increase within the AT as during weight gain also can lead to suppression of adiponectin expression in adipocytes; TNF-α and IL-6 being two such adipokines (Bruun *et al*, 2003). Additionally adiponectin may also decrease inflammation by reducing the inflammatory mediators TNF-α, IL-6, CRP (Zhou *et al*, 2008; Park *et al*. 2006; Thakur *et al*, 2006; Xu *et al*, 2003), suppressing the TLR-4 signaling pathway (Yamaguchi *et al*, 2005) as well as by increasing several anti-inflammatory cytokines for *e.g*. IL-10 (Choi *et al*, 2007; Engeli *et al*, 2003) and interleukin-1-receptor antagonist (Kumada *et al*, 2005; Wolf *et al*, 2004). The attenuation of the pro-inflammatory adipokines by adiponectin is thought in part to occur through NFκB activation (Wulster-Radcliffe *et al*, 2004; Ouchi *et al*, 2000). Furthermore,

adiponectin exerts anti-oxidant effects (it upregulates the uncoupling protein 2 expression) further reducing inflammation (Negre-Salvayre *et al*, 1997).

The aim of the chapter was therefore to look at the direct effects of individual human recombinant adipokines to ascertain the individual effect of such adipokines on BAT 1 expression in human differentiated Abd Sc adipocytes.

6.2 Research Design and Methods

6.2.1 Subjects

Human AbSc AT was collected from overweight non smoking female patients (age: 54.0 (mean±SD)±2.65yr; BMI: 28.43(mean±SD)±1.0 kg/m^2, undergoing elective or liposuction surgery with informed consent obtained in accordance with LREC guidelines and with ethics committee approval. The selected samples were from subjects of the same gender (females), postmenopausal so that any effect of sex hormones to be elucidated. In addition, the subjects were not smoking, had no other medical issues and were not on any medication. In total, 9 human non-diabetic primary AT samples were analyzed.

6.2.2 Cell Culture

In brief, human abdominal subcutaneous (Abd Sc) pre-adipocytes were differentiated as previously described in chapter 4. On day 12, the fully differentiated adipocytes were grown in normal DMEM/Ham's F-12 phenol-free medium containing only 2% serum (detoxification media) for 24 hr to remove effects of growth factors and other components in nutrition media. The selected treatments (leptin, resistin, and

adiponectin) were then placed in the fresh detoxification media for several different time points (0 24, 72 hr). rh Leptin and adiponectin were purchased from Sigma-Aldrich Corp. (Poole, UK), while rh resistin was purchased from Phoenix Pharmaceuticals, (Belmont, CA, USA).

6.2.3 Protein determination & Western blot analysis

The process that was described in previous chapters was also followed for these studies.

6.2.4 Statistical methods

For analysis of protein expression and gene expression data, statistical analysis was undertaken using unpaired t tests unless otherwise stated, where data were analyzed using nonparametric tests as previously stated in Chapter 3 with full details given in Chapter 2 and appendices. The threshold for significance was $P < 0.05$. The data in the text and figures are presented as the mean ± SEM unless otherwise stated.

6.3 Results

6.3.1 Effect of leptin on BAT1 protein expression in primary human Abd Sc adipocytes

Two different concentrations of rh Leptin were used for these studies; rh Leptin 0.5 μg/ml and 1.5 μg/ml, respectively. The BAT1 protein was estimated at two different time points (24 and 72 hr) in naive and treated cells. Previous studies have shown the effectiveness of the selected leptin concentrations and the particular time points in human

adipocytes (Ambatia *et al*. 2007; Ho *et al*. 2006; Cohen *et al*. 2001). Dose and time course studies were performed to assess BAT1 protein at 24 and 72 hr in controls and leptin-treated adipocytes. The analysis using t-test showed that BAT1 was significantly suppressed by the effect of rh leptin in comparison with control, baseline time zero (rh Leptin 24 hr, 0.5 μg/ml -29.8% (mean±SEM)±18.3%***; 72h 0.5 μg/ml -14.3%±22.8%***; 72h Leptin 1.5 μg/ml -5.8%±11.2%***; Total 0.5 μg/ml -21.2%±14.2%***, p-value: ***p<0.001, **figure 6.3.1**).

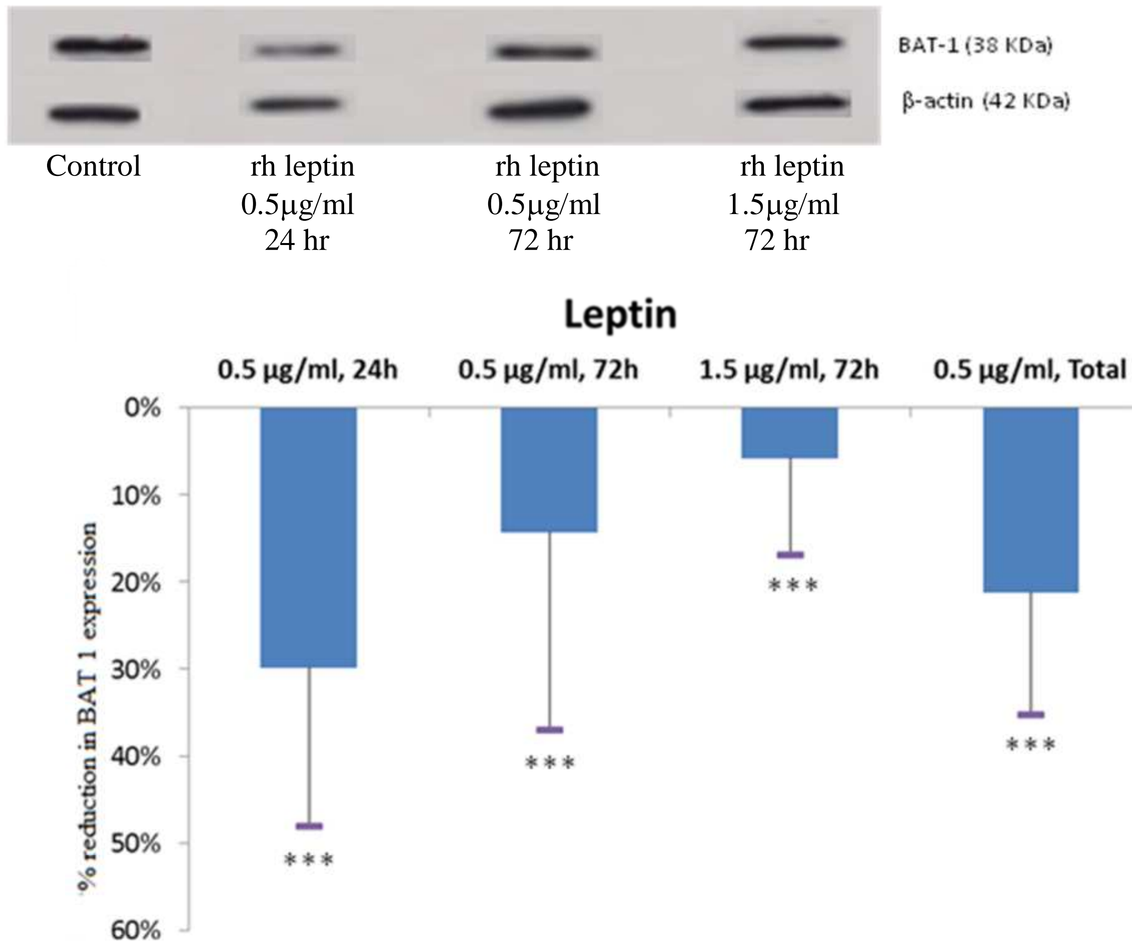


Figure 6.3.1 BAT1 protein expression in leptin-treated Abd Sc adipocytes (n=4-6), on different times (24 hr, 72 hr) and treatment concentrations (0.5µg/ml and 1.5 µg/ml). The overall (total) effect of leptin on BAT1 expression is also shown. Equal protein loading was determined by â-actin. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared to baseline time zero controls [(TRT-Control)%/Control], (p-value: ***p<.001).

6.3.2 Effect of resistin on BAT1 protein expression in primary human AbdSc adipocytes

Two different concentrations of rh resistin were used for these studies (50 ng/ml and 200ng/ml, respectively) at two different time points (24 hr 72 hr) based on previous studies of the research team (Kusminski *et al*. 2007). Dose and time course studies were performed to assess BAT1 protein at 24 and 72 hr in control and rh resistin-treated adipocytes. The analysis using t-test showed that the overall (total) effect of resistin on BAT1 protein expression was significantly reduced, although it varied depending on the selected dose and duration of treatment compared with baseline zero hr (24h rh resistin, 200 ng/ml: -16.4%(mean± SEM)13.0%; 72h rh resistin 200 ng/ml: -5.1% ±20.7%; 24h rh resistin, 50 ng/ml: -31.9% (mean± SEM)15.9%***; 72h rh resistin, 50 ng/ml: -11.9%±31.5%*; total resistin: 200 ng/ml -10.8% ±10.5%, total resistin 50 ng/ml: -20.8% ±18.2%***, p-values: *p<0.05, ***p<.001, **Figure 6.3.2**).

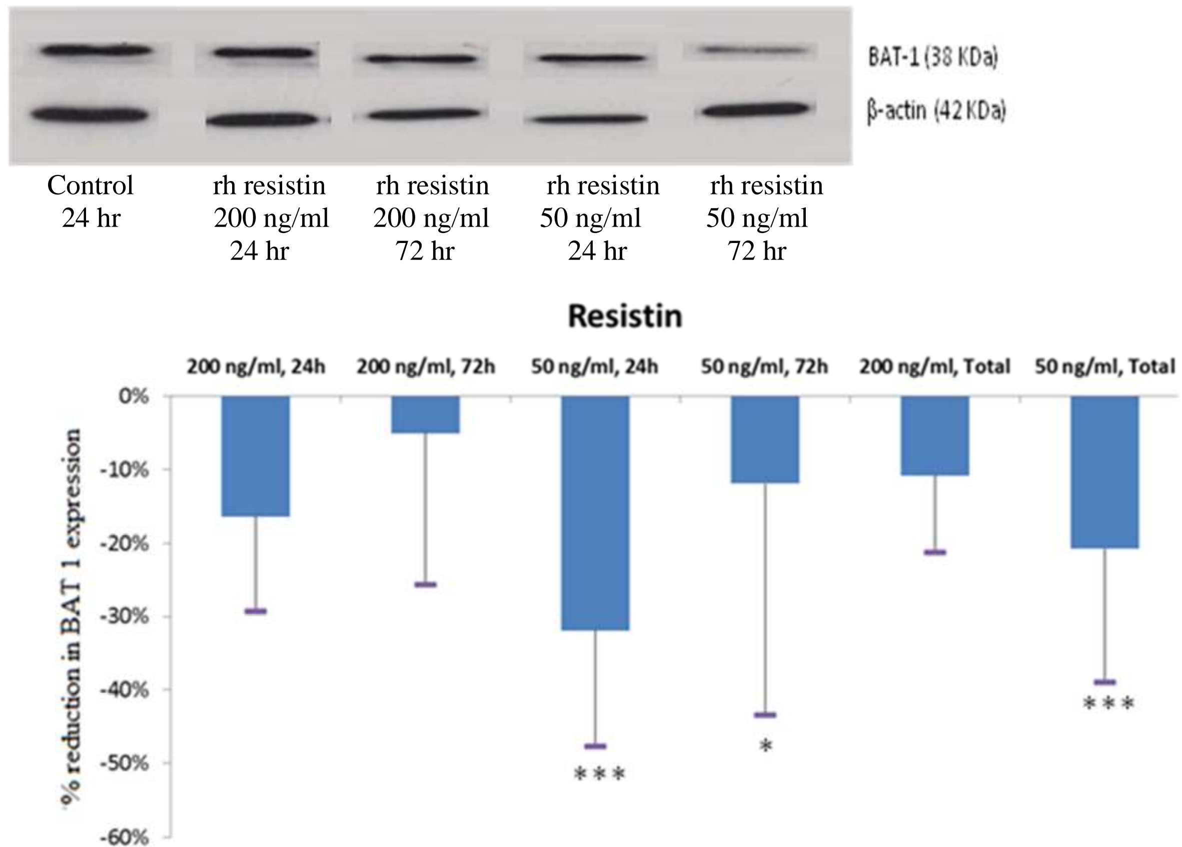


Figure 6.3.2 BAT1 protein expression in resistin (50ng/ml and 200ng/ml, respectively)-treated AbSc AT adipocytes (n=4-6), at two different time points (24 hr, 72 hr, respectively). The total effect of resistin on BAT1 expression is also shown. Equal protein loading was determined by â-actin. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared to baseline time zero controls [(TRT-Control)% / Control], (p-values: *p<0.05, ***p<.001).

6.3.3 Effect of adiponectin on BAT1 protein expression in primary human Abd Sc adipocytes

As adiponectin is considered to have anti-inflammatory properties (Zhou *et al*. 2008; Choi *et al*. 2007; Park *et al*. 2006; Thakur *et al*. 2006; Engeli *et al* 2003; Xu *et al*. 2003), it would seem that BAT1 expression should increase with adiponectin treatment. The abdominal subcutaneous adipocytes were treated with adiponectin (15 μg/ml); BAT1 protein in naive (control) and treated cells was estimated after 24 hours of treatment. Previous studies have shown the effectiveness of the selected adiponectin concentration and the particular time point in human adipocytes (Zoico *et al*. 2009; Ajuwon *et al*. 2005). According to this study adiponectin (15 μg/ml for 24h) had a negative effect on BAT1 expression when compared with control, baseline time zero: –45.5% (mean ± SEM)±17.0%, p-value: $p>0.05$, **figure 6.3.3**).

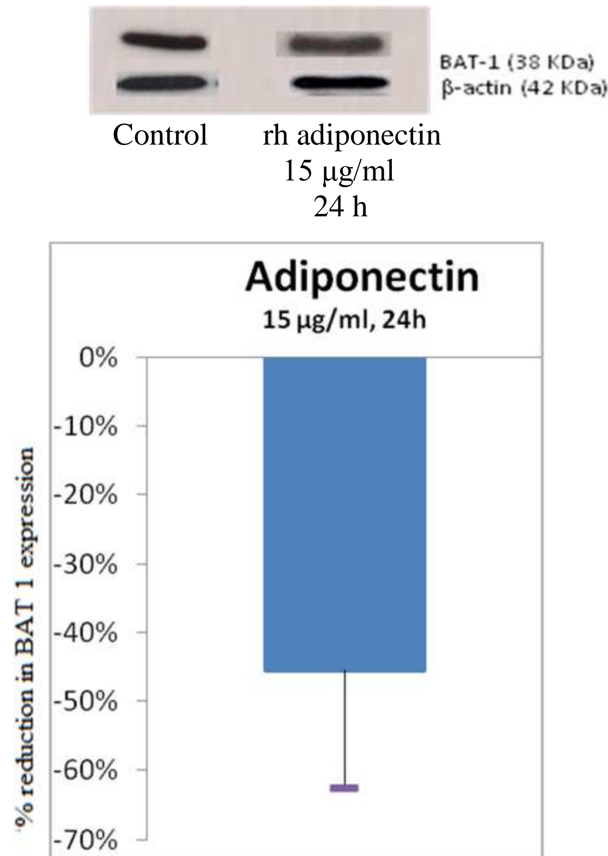


Figure 6.3.3 BAT1 protein expression in adiponectin (15µg/ml)-treated AbdSc AT adipocytes (n=4-6), for 24 hr. Equal protein loading was determined by â-actin. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared to baseline time zero controls [(TRT-Control)% / Control], (p-value: p>0.05).

Previous experiments, in chapter 5 showed that LPS was a significant repressor for BAT1 protein in human Abd Sc adipocytes. In this current chapter it was investigated whether adiponectin may reverse the negative effect of LPS on BAT1. Therefore, the cells were pre-treated with rh adiponectin (15µg/ml) for 24 hr and then incubated with LPS (25ng/ml) for a further 24 hr. Following this experiment BAT1 expression was estimated in the treated cells and compared with that of the LPS 25ng/ml (for 24 hours)-treated cells. This study showed that BAT1 gets suppressed by LPS despite previous

treatment with adiponectin (24h LPS 25 ng/ml: –45.6% (mean± SEM)±11.0 %***, 24h rh Adiponectin 15 μg/ml + LPS 25 ng/ml: –55.6%±17.2%***; p-value: ***p<.001, **Figure 6.3.3.1**).

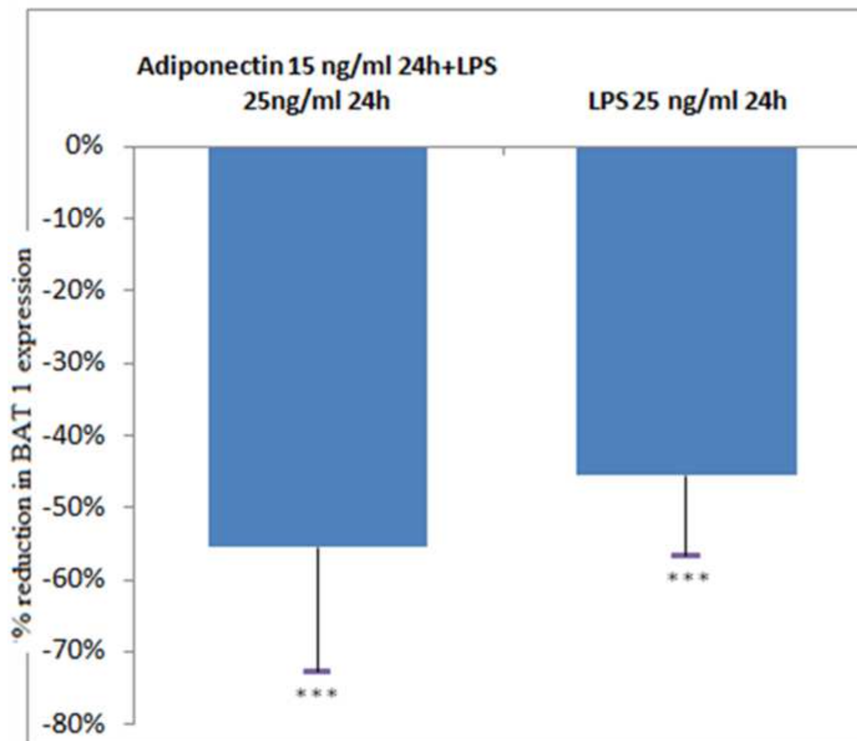


Figure 6.3.3.1 BAT1 protein expression in Abd Sc adipocytes (n=4-6) treated with LPS 25ng/ml for 24 hours or with adiponectin 15 μg/ml for 24 hours and then with LPS 25ng/ml for 24 hours. Equal protein loading was determined by â-actin. In the figure, values were measured by normalizing against the endogenous control â-actin (protein of interest/â-actin) and compared to baseline time zero controls [(TRT-Control)%/control], (p-value: ***p<.001).

6.4 Discussion

This study investigated the relationship between adipokines and BAT1 specifically pro-inflammatory (leptin, resistin) or anti-inflammatory (adiponectin) adipokines. Therefore, human adipocytes isolated from AbSc AT of overweight, non-diabetic, female subjects were used, that were cultured and differentiated as described in chapter 2; when fully differentiated, the cells were treated with the addition of the agent of interest (rh leptin, rh resistin or rh adiponectin) in the fresh detoxification media for pre-determined time points. At the end of the treatment duration, the cells were collected, the cellular protein content was isolated and the BAT1 protein expression was evaluated.

For this study the influence of rh leptin on BAT1 expression was determined, this showed leptin led to a direct reduction in BAT1 expression. This direct effect may be considered in the light of previous studies that indicate leptin can lead to an increase in the production of TNF-α in human AT (Lappas *et al.* 2005). As such taken these studies together suggests that despite leptin increasing TNF-α production leptin has a more dominant effect to repress BAT1 protein expression. This is an interesting finding considering that leptin increases with increasing adiposity, which means it could be responsible, at least in part, for the obesity-associated BAT1 down-regulation as observed in data in chapter 3.

These current studies also showed that rh resistin significantly suppressed BAT1 protein expression. In addition, it was noted that the duration of cellular exposure to rh resistin excess, the more potent the down-regulatory effects were on BAT1 protein expression. Findings from previous studies highlight the association between resistin and inflammatory response in morbidly obese individuals as well as positive correlation

between plasma resistin levels and other pro-inflammatory cytokines TNF-α, IL-6 and CRP (De Luis *et al*, 2010; Kunnari *et al*, 2006; Iqbal *et al*, 2005; McTernan *et al*, 2002; McTernan *et al*, 2002; Vidal-Puig & O'Rahilly, 2001). As noted for leptin, again resistin appeared to lead to a down-regulation of BAT1 protein expression.

Finally, it was investigated whether the anti-inflammatory adipokine, adiponectin may enhance BAT1 protein expression. This study highlighted a trend towards rh adiponectin reducing BAT1 expression although this did not reach statistical significance. This is however in contrast with the initial assumption according to which rh adiponectin would enhance BAT expression. However the increase in BAT1 expression may not have occurred as there was no inflammatory insult to respond to therefore a second set of experiments was undertaken to consider this point. Previous experiment, in chapter 5, investigated the effect of LPS on BAT1 protein in human Abd Sc adipocytes and showed that LPS was a significant repressor for BAT1, therefore in this current chapter cells were pre-treated with rh adiponectin (15 μg/ml) for 24 hr and then incubated with LPS (25ng/ml) for a further 24 hr. Following this experiment BAT1 expression was examined. This study showed that rh adiponectin failed to reverse the repressing effect of LPS on BAT1 protein expression. This highlights that the action of LPS with pre-incubation of rh adiponectin or co-current incubation didn't affect BAT1 protein expression. This suggests that LPS has more potent action on BAT1 reduction. Furthermore that rh adiponectin has little or no influence on regulating BAT1 protein expression in Abd Sc differentiated adipocytes.

In summary, this study highlighted that BAT1 appears to be regulated by inflammatory adipokines, whilst the anti-inflammatory influence of adiponectin to raise

BAT 1 expression was not observed. Taken together along with the observed effects of LPS following incubation with rh adiponectin it seems that BAT1 expression seems more profoundly regulated by inflammatory factors. BAT1 may represent a first line, non-selective, cellular protective signaling factor and is therefore affected by several different factors through common inflammatory pathways.

CHAPTER 7

Final Discussion

7.1 Discussion

We understand that obesity and T2DM are considered inflammatory disorders with common pathways by which several pathogenic components of obesity affect glucose metabolism, IR and the development of T2DM. BAT1 is a cellular DExD/H-box RNA-helicases which performs an essential role for cellular mRNA export by recruiting the adaptor proteins to spliced and unspliced mRNAs (Thomas *et al*. 2011). As such, this thesis has examined the impact that the molecule, BAT1, has during adipogenesis as well as the influence of nutrients and pro-inflammatory factors on its expression.

Whilst the role of BAT1 in the adipocyte has not been investigated, to date, we have clear evidence that BAT1 has anti-inflammatory properties which may be altered by metabolic states such as obesity and T2DM. This data has been derived from studies investigating monocytes and T-cell lines (Allcock *et al*; 2001), which suggest BAT1 acts an anti-inflammatory agent that downregulates several pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6. This suggests that BAT1 could play a protective role against the obesity-associated low-grade inflammatory state that contributes to diabetes development and hence has led to investigations into human adipose tissue (AT) and the adipocyte itself.

The rationale for examining BAT1 in AT has come from several piece of previous research examining the role of BAT1 in autoimmune disorders. BAT1 is coded by a gene located in the central part of the class III MHC genomic locus on chromosome 6, between TNFα and HLA-B genes localized in the nuclear speckle (Thomas *et al*. 2004; Alpert and Hashini 1993; Dias *et al*. 2010); this genomic region contains genes that affect susceptibility to immunopathologic disorders (Cheong *et al*. 2001; Ota *et al*. 2001; Price

et al. 1999). Further studies have shown that polymorphisms in BAT1 can lead to several auto-immune based disorders (Wong *et al*. 2003; Quiñones-Lombraña *et al*. 2008; Ramasawmy *et al*. 2006; Shichi *et al*. 2005; Price *et al*. 2004; Conrad *et al*. 1994; Bottazzo *et al*. 1985); whilst BAT1 can also be down-regulated by inflammatory cytokines (Van Harmelen *et al*. 1997). It is also apparent that an autoimmune condition can also occur concurrently with an inflammatory disorder, such as Type 1 diabetes (Chase *et al*. 2004); whilst a reduction in inflammation, in Type 1 diabetes can restore response to insulin without necessarily improvement in autoimmune pathology. Furthermore, emerging evidence supports the concept that IR, a consequence of inflammation, can precede the herald the onset of autoimmune diabetes (Razavi *et al*. 2006; Sherry *et al*. 2005; Betts *et al*. 2005; Fourlanos *et al*. 2004). Taken together, chronic inflammation may, at least, accelerate ß-cell death and lead to T1DM; concurrently polymorphisms of BAT1 have also been directly associated with T1DM which indicates BAT1 may be a crucial factor for the development of chronic inflammation.

AT is a critical tissue in the response to inflammatory insults, which can occur in many forms, as well as maintaining energy, satiety, blood pressure and homeostatic control through many cellular processes. AT has also been increasingly viewed as an important tissue to understand therapeutically, despite its complexity. Within this thesis, BAT1 was considered within the context that, based on other studies, it might be a suitable target to influence within adipose tissue. Therefore to understand the role of BAT1 in adipose tissue this thesis sought to investigate the expression and regulation of BAT1. Initial studies investigated BAT1 expression in *ex vivo* human AT which

highlighted that increasing adiposity and T2DM status reduced BAT 1 expression. In addition, that BAT 1 expression was altered by AT depot, for instance BAT 1 expression being increased in AbdSc AT taken from lean subjects compared with either Om lean AT or obese AT. Furthermore there was a gender influence in BAT 1 expression with, again, an increased expression noted in women.

As has long been known human AT contains many different types of cells besides adipocytes, including fibroblasts, macrophages, lymphocytes, pre-adipocytes and endothelial cells. In addition, some particular cell types increase with increasing adiposity *e.g.* macrophages (Weisberg *et al* 2003) and lymphocytes (Kintscher *et al* 2008; Wu *et al* 2007). As such, subsequent studies determined the expression of BAT1 in both human primary pre-adipocytes cells and the human pre-adipocyte cell line, Chub-S7, as a considered pure population of adipose cells. In both cell types BAT1 expression (mRNA and protein) was observed to increase with lipid accumulation, expressing a similar BAT 1 expression level in differentiating pre-adipocytes at day 6 compared with mature adipocytes. Once it was clear that the Chub-S7 pre-adipocyte cells differentiated well, and BAT 1 expression was comparable to primary human differentiated pre-adipocyte cultures, the effect of nutrients and inflammatory factors on BAT1 expression were examined, as well as the NFκB and or JNK pathways which may affect BAT 1 expression directly/indirectly. Both glucose and NEFA were shown to repress BAT 1 expression, which was in keeping with the *ex vivo* data determined in terms of AT from obese and T2DM subjects, indicating the impact of both factors on BAT1 expression. Furthermore that NEFA reduced BAT1 expression which appeared substantially more influenced by the JNK pathway, using inhibitor studies. However these studies also

indicated a synergistic action of both JNK and NFκB when used in combination to reduce BAT1 expression, indicating interconnectivity between JNK and NFκB pathways, as noted in other human AT studies examining other molecules (McGee *et al*, 2011).

Studies also showed LPS, a known systemic gut derived factor, reduced BAT1 expression. Such findings were, again, in keeping with the previous *ex vivo* AT data since LPS is raised in conditions of metabolic disease (Creely *et al* 2007). Further analysis of the potential paracrine influences of leptin and resistin on differentiated primary adipocytes highlighted BAT 1 repression whilst adiponectin appeared to have no significant effect alone to alter BAT 1 expression or reduce LPS induced BAT1 repression. Examining the current thesis data it appears that BAT1 is more influenced by glucose and NEFA than paracrine inflammatory or anti-inflammatory adipokines. BAT1 therefore represents a first line, non-selective, cellular protective signaling factor which is influenced by several different factors through common inflammatory pathways. As such, with BAT1 expression altered so readily by inflammatory factors this may suggest to potentially exploit BAT1 protective anti-inflammatory mechanism as a drug target, although further future studies would need to explore this in more depth.

7.2 Future Directions

This thesis has altered our understanding as to the factors that could affect BAT1 expression systemically or in a paracrine fashion within the adipocyte. Our current findings highlight that BAT1 is down-regulated by increasing adiposity, and detrimentally influenced by glucose, NEFA, LPS and adipocytokines. These findings could suggest that BAT1 suppression is an early event in the pathogenesis of a low

chronic inflammatory state and that early intervention to modulate BAT1 may either impede the inflammatory response or reduce its progression. Therefore future studies could examine two distinct areas, firstly, human studies to examine the impact of weight loss in BAT1 expression from AT as well as mononuclear blood cells to determine local cellular impact on BAT1 as well as systemic impact. This may highlight the potential for BAT1 expression to be reversed as the inflammatory insult reduces indicating that the effects are reversible. Secondly, other human AT depots could be examined with particular reference to epicardial fat an important reservoir. This depot, situated between the visceral layer of the pericardium and the anterior face of the myocardium, has been shown to provide non-esterified fatty acids (NEFA) to the myocardium (Marchington & Pnd 1990; Marchington *et al*, 1989). Therefore it would be interesting to examine whether BAT1 expression behaves differently in this depot due to the constant flux on fatty acids required by the myocardium. Does BAT 1 activity depend on its AT location, as noted with the AbdSc and OM data detailed? As epicardial AT from patients with and without coronary artery disease differs in their inflammatory status and BAT1 activation and could, this be manipulated to reduce the inflammatory status in this important AT site (Baker *et al*, 2009. Kostner *et al*, 2012).

Future studies could also use transgenic mouse models to overexpress BAT1 preferentially in adipose tissue to construct further data into the functionality of BAT1. This research may encourage the initiation of further trials to elucidate the beneficial effects of BAT1 and potentially lead to the development of new anti-inflammatory agents for the management of immunopathologic disorders as well as IR, T2DM and CVD.

7.3 Conclusion

BAT1 is expressed in human adipocytes at mRNA and protein level. It is down-regulated by several nutritional (saturated fats, high glucose concentration) and inflammatory factors (LPS) as well as by adipokines, including leptin and resistin. Adiponectin however has little or no influence on regulating BAT1 protein expression in AbdSc differentiated adipocytes. The BAT 1 protein has anti-inflammatory properties, as noted in other studies, and is repressed by many different molecules in AT; this could suggest that BAT1 represents part of a first line cellular protective pathway by which adipocytes respond to detrimental agents. As a first line cellular response to 'inflammation' this protective molecule, BAT1, may be affected by several different factors via common cellular pathways with the potential to exploit its anti-inflammatory properties.

APPENDICES

APPENDIX I: Buffers and Solutions

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

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

1.3 Phosphate Buffered Saline (PBS) (pH 7.6)

PBS 120 mM
NaCl 2.7 mM
KCL, 10 mM
Solution stored at RT.

1.4 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.

1.5 PBS/PBS-T solution for antibody preparation (0.5%)

X quantity 1.0% PBS-T (prepared as above)
X quantity PBS (prepared as above)

1.6 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; pH adjusted using HCl. Solution stored at RT.

1.7 TBS-T (1X)

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

1.8 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 Collagenase

50mls of Hank's Buffer Salt Solution

450mls of dH_2O
5mls Pen/Strep
Stored at -20° C

2.3 Transferrin
Transferrin is a serum protein, responsible for the binding and transfer of iron to cells. It has a molecular weight of around 80 kiloDaltons and contains two high-affinity Fe^{3+} binding sites. In the cell-culture media, tranferrin binds iron, and prevents its loss from the medium. It is also capable of binding other metal irons in the medium at concentrations which are toxic.

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

AI. 3 SOLUTIONS AND BUFFERS USED IN RT-PCR

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 CaCl2 10 mM MgCl2	200 mM Tris-HCl (pH 8.3) 20 mM MgCl2	50 mM EDTA

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

APPENDIX II: Reverse Transcription (RT) and Quantitative Real-Time Polymerase Chain Reaction

AII. 1 SYNTHESIS AND EXPRESSION OF mRNA

The genes encoding proteins are in nuclear chromosomes and are made of deoxyribonucleic acid (DNA). They contain coding (exons) and non-coding (introns) regions the number of which differs according to the gene. The genetic information contained in the genes, needs to be transferred in the cell cytoplasm to be decoded-translated into the specific polypeptide chain. This process is mediated by RNA polymerase that removes from the DNA of the gene all the introns, synthesizing messenger ribonucleic acid (mRNA) that contains only coding regions (exons). This process is called transcription. The so produced mRNA will be translated in cytoplasmic ribosomes to the specific protein. The assessment of mRNA expression, allows for estimations to be made as to the level of protein expression of a particular gene of interest.

AII. 2 RT-PCR

As the polymerase chain reaction (PCR) amplifies DNA sequences, DNA needs to be synthesized from the mRNA template. This process is termed 'reverse transcription', is catalyzed by the enzyme reverse transcriptase and the produced DNA is called complementary DNA (cDNA). The term complementary comes from the fact that the new-formed DNA (cDNA) contains the complimentary bases to that on the mRNA strand.

AII. 3 QUANTITATIVE REAL-TIME PCR

As mentioned in chapter 2, the quantitative real time polymerase chain reaction is a laboratory technique used in molecular biology that enables both detection and quantification, as absolute number of copies of a targeted DNA sequence. It uses fluorescence technology to monitor amplicon production during each PCR cycle; this enables the analysis of the amount of template rather than the amount of amplified product at the endpoint of the reaction. In this study, an ABI 7700 Sequence Detection system was used to analyze the mRNA levels. This system utilizes TaqMan chemistry for highly accurate quantification of specific mRNA levels.

TaqMan probes contain a fluorescent reporter dye and a quenching dye. The latter is usually on the 3' base, while the fluorescent reporter dye is usually on the 5' base; due to the close proximity of the two, the quenching dye prevents emission of any fluorescence as long as the probe is intact. During the Quantitative Real-Time PCR however, the probe anneals between the forward and reverse primer sites within the PCR product of interest. Thus, when the Taq DNA polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This results in removal of the fluorescent reporter dye from the proximity of the quenching dye allowing a florescent signal. Each amplification cycle adds more florescent signal of the targeted cDNA sequence, which becomes intense enough to be detected and quantitatively measured by a laser and charged coupled device (CCD) camera, used in Quantitative Real-Time PCR.

The principles of the TaqMan Sequence Detection Chemistry are demonstrated in **figure II.1**. The probe, containing both the fluorescent reporter and quencher dyes, is

attached to the targeted cDNA. The reporter dye is cleaved from the probe during the polymerization, which enhances the fluorescence of the reporter. Each amplification cycle enhances further the intensity of the fluorescence, allowing the monitoring of the reaction in real-time

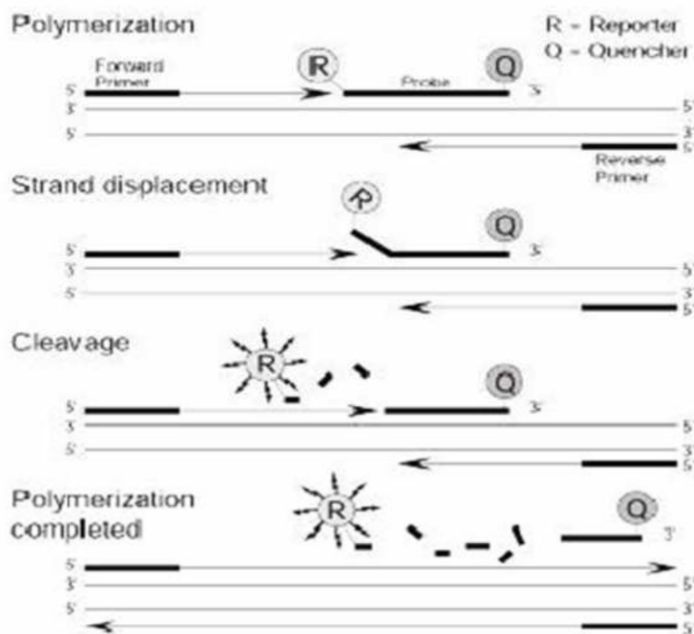


Fig II.1. Principles of the TaqMan Sequence Detection Chemistry. Attachment of the probe to the targeted cDNA. Cleavage of the dye from the probe during the polymerization and subsequent enhancement of the reporter's fluorescence.

APPENDIX III: Western Blotting (WB)

AIII. 1 CALCULATION OF THE SAMPLES' PROTEIN CONTENT FOR WESTERN BLOT ANALYSIS

A spectrophotometer at 655 nm was used to analyze the protein samples. The calculation of the optical densities converted to protein content (μg) each time samples were assayed, was made by the construction of a standard curve using bovine serum albumin (BSA) diluted in dH2O, (**Figure AIII.1.1**). To exclude any interference with calculated protein sample concentrations, a mixture containing only Reagent S, Reagent A and Solution B, had no optical density (protein signal).

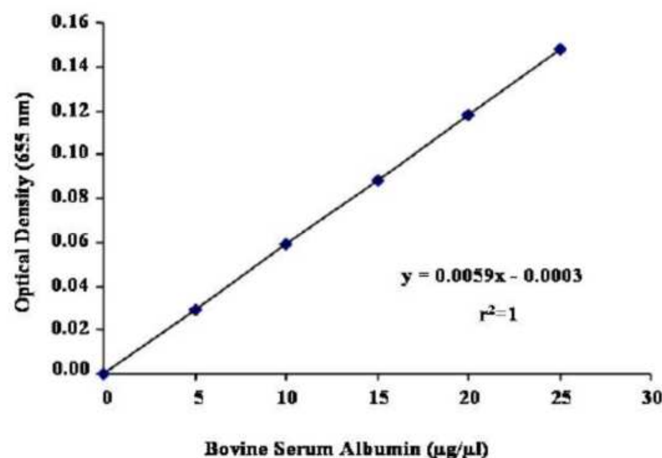


Figure III.1. The calculation of the protein content in proteins samples extracted from adipose tissue and isolated adipocytes was made used as a reference standard curves at the one shown in the graph. Bovine serum albumin was diluted in dH2O to known concentrations and absorbance read at 655 nm on a spectrophotometer.

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ABSTRACT LIST:

1. **I. Kyrou, G. Osei-Assibey, C. Baker, D. Kendrick, K. Lois, P. Saravanan, S. Kumar** 2009 Impact of weight loss on anxiety and depression in obese patients with type 2 diabetes mellitus. Vienna, EASD.

2. **Konstantinos Lois, Saif Alhusaini, Elham Youssef, Gyanendra Tripathi and Sudhesh Kumar** 2009. Expression of the anti-inflammatory gene BAT1 is suppressed in adipose tissue of obese human subjects. MRS / AMS / RCP Clinical Scientists in Training meeting. London, UK.

3. **Saif Alhusaini, Konstantinos Lois, Christine M Kusminski, Phillip G McTernan, Sudhesh Kumar and Gyanendra Tripathi** 2008 Obesity is associated with activation of innate immune pathway and ER stress in human abdominal subcutaneous adipose tissue. Uk Adipose Tissue Discussion Group Meeting (ATDGM), University of Warwick, UK.

4. **Konstantinos Lois, Saif A Alhusaini, Philip G McTernan, Sudhesh Kumar and Gyanendra Tripathi** 2008 Expression of anti-inflammatory gene BAT1 is suppressed in adipose tissue of obese human subjects. PG Symposium, University of Warwick, UK

5. **AIF Alhusaini, Konstantinos Lois, Christine M Kusminski, Phillip G McTurnan, Sudhesh Kumar** 2008 Obesity is associated with activation of innate immune pathway and ER stress in human abdominal subcutaneous adipose tissue. UK Adipose Tissue Discussion Group Meeting, University of Warwick, UK

6. **A.A.Kumar, K.Lois, J.McMoran, M.Wallace, M.Mistry, L.Dodd, J.Hancox, A. Anwar** 2007 Assessment Of Diabetes Intervention Strategies In Clinical Practice**.** Spring Meeting of the BRITISH Clinical Diabetologists (ABCD), Chester, UK.

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