Sensing and control of adipocyte function

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Summary

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Obesity has become a global epidemic. Approximately 15% of the world population is either overweight or obese. This figure rises to 75% in many westernised countries including the United Kingdom. Health costs in the UK to treat obesity and associated disease are conservatively estimated at 6% of the National Health Service (NHS) budget equating to 3.33 billion Euros. Excess adiposity, especially in visceral depots, increases the risk of type 2 diabetes, cardiovascular disease, gall stones, hypertension and cancer. Type 2 diabetes mellitus accounts for >90% of all cases of diabetes of which the majority can be attributed to increased adiposity, and approximately 70% of cardiovascular disease has been attributed to obesity in the US. Weight loss reduces risk of these complications and in some cases can eliminate the condition. However, weight loss by conventional non-medicated methods is often unsuccessful or promptly followed by weight regain.

This thesis has investigated adipocyte development and adipokine signalling with a view to enhance the understanding of tissue functionality and to indentify possible targets or pathways for therapeutic intervention. Adipocyte isolation from human tissue samples was undertaken for these investigative studies, and the methodology was optimised. The resulting isolates of pre-adipocytes and mature adipocytes were characterised and evaluated.

Major findings from these studies indicate that mature adipocytes undergo cell division post 'terminal' differentiation. Gene studies indicated that subcutaneous adipose tissue exuded greater concentrations and fluctuations of adipokine levels than visceral adipose tissue, indicating an important 'adiposensing' role of subcutaneous adipose tissue. It was subsequently postulated that the subcutaneous depot may provide the major locus for control of overall energy balance and by extension weight control.

One potential therapeutic target, 11β-hydroysteroid dehydrogenase (11β-HSD1) was investigated, and prospective inhibitors of its action were considered (BVT1, BVT2 and AZ121). Selective reduction of adiposity of the visceral depot was desired due to its correlation with the detrimental effects of obesity. However, studies indicated that although the visceral depot tissue was not unaffected, the subcutaneous depot was more susceptible to therapeutic inhibiton by these compounds. This was determined to be a potentially valuable therapeutic intervention in light of previous postulations regarding long-term energy control via the subcutaneous tissue depot.

Obesity, Type 2 Diabetes Mellitus, Adipocytes, Pre-adipocytes, Adipokines, 11β-HSD1, adipocyte development.

"A little hope, two wings and some sky that's all we really need to fly".

For my parents; Bob and Julie Hubber, whose unfailing love and support has given me inspiration and determination throughout my life.

Acknowledgements

Abbreviations

11-βHSD	11-beta hydroxysteroid dehydrogenase
ACAT	acyl-CoA: Cholesterol acyltransferase
ACC	Acetyl-CoA carboxylase
ACL	ATP – citrate lyase
ACTH	adrenal corticotrophic hormone
AdipoR1	Adiponectin receptor 1
AGRP	agouti-related protein
AMP-K	Adenosine monophospahte – kinase
ANOVA	analysis of variance
ARC	Arcuate nucleus
ASP	Acylation stimulating protein
AZ	AstraZeneca
β-AR	Beta – adrenergic receptor
BIA	Bio-electrical impedance
BMI	Body mass index
BVT	Biovitrum
CAD	Coronary artery disease
cAMP	cyclic adenosine monophosphate
CART	Cocaine and amphetamine regulated transcript
CB_1	Cannabinoid receptor
CCK	Cholecystokinin
CPT-1	Carnitine palmitoyl – transferase

CRH	Corticotrophic releasing hormone
CRH	Corticotrophin-releasing hormone
CVATT	Critical visceral adipose tissue threshold
CVD	Cardiovascular disease
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DNL	*De novo* lipogenesis
DPP	Diabetes prevention program
EASO	European Association for the study of Obesity
ECM	Extracellular matrix
FATP	Fatty acid transporter protein
FFA	Free fatty acid
FIZZ	'found in inflammatory zone'
Glut-4	Glucose transporter – 4
GLY	Glycyrrhetinic acid
GOI	Gene of interest
GPAT	Glycerol-3-phosphate acyltransferase
GPCR	G-Protein coupled receptor
GPR41	G-protein receptor 41
GR	Glucocorticoid receptor
GRE	Glucose response elements
HDL-c	High density lipoprotein - Cholesterol
HOMA	The homeostasis model assessment
HPA	Hypthelamic pituitary- adrenal

HSL	Hormone sensitive lipase
IARC	International agency for research on cancer
IBMX	3-Isobutyl-1-methylxanthine
IGT	Imaired glucose tolerance
IL6	Interleukin 6
IOTF	International obesity task force
IR	Insulin resistance
LCAT	Lecithin: cholesterol acyltransferase
LDL-c	Low density lipoprotein – Cholesterol
LHA	Lateral hypothalamic area
LPL	Lipoprotein lipase
MC	Melanocortin
MCH	Melanin concentrating hormone
MG	Mono-acyl glycerol
MONW	Metabolically obese normal weight
MR	Melanocortin receptor
mRNA	messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
MUFA	Monounsaturated fatty acids
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCEP-ATP	National cholesterol education program – Adult treatment panel
NEFA	Non-esterified faty acids
NFκ-B	Nuclear factor kappa B

NGT	normal glucose tolerance
NHANES	National health and Nutritional examination survey
NOA	National obesity audit
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
PAI-1	Plasminogen activating factor inhubitor -1
PCOS	Polycystic ovarian syndrome
PEPCK	phosphor-enolpyruvate carboxylase
PFA	Perifornical area
PHP	Primary human pre-adipocytes
POMC	Propiomelanocortin
PPARα	Perioxisome proliferators – activated receptor alpha
PVN	Paraventricular nucleus
PVN	Paraventricular nuclei
RELM	Resistin-like molecules
RT-PCR	Reverse transcriptase – polymerise chain reaction
SCD1	Stearoyl-CoA desaturase
SES	Social economic status
SNS	Sympathetic nervous system
SREBP-1	Stearol regulatory element binding protein
T2DM	Type two Diabetes Mellitus
TG	Triglyceride
TNFα	Tumor necrosis factor alpha
TZD	Thiazolidinediones

US	United States
VLDL	very low densisty lipoprotein
WAT	White adipose tissue
WHO	World health organisation

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Chapter 1: Introduction

1.1 Prologue

This thesis concerns a programme of experimental studies to identify potential new therapeutic approaches against obesity and its detrimental effects. There is now a global epidemic of obesity and the excess of adipose tissue is well recognised to be an underlying cause and an aggregating factor in many other clinical conditions. Unfortunately, lifestyle measures are rarely adequate, due in part to the complexity of the adipocyte which can no longer be regarded as a rather passive organ of triglyceride storage. Moreover there is no effective and safe therapeutic intervention to prevent or reverse obesity. Comparatively little work has been conducted into the activities of adipocytes beyond lipid storage, particularly in human adipocytes. There is emerging evidence that different depots of adipocytes contribute in a differing manner to the sensoring and control of energy homeostasis and accompanying conditions. Most notably excess visceral fat, which represents only 20% of total body fat, appears to be strongly associated with most of the metabolic vascular complications of obesity, whereas excess subcutaneous fat shows a much weaker association with these complications. Little is known about the characteristics that distinguish between these adipose depots and contribute to their different pathophysiological roles.

Insulin resistance is now emerging as a fundamental mechanism through which obesity increases susceptibility to a spectrum of metabolic vascular disorders. Insulin resistance has been implicated in the development of IGT, type 2 diabetes, raised blood pressure, dyslipidaemia, all of which conspire to promote atherogenesis and the development of arterial vascular disease. The clustering together of these disorders within the same individual is a common occurrence, and is now referred to as the metabolic syndrome.

First, intervention against obesity, especially excess visceral adiposity should provide a basis for reducing the attendant metabolic and vascular complications. With this aim in mind the present research project set out firstly to establish methods applicable to human tissue secondly, to identify growth and general differences between visceral and subcutaneous fat thirdly, to identify genetic and functional differences between visceral and subcutaneous fat and finally to exploit potential sites in order to decrease adipocyte metabolism especially in visceral fat and to decrease the opportunity for adipose tissue to create insulin resistance.

Thus this thesis investigates the hypothesis that human visceral and subcutaneous adipocytes exhibit functional and metabolic differences. This thesis characterizes the tissues and then explores expression differences between tissue depots, gender and adiposity. Further investigation into 11β-HSD1 activity in both tissue depots with a view to selective inhibition of this local adipokine as a potential method of reducing adiposity was explored. Selective reduction of adipose tissue by depot could reduce insulin resistance associated with obesity which underpins many of the accompanying metabolic and vascular complications.

1.2 Obesity

1.2.1 Definition

The WHO (2000) defines obesity as 'abnormal or excessive fat accumulation, to the extent heath is impaired'. This is echoed by Lowell (2000) who defines obesity as 'an increase in weight stored as fat, above an ideal body weight at which life expectancy is maximal'. Several techniques have been employed to quantify the measurement of obesity, including total body weight, skin fold thickness (McNulty, 1999) and various estimates of total body fat such as weighing submerged in water and bio-electrical impedance (BIA). BIA methods of measuring body fat mass are still in their infancy but should eventually enable determination of medical risks associated with excess fat on a person by person basis. However, currently the equipment is expensive and results vary between different models and makes.

The current standard measure of obesity in routine clinical practice is via body mass index (BMI) calculated as a ratio of weight (kg) to height squared (m^2). Recently there has been a call for ethnic-specific cut-off bands due to subtle variations in the relationship of BMI, body fatness, mortality and morbidity in different ethnic groups (Deurenberg 2001). Taking this into account BMI offers a relatively reliable quantification for most cases of obesity, except at extremes of age, height and musculature (McNulty 1999). However BMI does not take account of the distribution of excess fat and different fat depots exhibit differenced in their functional properties and clinical risk.

The currently accepted classifications of overweight and obese as applied to Europid Caucasians are outlined by the WHO technical report and have been presented in table 1.1 below.

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Table 1.1: WHO classification of Overweight in adults according to BMI

(WHO, 2000 and National Institute of Health, 1998) Modified from Ravussin and Bouchard, 2000)

Classification	Body Mass Index (BMI*)	Risk of co-morbidities
Underweight	<18.5	Low (Risk of other clinical.problems increased)
Normal	18.5-24.9	Average
Pre-Obese	25.0-29.9	Increased
Obese class I	30.0-34.9	Moderate
Obese class II	35.0-39.9	Severe
Obese Class III	$\geq$40.0	Very severe

*BMI = Weight (kg) / Height^2 (m) There is an increased risk of metabolic complications with a waist circumference $\geq$ 98cm in men and $\geq$80cm in women. This risk is substantially increase with a waist circumference $\geq$102 cm in men and $\geq$88cm in women (see NIH guidelines)

1.2.2 Epidemiology

The prevalence of obesity in westernised and westernising countries has doubled over the last decade (James 2004). Nearly one third of all adults are classified as obese in the US alone (Ogden *et al* 2003). Globally there are over 1 billion overweight adults and of these 300 million are classified as clinically obese: this constitutes around 5% of the population (WHO: Puska *et al*, 2003). In the EU up to 135 million people are affected in some countries of the European community over 50% of people are over weight and up to 30% are clinically obese (IOTF and EASO 2002). Obesity not only affects adults: children are becoming increasingly at risk (James 2006). Data from 79 developing countries indicate that approximately 22 million children under the age of 5 years are overweight (WHO: Puska *et al* 2003). In the United States, where over 55% of the population are either overweight or obese; there has been an increase of 30% in the prevalence of obesity over the last 50 years (Willet *et al* 1999). Recent estimates suggest that in the USA 300,000 (aprox.) deaths per year are attributable to obesity (Harrold, *et al* 2004). An increase in the prevalence of obesity has been observed throughout Europe especially in the UK which is now believed to

have the fastest growing prevalence of obesity in Europe (EASO 2003). Other countries undertaking obesity surveys including Australia, Brazil, China, Japan, Mauritius, and Western Samoa all of which have shown an increasing prevalence of obesity (Flegal *et al*, 1998).

1.2.2.1 UK

In England, obesity has trebled over the last 20 years (National Obesity Audit, 2001) and current data shows that >65% of people are classified as overweight of whom 20% (men) and 25% (women) are clinically obese (EASO 2003). In 2001 it was estimated that approximately 2.25 billion pounds sterling is expended per annum on the costs of managing obesity and its complications. Furthermore, as the prevalence of obesity increases so will the overall costs of treatment (National Obesity Audit: Bourn J, 2001). In the IOTF and EASO report (2003) this is reported as almost 554 million pounds sterling (816 million euros) on direct costs and a further 2.3 billion pounds sterling ($\approx$ 3.33 billion euros) on indirect costs surmounting to direct costs of 1.5% of total health expenditure and 6% of total health expenditure budget including indirerct costs.

Obesity is not a simple disease as there are multiple influencing factors driving the pandemic including genetic pre-disposition, economic growth, modernisation, urbanisation and globalisation of food markets (WHO: Puska *et al*, 2003).

1.2.2.2 Childhood Obesity

Childhood obesity is defined under the same terms as adult obesity mentioned above. However, when determining overweight or obese status in children under the age of 18 years BMI references have been revised to represent age and gender differences. The BMI cut-off

points designated by the IOTF were designed to complement existing adult BMI reference values. The charts were generated from the NHANES I study, which incorporated data from 6 countries (Cole *et al* 2000). Sadly childhood obesity is on a sharp increase (Caterson and Gill 2002). There is an immediate associated increased risk of T2DM that had previously only been connected with adults. This risk is mostly associated with children in westernised and developed nations however it is also increasing in countries in a state of nutritional and economic transition.

1.2.2.3 Gender

Women have a higher incidence of obesity than men in the majority of ethnic groups (James 2004, Caterson *et al* 2004). However men have a higher incidence of being overweight that does not necessarily progress to obesity. It has been suggested that this may be due to an increased ability of men to lay lean body mass during times of energy imbalance (James 2004) Co-morbidities of obesity risks differ by both gender and ethnic group; for example there is a higher risk of T2DM than hypertension in Caucasian women compared with both women of other ethnic backgrounds and men. In African American men the relative risk of these co-mobidities is reversed with hypertension being of greater risk than T2DM (Caterson *et al* 2004).

1.2.2.4 Age

The medical risks associated with obesity, particularly CVD, increase with advancing age. The onset of obesity co-morbidities is apparent earlier in life, consistent with the earlier onset of obesity (Caterson *et al* 2004). This risk may be due in part to a change in body composition. As age advances there is an increased propensity to store fat rather than generate lean body mass (Caterson *et al* 2004).

1.2.2.5 Ethnicity

As previously mentioned BMI varies with ethnicity. In Asian populations it was noted that associated risk of some metabolic diseases occurs at a lower BMI than observed in Europid caucasians. For example in Japanese populations risk of hypertension is three-fold greater at a BMI of 24.9 and risk of T2DM is increased at a BMI of 22-23 (Caterson *et al* 2004). In the NHANES 1999-2000 survey, people older than 20 years show ethnic differences were greater in women versus men (Ogden *et al* 2003). 50% of obese non-Hispanic black population were classified as obese compared with 30% of the non-Hispanic white population. Both the Aboriginal and Chinese populations have greater risk of T2DM within the range defined as 'healthy' (BMI < 25). As a result of these differences associated with ethnicity the BMI cut-offs for recommended intervention have been adapted for each ethnic population (Caterson *et al* 2004).

1.2.3 Genetic and environmental implications

1.2.3.1 Genetic

The thrifty gene hypothesis attempts to explain from an evolutionary perspective how *Homo sapien* has become predisposed to excess adiposity (Neel 1962).

It proposes that exceptional efficiency in food utilization provided a selective advantage during the evolution of human beings. The most efficient mechanism was a prompt increase of insulin after a meal, which minimized hyperglycaemia, prevented glucosuria and cellular uptake and storage of nutrient fuels. Those better adapted to storing energy would be selected for in times of the inevitable famines because they would be able to sustain life via the breakdown of energy dense stores of fat. Thus adaptive pressure reflecting repetitive famine favoured those with the so-called thrifty genotype (Lev-Ran 1999). In current "western" society there is an abundance of palatable food and therefore a propensity and temptation to over consume, ultimately resulting in obesity. The postprandial surge of insulin has also been preserved, and prolonged periods of raised insulin concentrations (hyperinsulinaemia) have lead to the secondary insulin resistance. Excessive insulin release may also contribute to β-cell failure of T2DM (Chiasson and Rabasa-Lhoret 2004). In addition, more efficient lipid storage in adipose tissue selected for throughout history would have conferred resistance to famine and relative protection against lipotoxicity of non-adipose tissues. Moreover the genes for both thriftiness and postprandial hyperinsulinaemia continue to be conserved as there are limited or no environmental evolutionary pressures that are beyond the capability of man to overcome in today's society (Lev-Ran 1999). In addition to evolutionary pressures conserving the thrifty genotype, environmental and lifestyle pressures of the hunter-gatherer existence adopted by most Paleolithic humans would have prevented obesity. Their societies were exceedingly mobile; this facet of life introduces a selective pressure against obesity, and is one that is

becoming increasingly redundant in modern times of vast technological advance. It appears that modern humans have an individual "calorie-stat" that is present early in life. This may have been due to a more condensed growth period than our ancestors and therefore may have contributed to the early onset of some pathophysiological changes associated with obesity, diabetes and hypertension (Lev-Ran 1999). However, until relatively recently the full clinical expression of these pathophysiological changes has been delayed until adulthood.

1.2.3.2 Set point theory

In 1976 Jules Hirsch developed the set point theory, where adjustment towards a 'prefered' weight is continually attempted by inherent physiological mechanisms of nutrient homeostasis (Hirsh 1976). He showed that if weight was gained, the efficiency of metabolism would decrease in a compensatory fashion and that the converse was also true. There are two regions of the hypothalamus that are implicated in the regulation of body weight. The feeding centre is located in the lateral regions and the satiety centre in the ventromedial regions of the hypothalamus (Wynne *et al* 2005).

The set point theory requires that there is a point at which the body prefers to maintain body fat mass and body weight referred to at the calorie-stat. It is determined mainly by genetic factors, yet it is also influenced by early environmental factors such as intrauterine temperature and growth (Wilding et al 1997). However, the calorie-stat point is not fixed and so can be altered by the level of physical activity or prolonged over/under feeding (Wilding et al 1997). It is hypothesised that this effect could be due to the adjusted hypothalamic response to long term satiety signals. For example, in periods of long term weight gain due to lack of exercise and over feeding the hypothalamus adjusts to accept a higher set point (Friedman and Leibel 1992). The discovery of leptin (Friedman 1994) provided a viable molecular link

between the satiety mechanisms in the brain and the level of adiposity, thus a probable metabolic function for the explanation of this theory.

1.2.3.3 Environment

Environmental influences of diet and lifestyle influence body weight by propagating an imbalance between energy intake and energy expenditure. In western diets, one cause of such an imbalance is the propensity to high fat intake. Fat is 2 ¼ times more energy dense than carbohydrate and protein, thus significant overindulgence in this energy source can lead to overweight and obesity (Keller and Lemberg 2003). In addition, most people have a taste preference for food rich in fat (Lissner and Heitmann 1995) making it difficult to maintain a "healthy" diet in modern times of gastronomic affluence.

This is reflected and further compounded by 'fad diets' such as the Atkins diet, GI diet, 5-a-day plan, low carbohydrate diet, Ornish, low fat diet, Sugar busters, and other calorie restriction plans (Dansinger et al 2005, Anderson et al 2001, Parikh et al 2005). These diets may not be mutually exclusive but are not all endorsed by the majority of the medical profession. This is most notable for the Atkins diet, which is high in saturated fat and is predominantly used as a binge diet prior to a social event. It is rarely maintained and usually ends with weight re-gain (Riebe *et al* 2004). The confusing and overwhelming quantity of diet information leads to a nation of binge dieters and swing dieters. The decline in food prepared from fresh and natural ingredients in part due to a decrease in home cooking and an increase in availability and variety of instant meals nutritionally high in energy dense ingredients, promotes a diet high in fats and sugars.

Industrial and technological advances have provided increased refinement of ingredients and subsequently decrease in energy expenditure by the body to digest a meal. Energy dense food

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substances are compacted into smaller portions providing stimulation by fullness only on increased intake, thereby encouraging an increase in energy consumption. Eventually these factors will result in weight gain, especially if lifestyle is not adapted to compensate (MacLean et al 2005, Hambrecht and Gielen 2005).

In modern society especially in western civilisations lifestyle has become increasingly sedentary (Hu et al 2003). The advancement of technology such as cars, computers, television and more recently the internet has decreased the necessity for movement. Levels of voluntary exercise have decreased, and there has been a corresponding increase in average weight.

Work patterns and increasing demand for longer hours in sedentary occupations decreases the amount of time devoted to voluntary exercise and an increase in convenience food in the workplace perpetuates the weight problem (Jebb 1997). Eating habits are also influenced by psychological pressure of self image. This is impressed upon people young and old primarily by the instigated by the media, filtering through to family values and peer pressure. Fad diets increase the epidemic of binge dieting and yo-yo weight. Constant overeating results from social meal eating or boredom and depression. Similar psychological influences to anorexia nervosa are impinged upon with the obese. Negative self image and low self-esteem often drive comfort eating and perpetuate a destructive eating pattern typically with foods high in fat and sugar. Binge eating results in weight cycling leading ultimately to further weight gain (Munsch et al 2003).

1.2.4 Socio-economic effects on obesity

It has been reported that as increased social economic status (SES) reduces the risk of obesity increases in most western societies (Reidpath, 2002). Consumption of energy rich food from fast food outlets is higher in poorer regions (WHO 1998). Fast-food outlets are more numerous in areas of low SES (Reidpath 2002) and there is a strong correlation between income inequalities, neighbourhood deprivation and obesity (Kahn 1998, Ellaway 1997). In short, any preventative dietary modification to this target population of obese individuals will be increasingly difficult due to their 'obesogenic' environment.

1.2.5 Economic effects

Economic development aims to include improvements in food supply in quantity and variety to address dietary deficiencies and improve nutritional status (WHO 2002). Changes in diet, patterns of work and leisure are often referred to as "nutritional transition", characterisation of which includes quantitative and qualitative changes in diet (WHO 2002). The current 'nutritional transition' trend in occidental and developing countries has promoted an adverse dietary outcome in the form of obesity attributable to a higher energy dense diet involving increased fat, added sugars, reduced complex carbohydrates and dietary fibre and in some communities reduced fruit and vegetable intakes (Drewnoski 1997).

1.2.5.1 Cost of co-mobidities

Co-morbidities attributable to obesity certainly include CVD, gall bladder disease, colonic and breast cancer, hypertension and T2DM (Colditz 1992). The cost of treating the co-morbitities attributable to obesity in the USA was estimated to be in the region of 39.3 billion dollars in 1986. In 1990, with inclusion of musculoskeletal diseases, the estimated cost increased to around 45.8 billion dollars: this comprised 6% of total heath care expenditure. In the UK the

costs were determined using a similar method and were estimated at £195 million, of which 15% was directs costs of treating obesity and 44% was dietary products (West 1994). Data outlined in the EASO report (2003) estimate the indirect costs of treating obesity in England at 2.24 million and direct costs of approximately 5.6 million pound sterling.

1.3 Clinical implications

As noted above, obesity introduces chronic morbid disorders (table 1.2) the incidence and severity of which are dependent on the severity, duration and cause of the obesity (Flegal, 1998). The greatest risk associated with severe obesity, eg. where BMI is $>35kg/m^2$, is T2DM, with a 93-fold increase in females and a 43-fold increase in males (Jung 1997).

Table 1.2: Morbidity in obesity

Cardiovascular	Hypertension, Coronary heart disease, Cerebrovascular disease, Varicose veins, Deep vein thrombosis	**Breast**	Breast cancer, Male gynaecomastia
Respiratory	Breathless, Sleep apnoea, Hypoventilation syndrome	**Uterus**	Endometrial cancer, Cervical cancer
Renal	Proteinuria	**Urological**	Prostate cancer Stress incontinence
Metabolic	Hyperlipidaemia, Insulin resistance, Diabetes mellitus, Polycystic ovarian syndrome, Hyperandrogenisation, Menstrual irregularities	**Skin**	Sweat rashes, Fungal infections, Lymphoedema, Cellulitis, Acanthosis nigracans
Neurology	Nerve entrapment	**Orthopaedic**	Osteoarthritis, Gout
Gastointestinal	Hiatus hernia, Gallstones and choleithiasis, Fatty liver and cirrhosis, Haemorrhoids, Herniae, Cancer colorectal	**Endocrine**	Growth hormone and IGF reduced, Reduced prolactin response, Hyperdynamic ATCH response to CRH, Increased Urinary free cortisol, Altered sex hormones
Pregnancy	Obstetric complications, Caesarean operation, Large babies, Neural tube defects		

Adapted from Jung 1997

1.3.1 Cardiovascular Risk

The Nurses' health study in the USA found that the risk of coronary artery disease increased 3.3 fold with a BMI >29 kg/m^2 when compared with a BMI of <21 kg/m^2 in a cohort of women (Bray 2004). In addition to the physical effect on the heart of chronic excess weight gain, namely cardiac hypertrophy, there is a harmful molecular affront caused by adipokine release directly correlated with adipose tissue mass gain particularly in the visceral region. Examples of these chemokines include adiponectin, resistin, leptin, plasminogen activating factor inhibitor 1 (PAI-1), tumor necrosis factor α (TNFα) and IL-6 (Sowers 2003). Adiponectin is regarded as an antiatherogenic adipokine and is decreased in obesity (Matsuzawa 2005). Levels of adiponectin are inversely correlated with insulin resistance, of which the latter is an independent risk factor for CVD.

Table 1.3 Cardiovascular risk factors associated with visceral obesity

- Insulin resistance/hyperinsulinemia
- Low serum levels of HDL-C
- High serum triglyceride concentrations
- Increased apolipoprotein B serum concentrations
- Small, dense LDL particles
- Increased serum fibrinogen concentrations
- Increased production of plasminogen activator inhibitor
- Increased serum levels of C-reactive protein
- Increased production of tumor necrosis factor-α
- Increased production of interleukin-6
- Microalbuminuria
- Increased blood viscosity
- Increased systolic and pulse pressure
- Left ventricular hypertrophy
- Premature atherosclerosis
- Microalbuminuria

HDL-C: high-density lipoprotein cholesterol; LDL: low-density lipoprotein.

Adapted from Sowers 2003

Resistin is positively associated with insulin resistance but its function and molecular actions in humans are not yet clearly characterised (Borst et al 2005, Steppan and Lazar 2002). Overexpression of TNFα and IL6 occurs in obesity, correlating with the increase in adipose tissue mass (Aldhahi and Hamdy 2003). Both TNFα and IL-6 are regulators of the acute-phase response, and PAI-1 promotes a pro-coagulatory state. Combined effects of these characteristics increase the risk of atherogenesis and cardiovascular events.

It is important to stress that although each risk factor (see table 1.3) is an independent predictor of cardiovascular disease, these risk factors tend to cluster together in individuals, with visceral obesity and insulin resistance playing a central role (Aizawa et al 2005). A procoagulant state combined with dyslipidaemia for example markedly increases the risk of atherogenesis and cardiovascular events. In an insulin resistant state the increase in insulin also promotes atherogenesis (DeFronzo 1997). The increased risk results invariably in

increased morbidity and mortality from stroke and myocardial infarction (O'Brien and Dixon 2002).

1.3.2 Diabetes

Diabetes mellitus is a disorder of fuel metabolism, characterized by hyperglycaemia. There are two main forms of diabetes mellitus; type 1 (T1DM) and type 2 (T2DM). T1DM patients are unable to produce insulin, mostly consequent due to an autoimmune response that destroys the β-cells of the pancreatic islets of Langerhans. These patients require treatment with an exogenous source of insulin. Patients with T2DM typically develop progressive insulin resistance that is initially compensated by increased insulin concentrations before leading to impaired insulin secretion (Bailey 1999).

1.3.2.2 Type 1 Diabetes (T1DM, Juvenile onset/insulin dependent):

This form of diabetes is generally considered as a young person's disease because onset is usually in childhood. It is still unclear exactly what triggers autoimmune pancreatic β-cell destruction. Viral infections or other environmental factors may trigger an auto immune reaction in genetically susceptible individuals, via an inflammatory infiltration of the islets.

1.3.2.3 Type 2 diabetes (T2DM, adult onset/ insulin independent)

T2DM is a complex disease, resulting from a progressive imbalance in insulin secretion (β-cell dysfunction) and insulin sensitivity (insulin resistance) (Chiasson and Rabasa-Lhoret 2004). Approximately 95% of diabetics are type 2 and of those 90% are (or have been) overweight or obese (Albu and Pi-Sunyer HBO). The WHO predicts that this trend will continue and by 2020 the number of T2DM patients will have doubled (Waldhäusl 2003). At present more than

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3% of the population worldwide is estimated to have diabetes, which is about 190 million of the 6 billion people on earth, this number is set to rise according to the WHO prediction to 300 million (WHO 2003). In the past T2DM has most commonly been associated with people over the age of 40; however with the constant increase in prevalence of obesity throughout the population it is becoming progressively more common in children and young adults. The pathogenic factors involved in the development of T2DM are both genetic and environmental. Primary studies have discovered potential genes involved such as calpain-10 and other genetic hotspots have been found, these will not be elaborated here but have been succinctly reviewed by Leahy (2005). Environmental factors that predispose to T2DM include obesity, inappropriate diet (section 1.2.2.6c) and sedentary lifestyle all of which decrease glucose utilisation. Eventually it is IR and β-cell dysfunction that causes the downward spiral to T2DM, this is discussed in section 1.3.6.

In section 1.3.6 the progression from IGT to T2DM is discussed. 11% of patients diagnosed with IGT in the US progress to full T2DM annually (Leahy 2005). The extent to which insulin resistance is the cause or the result of β-cell dysfunction in T2DM, and the extent to which there are independent factors are still not clear. However, current opinion favours a duel-defect, neither preceding but indeed occurring simultaneously and perhaps having a genetic link (Leahy 2005). What is clear is the effects of IR and progression from IGT to T2DM can be reversed via weight loss and exercise, as corroborated by the Diabetes prevention program trials (DPP research group 2001); reducing IR and promoting glucose utilization, in essence rebalancing the adipo-insulin axis (Leahy 2004).

1.3.3 Dyslipidaemia

The plasma lipid profile of an individual is strongly determined by the level of expression of liver genes (apoliporoteins, lipoprotein lipase, lipoprotein receptors, hepatic lipase and lecithin:cholesterol acyltransferase (LCAT)) involved in lipid metabolism, synthesis, packing and export. Excessive glucocorticoid levels promote hyperlipidaemia and induce insulin resistance (Peeke and Chrousos 1995). Indirectly, glucocorticoids affect lipid metabolism via key transcription factors. PPARα is also the molecular target for the hypolipidaemic fibrate drugs (Krey et al 1997, Peters et al 1997). It has been demonstrated that 11β-HSD1 deficiency in mice opposes the pathogenic lipid and lipoprotein profile found in disease states. This is proposed to be due to increased lipid catabolism, reduced intracellular glucocorticoid concentrations and increased hepatic insulin sensitivity (Morton et al 2001). Obesity associated dyslipidaemia is inextricably linked to insulin resistance (Albrink and Meigs 1965, Howard et al 2003). The predominant dyslipidaemia associated with obesity is hypertriglyceridaemia; in which order the two disorders present is still controversial.

1.3.4 Procoagulant state

As previously mentioned in section 1.3.3 a dyslipidaemic profile in conjunction with other components of the metabolic syndrome induce proinflammatory changes which leads to atherogenesis (Howard *et al* 2003). Obesity increases risk of venous thromboembolism by about 2 fold (Abdollahi 2003). Obese individuals have higher levels of clotting factors VIII and IX but not fibrinogen, providing a procoagulant state that increases risk of deep vein thrombosis and embolism (Abdollahi 2003). Adipose tissue produces adipokines which influence the coagulative state. TNFα is associated with the stimulation of NFκ-B which in

turn enhances production of other cytokines creating an increasing spiral of inflammatory effects and increasing oxidative stress (Sonnenberg et al 2004)

In summary; the pro-coagulant state, often exacerbated by hypertriglyceridaemia occurs due to the presence of several abnormalities. These are summarised by Miller (1999):

1. Activation of endothelia cells, promoting production of thrombin and fibrin

2. LDL oxidation causing activation of macrophages

3. Predisposition of microthrombin via enhanced platelet aggravation

4. Activation of factor VI

5. Increase in factor IX, X and prothrombin

6. Increase concentrations of PAI-1 causing a decrease in plasma fibrinolytic activity

In addition to the above, adipose tissue expressed numerous other cytokines purported to be involved in low grade inflammatory response including TNFα, interleukins (IL) 1β, 6, 8, and 10, and IL-1 receptor antagonist and macrophage inflammatory protein 1α (Kershaw and Flier 2004, Gimeno and Klaman 2005).

1.3.5 Impaired Glucose Tolerance (IGT)

IGT is determined by measuring plasma glucose concentrations before and after a preset glucose load. IGT is closely linked to the development of T2DM and insulin resistance (IR). It is a component of metabolic syndrome and may predispose to other features of metabolic syndrome (Costa et al 2002). Individuals with IGT can manifest abnormalities in insulin secretion and insulin action, predisposing to future development of T2DM (Pratley and Weyer 2002)

Greater first phase response

Insulin secretion (y-axis)

Insulin sensitivity (x-axis): Less sensitive ... More sensitive

Legend:
— NGT
········· IGT
— — — Diabetes
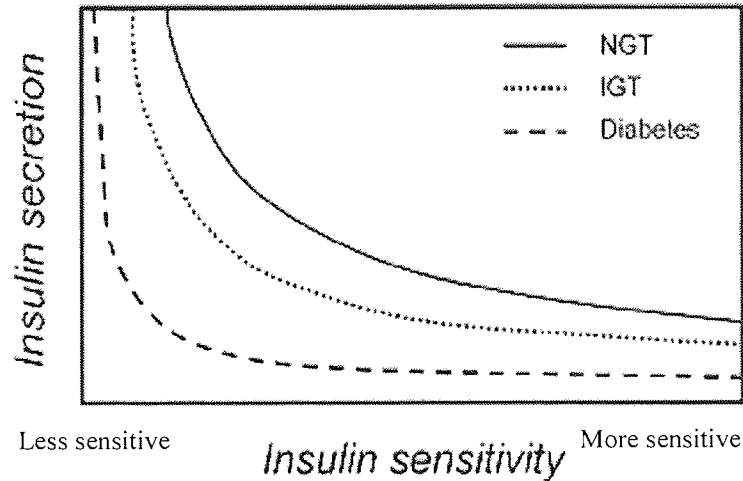
Figure 1.1: Insulin sensitivity

The hyperbolic relationship between insulin sensitivity and insulin secretion under differing glucose tolerance as adapted from Kahn et al 1993 and depicted in Chiasson and Rabasa-Lhoret 2004

IGT is proportionally correlated with insulin sensitivity and reciprocally correlated with insulin output. The latter correlation is weaker than the former (Chiasson and Rabasa-Lhoret 2004). It is the compensatory effect of insulin secretion for IR at a given plasma glucose level that has to remain in balance to keep glucose tolerance constant. IGT occurs when these compensatory processes can no longer counteract the effects of IR on plasma glucose (Chiasson and Rabasa-Lhoret 2004). However, to complicate medical intervention, IGT can present before measurable impairment of insulin secretion and IR.

1.3.6 Insulin resistance (IR)

IR is usually defined as impaired insulin-mediated glucose clearance into mainly muscle tissues (Leahy 2005) although it could equally indicate reduced insulin action on a range of

vascular and metabolic effects. IR appears to be the linking factor between the components of the metabolic syndrome. IR is profoundly associated with obesity and is regarded as the underpinning cause of the escalating incidence of T2DM in the overweight and obese (Bailey 1999). The cause of this resistance is multifaceted with no distinct metabolic imbalance taking priority (Kahn 2003). IR is correlated with the incidence and degree of IGT (see section 1.3.6) and is a marker for future incidence of T2DM. However, one of the major contributors to the increase in IR within the population is obesity, particularly visceral adiposity. There is a powerful positive correlation between visceral adiposity and IR which is reinforced by the inverse correlation seen with weight loss (Kahn 2003). Adipose tissue is normally highly insulin sensitive, but the effects of excess free fatty acids have a negative effect on insulin sensitivity by perpetuating an increase in plasma glucose via gluconeogenesis in the liver (Reaven 1995). Excess fatty acids contribute to IR through other 'lipotoxic' mechanisms considered later. It has been argued that increased visceral fat increases fatty acid flux via the portal vein to the liver thus triggering and compounding insulin resistance, and extension of this promotes development of IGT and T2DM (Kahn and Flier 2000, Reaven 1995, DeFronzo 1997). This adipo-insulin (Kahn and Flier 2000) is just beginning to be understood and the order of appearance of the metabolic symptoms related to IR, obesity and T2DM are yet to be resolved.

Further manifestation of insulin resistance in obesity and T2DM is promoted by a decrease in insulin-stimulated glucose transport and metabolism within target tissues including adipose tissue and skeletal muscle (Kahn and Flier 2000).

1.3.7 Metabolic Syndrome

In this project the metabolic syndrome and insulin resistance syndrome are referred to as if they are interchangeable, however there are subtle differences as outlined in a recent review by Reaven (2004).

Initially proposed by Reaven in 1988 in the Banting Lecture of the American Diabetes Association, the metabolic syndrome (insulin resistance syndrome, syndrome x) is characterised by a cluster of metabolic risk factors which are commonly found in the same individual. Recent criteria for inclusion in the syndrome are listed in table 1.4. The NCEP-ATPIII panel decided that classification of metabolic syndrome would be given to those presenting with 3 or more of the risk factors (Sorrentino 2005). The principal conditions associated with metabolic syndrome are T2DM, obesity, atherosclerosis, coronary artery disease (CAD) and cardiovascular disease (CVD). Although IR is regarded as the linchpin connecting these conditions, it is not listed in the clinical criteria as it is not readily quantified in routine clinical practice.

Table 1.4: Metabolic syndrome criteria

Factor	Parameter
Abdominal (Central) adiposity*	Waist circumference >102cm (men) >88cm women
Atherogenic Dyslipidaemia* (Hypertriglyceridaemia)	≥150 mg/dl (1.6955 mmol/L)
Raised blood pressure*	≥130/85 mm Hg
Low HDL-C*	< 40 mg/dl (1.03 mmol/L) (men) <50mg/dl (1.295mmol/L) (Women)
High fasting glucose*	≥110mg/dl (6.1 mmol/L)
Insulin resistance[ψ]	
Prothrombotic and preinflammatory state[ψ]	Increased fibrinogen, PAI-1, C-reactive protein and TNFα

* As proposed by the NCEP/ATPIII report. ψ considered but not recommended by the NCEP/ATPIII panel (adapted from Sorrentino 2005)

Most of the insulin resistance in obesity can be negated by weight loss and increased exercise. The literature suggests that the definition of metabolic syndrome is uncertain and changeable.

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The official definition has excluded some factors including microalbuminurea and some publications have considered inclusion of precariously correlated factors such as IGF (Laaksonen *et al* 2002, Ford *et al* 2005). However, the essentially agreed components include glucose intolerance, obesity, hypertension, dyslipidaemia and T2DM (Eckel *et al* 2005). It must be noted that obesity is both a cause and a result of metabolic syndrome which may cause some confusion.

The aetiology of metabolic syndrome although yet to be finalised is generally accepted as a manifestation of IR as a major, if not the major, causative influence. Alternative theories suggest hormonal change may be responsible for the development of IR increasing risk of abdominal obesity (Deen 2004), the hormonal imbalance being the result of age or stress. Nevertheless the incidence of metabolic syndrome is increasing in all age groups and ethnicities. It is estimated that there is an increase of 17% per year presenting with metabolic syndrome in the US alone (Ford *et al* 2002) and many more individuals only just fall short of the required criteria.

1.4 Adipose tissue

Adipose tissue is the body's largest store of reserve energy (Arnet and Eckel HBO). In the following chapter relevant aspects of its morphology, biochemistry, endocrine properties and role within the metabolic syndrome will briefly describe.

Adipocytes store energy as fat in the form of triglycerides. Triglycerides are acquired from the diet directly or synthesised from fatty acids and glycerol. The purpose of adipocytes is multifaceted acting as mechanical cushioning, thermal insulation, metabolic energy storage and an endocrine organ (Frayn 2003)

1.4.1 Morphology

Adipose tissue is comprised of four main cell types. Mature adipocytes, which store triglyceride, pre-adipocytes, which can be differentiated into mature cells, endothelial cells lining blood vessels infiltrating the adipose tissue mass and macrophages. Adipose tissue can be divided into two types; white adipose tissue (WAT), distinguished by its characteristic single lipid droplet and relatively small amount of cytosolic material which is forced to the edge of the cell by the lipid droplet. By contrast brown adipose tissue (BAT) has multiple lipid droplets and given its characteristic brown colour by the presence of numerous mitochondria providing a large capacity for oxidation.

In this thesis I shall be concentrating on the former of the two (WAT), however novel and exciting proposals have been discussed about the latter by Sell *et al* (2004).

1.4.1.1 White adipose tissue

White adipose tissue controls the synthesis (lipogenesis) and breakdown (lipolysis) of fatty acids; these are discussed further in section 1.4.2.1 and 1.4.2.2. Briefly, fat is stored as triglyceride and released as non-esterified fatty acids (NEFA). Due to its low oxygen consumption adipose tissue is sometimes described as metabolically inert; however fatty acid flux into and out of the adipocyte mass represents a large proportion of the body's energy metabolism.

1.4.1.2 Pre-adipocytes

Due to the extensive characterisation and availability of murine adipocyte cell lines, Primary Human pre-adipocytes (PHP) isolation and study is in its infancy. PHPs have low proliferative ability and there is a great decrease in differentiation capacity post sub-culturing (Darimont

and Mace 2003). For this reason little is known about the characteristics of these cells. *In vitro* the cells form a fibroblast-like monolayer: these can either self differentiate upon confluence or be induced using 'chemicals' such as IBMX, insulin and cortisol. In the literature, cells isolated from adipose tissue are also referred to as human mesenchymal cells, and adipose stromal cells (Kim *et al* 2005). These cells are described as 'multi-potent' and using different chemical and metabolic inducers have been differentiated into many lineages including bone cartilage, fat, muscle, neuronal, blood (Kim *et al* 2005), and hepatic (Seo *et al* 2005).

1.4.1.3 Formation

In vivo the extracellular matrix (ECM) of adipose tissue interconnects the cells to form fat cell clusters and fat lobules (Bailey 1978). Individual mature cells are spherical in shape both *in vivo* and *in vitro*. By contrast the pre-adipocytes containing no fat droplets conform to a fibroblast like shape and growth pattern *in vitro*. Pre-adipocytes form tiny pockets of cells within the adipose tissue mass and remain undifferentiated until required. This is considered in more detail in section 1.4.1.2.

1.4.1.4 Innervation and Vascularisation

Both sympathetic (adrenergic) and parasympathetic (cholinergic) stimulation affect adipose tissue lipolysis (Frayn 2003). Much of the preliminary work described in literature and conducted in this study has been carried out on rodents; little work to date has extended to humans. Previously it was thought that the sympathetic nerves terminated in the walls of the blood vessels, however, more recent studies show direct contact with adipocytes in addition to blood vessel innervation; this appears to be subject to regional differences. (Youngstrom and Bartness 1995, 1998).

1.4.2 Development, differentiation and deposition

Excess adiposity is determined by both increased volume (hypertrophy) of the adipocyte an increased number (hyperplasia) of adipocytes. Hypertrophy of adipocytes requires an imbalance of adipocyte metabolism; increased lipogenesis and/or decreased lipolysis. Hyperplasia occurs when there is an increase in cell acquisition versus cell loss. Cells are acquired via proliferation and differentiation of pre-adipocytes and mature adipocytes (see chapter 3) and cells are lost via apoptosis of both pre-adipocyte pools and mature adipocyte pools and possibly by a process of adipocyte de-differentiation (Prins 1997).

1.4.2.1 Development

Adipocyte formation begins before birth and expands rapidly after birth due to an increase in both cell number (hyperplasia) and size (hypertrophy) (Gregoire *et al* 1998). New cells can be acquired from fat cell precursors throughout life, and thus predisposing a nutritionally affluent society to excess weight gain. An increase in adiposity is determined by both hypertrophy and hyperplasia of cells. Hypertrophy of adipocytes occurs in periods of metabolic imbalance favouring increased lipogenesis and decreased lipolysis. Hyperplasia of adipocytes occurs when cell acquisition outweighs cell loss. Cells can be acquired via proliferation of pre-adipocytes, mature adipocytes and differentiation (Prins 1997). It is understood that the *in vivo* adipose lineage was derived from an embryonic stem cell precursor and thus can potentially differentiate into a number of cell types (see section 1.3.1) such as adipocytes, chondrocytes, osteoblasts and myocytes (Gregoire *et al* 1998).

1.4.2.2 Differentiation

Over the last 20 years, *in vitro* cell systems have been utilised. Of the cell lines mentioned 3T3-F442A and 3T3-L1 cell lines; clonally isolated from Swiss 3T3 cells derived from disaggregated 17-19 day mouse embryos (Green and Kehinde 1979; Green and Meuth 1974) are the most frequently employed.

Stem cell   Adipoblast   Preadipose   Immature adipose cell   Mature adipose
(multipotent)   cell   cell
                                            (containing small lipid
                                                droplets)   (lipid filled)
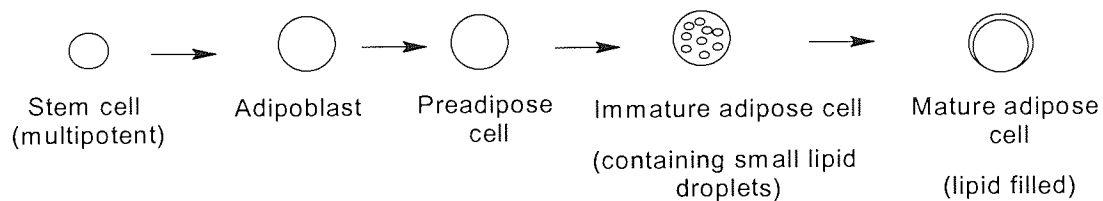
Figure 1.2 Adipocyte development (modified from Gregoire *et al* 1998)

Cell development is shown in figure 1.4; this outlines the progression by which an adipocyte develops *in vitro*, which closely relates to its progression *in vivo* (Ailhaud and Hauner 1998). The accumulation of lipid droplets into the cell instigates the morphological change to spherical shape; simultaneously the latter stages of differentiation initiate a change in gene expression, the products of which are discussed in section 1.3.5.

Differentiation has numerous triggers which are not all completely understood. *In vitro* it is usually induced using a cocktail of chemicals including IBMX and insulin. *In vivo* there are an array of differentiation-inducing molecules including insulin, NEFA and cortisol. Cortisol has been deemed essential for adipocyte differentiation (Hauner 1987) introducing the importance of the regulatory enzyme 11B-hydroxy steroid dehydrogenase (11B-HSD) since this enzyme is pivotal in my research it is discussed in detail in chapter 6.

Once committed, the pre-adipocyte maintains capacity for growth but must resign from the cell cycle prior to maturation allowing the acquisition of the adipocyte phenotype. Cells pass through four stages of growth and differentiation: growth arrest, clonal expansion, alterations

in gene expression and terminal differentiation are elegantly expanded by Gregoire *et al* (1998) and will not be depicted further here.

After terminal differentiation the cell functions as a lipid store and a source of adipokines. The metabolic balance of NEFA in the plasma is primarily controlled by adipose tissue via lipogenesis and lipolysis (see section 1.3.3).

1.4.2.3 Deposition

Adipose tissue is deposited throughout the body; viscerally and more peripheral depots under the skin (subcutaneously). These two main depots are further defined by region in the body and proximity to organs. Each region may have both subcutaneous and visceral deposits, for example glutofemoral depots are situated around the thighs, buttocks and hips. This deposition of fat is usually subcutaneous and gives a classic 'pear' shaped figure. In contrast abdominal or central deposits, usually viscerally, give an 'apple' shape (Albright and Stern 1998). In both cases the deposition can be both visceral and subcutaneous. Subcutaneous fat, situated between the dermis and the muscle, particularly at the extremities provides an insulatory role preventing heat loss. Visceral fat situated around the organs also provides protection against heat loss and provides a shock absorbing function for organs during body movement. Both depots reduce damage from bruising caused by external influences. In addition to its structural role; visceral and subcutaneous depots provide paracrine, endocrine, cytokine and adipokine cellular interaction molecules (see section 1.3.5).

Excess visceral adipose tissue is closely associated with deleterious medical effects of metabolic syndrome, including cardiovascular disease and T2DM. When visceral adipose tissue depots are reduce via either surgery or diet and exercise the risk of these effects are also reduced and sometimes can be reversed (e.g. T2DM). However, the removal of subcutaneous

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adipose tissue does not confer the same metabolic benefits (Klein 2004). There is a wealth of *in vivo* human evidence demonstrating that there is substantially greater triglyceride turnover in visceral adipose tissues when compared with peripheral adipose tissue (Jensen 1997). There is a greater lipolytic response to chatecholamines in visceral tissue, increased numbers of adrenergic receptors and decreased response to α_2-adrenergic receptor agonists (Montague 2000). Thus visceral tissue depots are programmed to have a greater capacity for lipolysis. In addition it would seem that due to this drive for fatty acid turnover, visceral depots are more susceptible to insulin resistance; showing less affinity for insulin than their subcutaneous counterparts. Therefore it would appear that the two depots have defined metabolic functions which differ from one another. One hypothesis of adipose depot control mechanisms was proposed by Freedland (2004). He suggested that there was a critical visceral adipose tissue threshold (CVATT). The threshold is unique to each individual, representing the point at which metabolic dysfunction ensues with an increase accumulation of fat mass in the visceral depot. It proposes that the threshold is breached at the onset of insulin resistance which will have developed to protect cells with impaired fatty acid oxidation. The insulin resistance allows the cells to oxidise fat already within the cells and prevent damage by lipotoxicity (Unger 2003, Eckel 2003). This approach would explain why some extremely obese individuals do not exhibit symptoms of metabolic syndrome and why metabolically obese individuals with normal weight (MONW) exhibit the detrimental effects associated with metabolic syndrome (Karelis *et al* 2004).

1.4.3 Biochemistry

1.4.3.1 Lipogenesis

White adipose tissue stores lipid from the circulation. An increase in stored lipid occurs when non-esterified fatty acid are transported into the blood via the lipoproteins mainly chylomicrons, very low density lipoproteins (VLDL) and low density lipoproteins (LDL). The transporter lipoproteins interact with lipoprotein lipase (LPL). LPL located on the surface of the adipocyte is particularly important for plasma fatty acids to be taken up by adipocyte. Albumin-bound non-esterified fatty acids are also present in the plasma, usually at a concentration of 100-1000 μmol/L for transit to adipose tissue. In order for this transfer occurs the unesterified component is dependent on a 'carrier' protein. The LPL is stimulated by increased plasma insulin. Inside the adipocyte the components of triglyceride are reassembled via esterification usually through the phosphatidic acid pathway. The re-esterified triglycerides (TG) are added to the lipid droplet in the cell within the perilipin membrane.

1.4.3.2 Lipolysis

Lipolysis is the mobilisation of triglycerides. This process is regulated by hormone sensitive lipase (HSL). Increase HSL is mainly regulated by adrenaline and noradrenaline. Although cAMP controls HSL phosporylation, insulin reduces HSL and so reduces lipolysis. Upon stimulation at low insulin concentrations inactive HSL is activated via phosphorylation and translocated from the cytosol to the lipid droplet surface. The perilipin coating the droplet is phosphorylated and peels away exposing the lipid store allowing HSL access. HSL catalyses the hydrolysis of esterbonds of two fatty acids to form Diacylglycerol (DAG) and monoacylglycerol. Monoacylglycerol lipase (MAGL) is responsible for hydrolysing the 3rd

fatty acid. When completely hydrolysed triglyceride yields 1mole of glycerol to 3moles of free fatty acid (FFA).

After hydrolysis the fatty acids can be either re-eserified, undergo beta-oxidation or be released for use as energy substrates by skeletal muscle, cardiac muscle and liver (Albright and Stern 1998). NEFA are bound to albumin for transportation to tissues.

The above is a simplified account of lipid mobilisation. Many other genes and proteins are involved with regulation and liberation of fatty acids most will be discussed further in section 1.3.5

1.4.3.3 *De novo* lipogenesis

In humans *de novo* lipogenesis (DNL) rarely occurs and in general would not contribute greatly to the overall metabolism. Nevertheless, it may greatly impact on the metabolism in instances of over-feeding on a high carbohydrate diet (Frayn *et al* 2003) and the adipocyte may be the primary site of action. For this reason I shall briefly outline its process here.

De novo lipogenesis is the synthesis of lipid from sources other than fatty acid and triacylglycerol, in particular glucose. Fatty acids are synthesised from acetyl-CoA which is derived from glucose taken into the cell and converted by PDH in the mitochondria. The acetyl-CoA is converted to malonyl-CoA in the presence of the rate limiting enzyme Acetyl-CoA carboxylase (ACC) and insulin and finally into fatty acid by fatty acid synthase. This occurs in the same way in the liver. Much debate over the accountability to the total lipid pool by *de novo* lipogenesis on constant over-feeding on a high carbohydrate diet still exists but position of the majority supports its irrelevance to over all metabolism.

1.4.3.4 Free Fatty Acids (FFA)

FFA concentrations of plasma increase exponentially with fat mass and therefore create an increased flux of FFA to non-adipose tissue (Robinson *et al* 1998). This increase in FFA concentration is especially detrimental from the visceral depots. Located in the central or abdominal region, the visceral fat depot has increased fatty acid turnover due to an increased sensitivity to catecholamine β-AR stimulation and less sensitive to the anti-lipolytic effects of insulin. This fat depot has a direct link to the liver via the hepatic portal vein and so excess FFA flux caused by visceral adiposity results in an accumulation within the liver. Despite the fact that only an estimated 10% of fat is visceral, 80% of the blood supply to the liver is delivered via the hepatic portal vein (Campra and Reynolds, 1982). These increased concentrations of circulating FFA are diverted toward other organ tissues including the heart, muscle, liver and pancreatic β-cells. Exaggerated effects of which have been observed in animal models with congenital lipoatrophy, in which TGs accumulate massively in the tissues provoking manifestation of consequences associated with extreme insulin resistance (Gavrilova *et al* 2000).

1.4.3.5 Lipotoxicity hypothesis

The theory states that insulin resistance develops due to the build up of excess lipid in insulin-sensitive cells other that adipocytes, most predominantly liver and muscle cells. This atypical storage of lipid inhibits the action of insulin, the precise mechanism by which this transpires is unknown although molecules suspected to play an important role include fatty acyl-CoA, diacylglyceral and protein kinase C (Friedman 2002). Support for this theory arises from lipodystrophic patients, whose absence of fat storage capabilities results in the build up of

lipid in the non-fat storing cells of the liver and skeletal muscle. The major effect of the disease is a profound insulin resistance as would be expected in lipotoxicity.

1.5 Control of energy homeostasis

The body must maintain energy homeostasis, providing adequate storage of both short term and long term energy 'stores' to cope with the body's demand over time. Maintenance of energy homeostasis is controlled centrally by the brain and peripherally by the adipocytes, pancreas and liver.

Adipose tissue secretes many regulatory cytokines influencing the metabolic activity of cells at many sites including the brain, liver and skeletal muscle (Mohamed-Ali *et al* 1998). Biochemical signalling molecules produced by adipose tissue include TNFα, Leptin, IL-6, FFA and acylation stimulating protein (ASP), adipsin, apolipoprotein lipase, retinol binding proteins, angiotensinogen, transforming growth factor β (TGFβ), type-1 plasminogen activating factor (PAI-1) and agouti protein (Rehman, 2000).

Energy levels are tightly regulated in the body and information regarding level of stores is relayed by insulin and leptin; insulin acts as a signal of carbohydrate repleteness whilst leptin acts as a signal of lipid store level (Frayn 2003).

1.5.1 Central control

In simple terms there are two systems governing the regulating the quantity of food intake; short term and long term regulation. Short term regulation prevents excessive intake during meals via the signalling of gut peptides such as cholecystokinin and ghrelin to the brain. Long term regulation manages the body's energy stores; glycogen and fat through mediators including leptin and insulin (Konturek *et al* 2004, 2005).
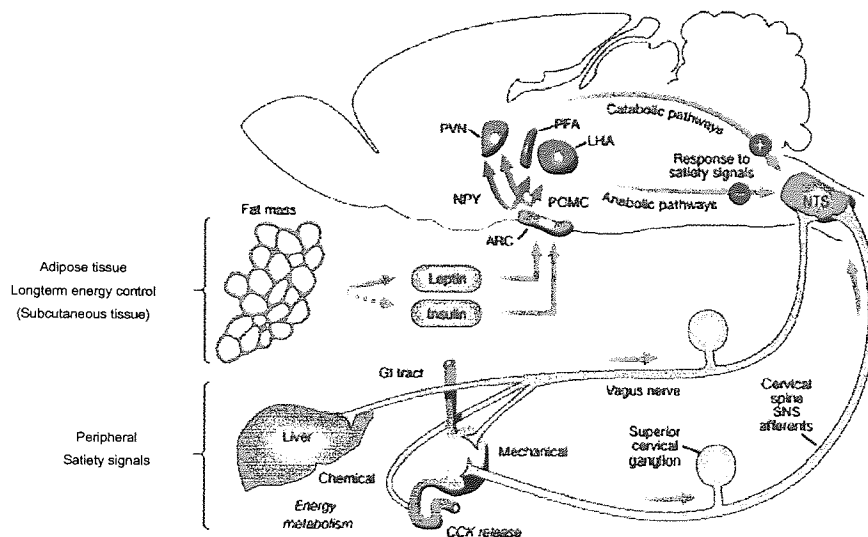
Figure 1.3 Central and peripheral control of energy homeostasis.

(Adapted from Woods *et al* 2004) List of abbreviations: Hypothelamic arcuate nucleus (ARC), Gastrointestinal (GI), Cholecystokinin (CCK), Nucleus of the solitary tract (NTS), Sympathetic nervous system (SNS), Paraventricular nerves (PVN), Lateral hypothalamic area (LHA), perifornical area (PFA), propiomelanocortin (POMC), Neuropeptide Y (NPY)

Most mechanisms controlling body energy stores encourage weight gain rather than loss (Druce *et al* 2004). The peripheral signals act centrally mainly in the hypothalamus integrated in the arcuate nucleus, in which there are two subsets of neurons those which stimulate (orexigenic) and those which inhibit (anoreigenic) food intake. Orexigenic peptides include neuropeptide y (NPY), agouti-related protein (AGRP) and Melanin concentrating hormone (MCH). Anorexigenic peptides include α-melanocyte-stimulating hormone (α-MSH), cocaine and amphetamine-regulated Transcript (CART) and corticotrophin-releasing hormone (CRH) (English and Wilding 2002).

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1.5.1.1 Orexigenic peptides

NPY is a 36 aminoacid peptide found in the hypothalamus. It is expressed in the hypothalamic arcuate nucleus (ARC) which is adjacent to the base of the 3rd ventrical and has connections with the paraventricular nucleus (PVN) (Frankish *et al,* 1995). This area is known to be important in the control of hunger and satiety (Stanley, 1986) NPY is a potent stimulator of food intake, with a preference for carbohydrate intake (Bray 2001). Increases in NPY occur when leptin levels decrease leading to a positive energy state resulting in an increase in food intake and stimulation of lipogenic enzymes for fat storage in WAT (Hillebrand *et al* 2002). AGRP is a 132 amino acid neuron signalling molecule and is inhibited in the presence of leptin (Hillebrand *et al* 2002). Over expression of AGRP in mice creates an obese phenotype, which can be augmented by co-administration of NPY. In addition AGRP is known to be involved with food selection specifically enhancing the intake of high fat dietary products (Hillebrand *et al* 2002).

Melanin concentrating hormone (MCH) plays an important role in food intake behaviour. It is considered to control feeding by acting downstream of leptin and melanocortin pathways. Studies show that intracerebroventicular injections of MCH induce hyperphagia and decreasing energy expenditure (Ludwig *et al,* 2001).

Table 1.5 Hormones, neurotransmitters and peptides involved in energy balance and obesity

Molecule	Effect on food intake
Neuropeptide Y (NPY)	Increase
5-Hydroxytryptamine (5HT)	Decrease
Melanocortin hormone (MCH)	Increase
Corticotrophin releasing factor (CRH)	Decrease
Galanin (GAL)	Increase
Orexerin	Increase
Propiomelanocortin (POMC)	Increase

1.5.1.2 Anorexigenic peptides

Melanocortin (MC) peptides are cleaved from the precursor molecule proopiomelanocortin (POMC). The POMC gene encodes for a 36-kDa pre-prohormone (MacNeil *et al* 2002). Seven mature peptide hormones (see table 1.6) are derived from the POMC gene via post translational cleavage by several hormone convertases. The effects of the melanocortins are mediated by the 5 MC receptors, of which MC3 and MC4 receptors are found in the brain. The MC4R holds high affinity for α-MSH an increase of which results in decreased food intake and increased energy expenditure (Hillenbrand *et al* 2002).

Table 1.6 Mature peptide hormones cleaved from the translational product of the POMC gene

Melanocortin	Length of peptide (aminoacids)	Brain region of processing
Adrenocorticotropic hormone [ACTH]	39	Anterior lobe of pituitary
α-melanocyte-stimulating hormone [α-MSH]	13	Intermediate lobe
β- melanocyte-stimulating hormone [β-MSH]	12	Processed from β-lipotrophin
γ-melanocyte-stimulating hormone [γ-MSH]	12	Intermediate lobe
Corticotropin-like intermediate lobe peptide [CLIP]		
β-lipotrophin		
β-endorphin		

In humans mutations of the MC4R result in an obese phenotype (Yeo *et al* 1998). CART is a 116 amino acid which is spliced to form two smaller peptides of varying length which are packaged to form neurotransmitting vesicles (Thim *et al* 1998). Chronic central administration of CART results in a decrease in food intake and subsequent decrease in body weight. CRH is a 41 amino acid peptide known for its role on the HPA axis. The effects of CRH are mediated by G-protein coupled receptors (GPCR). CRH acts down stream of leptin and NPY to decrease food intake and increase energy expenditure and lipolysis (Hillenbrand *et al* 2002).

1.5.2 Peripheral Control

Peripheral control of energy is provided by numerous hormones and cytokines such as insulin and leptin. In addition to this control there are various cytokines that influence metabolism as described below.

1.5.2.1 Insulin

Insulin is produced by the pancreas, specifically the β-cells of the islets of Langerhans. It is required for the regulation of carbohydrate, lipid and protein metabolism (Leong and Wilding 1999). In the liver, insulin inhibits gluconeogenesis and glycogen breakdown, mediates glucose uptake and promotes glycogen storage. In fat, insulin mediates glucose uptake and prevents lipolysis whilst promoting lipogenesis to form the fat storage molecule, triglyceride. In muscle insulin cause glucose uptake for use in glycolysis to supply energy to the cells.

An important role of insulin is to prevent lipolysis and thus the release of NEFAs. Lipolysis is sensitive to insulin and can be switched off even at basal levels of insulin in normal individuals. In obese patients NEFA levels are elevated and in T2DM patients this is accompanied by hyperglycaemia. This causes impaired glucose metabolism by reducing insulin stimulated glucose uptake, especially in skeletal muscle (Bonadona *et al* 1990). Increased NEFA levels in the liver interfere with the glucose-fatty acid cycle (Randle cycle: Randle *et al* 1963) increasing hepatic glucose output. Also NEFA stimulate insulin secretion, consequently obesity could be considered to be predisposing to T2DM. Over stimulation of insulin by elevated NEFA can ultimately cause β-cell failure by exhaustion. It has been suggested that enlargement of adipose tissue mass may protect against insulin resistance and diabetes in some circumstances. This "sink" action of adipose tissue (Frayn 2002) as a form of protection of other tissues from toxicity is supported by over expression of GLUT-4 in adipose

tissue which occurs with increased adipose tissue mass and an over all increase in insulin

sensitivity (Shepard *et al* 1993; Tozzo *et al* 1997).

1.5.2.2 Leptin

In 1953 Kennedy proposed the presence of a circulating factor that was generated in

proportion to adipose tissue and that could influence the appetite and energy expenditure. In

1973 Coleman provided the evidence of this factor and in 1994 it was cloned by Zhang *et al*

(1994) from the ob gene and was found to be a 16kDa protein. It was called Leptin and is

sometimes referred to the ob protein. It is expressed in white adipocytes, the stomach, placenta

and the mammary glands (Van Gaal 1999).

Leptin acts as part of a feedback mechanism proposed to been an internal mechanism to

prevent obesity. It functions as a "lipostat" providing information to the brain; during periods

of increased energy intake leptin is increased and in periods of decreased energy and

starvation leptin levels are decreased.

There are 2 mutations associated with the ob gene. The first causes a truncation of the ob

protein leaving it inactive and the second abolishes leptin gene transcription. These mutations

in the homozygous form, lead to the now classical *ob/ob* phenotype. Homozygous mutations

in the receptor gene lead to early onset obesity, absence of pubertal development, dysfunction

of growth and thyroid axes hormone activity (Clement *et al* 1998). In rodent models the db/db

mouse has a mutation in the leptin receptor resulting in its inability to bind leptin and thus is

unaffected by an exogenous source. Leptin binds to its transmembrane receptor causing

dimerisation and subsequent cells signalling (Rehman, 2000).

In *ob/ob* mice obesity models the leptin signal is absent or defective resulting in the perception

that fat mass is low and subsequently hyperphagia, reduced energy expenditure and weight

gain. When fat mass is increased circulating levels of leptin also increase (Guerre-Millo 1999). Leptin concentrations show a diurnal pattern and tend to be higher in women than in men regardless of adiposity (Rosenbaum *et al* 1996).

Leptin is commonly regarded as a satiety factor due to its effects on food intake. However, other effects of leptin have been documented such as; stimulatory effects on energy expenditure, effects of leptin within the reproductive system correlate with infertility and promotion of sexual maturation in young mice; inhibition of insulin secretion and stimulation of glucose utilisation; and simulation of lipolysis in adipocytes (Trayhurn *et al* 1999). Interestingly glucocorticoids stimulate leptin production whilst insulin sensitizers (thiazolidinediones), along with noradrenaline and isoproteronol reduce circulating leptin levels via decreased ob gene expression.

1.6 The "Secretome": Adipokines and enzymes

White adipose tissue was formerly regarded as a passive store for tryglycerides to provide energy in during starvation and diurnal periods of fasting. However, since the discovery of leptin is has emerged as a highly active endocrine organ providing regulatory physiological signals and pathologic processes of inflammation and immunity (Fantuzzi 2005).

There are over 50 different protein molecules (adipokines) secreted by the white adipocyte in their entirety they are termed the 'adipokinome'. In addition to these there other lipid and enzyme moeties secreted, together these form the adipocyte 'secretome' (Trayhurn and Wood 2004, 2005). In the following section a selection of these adipokines and enzymes have been discussed, further information can be found in a recent review by Trayhurn (2005).

1.6.1 Enzymes of triglyceride synthesis

1.6.1.1 Stearoyl-CoA desaturase 1 (SCD1)

SCD is an endoplasmic reticulum enzyme which catalyses the biosynthesis of monounsaturated fatty acids (MUFA) from saturated fatty acids (Dobrzyn and Ntambi 2005). In knock out experiments SCD1-deficient mice show a 50% decrease in epididymal fat pad weight and a subsequent signal directs fatty acid into the oxidation pathway in preference to synthesis (Ntambi et al 2002). Further investigation by the Ntambi laboratory (2004) proposed SCD1 to be an important control point in lipogenesis. In the absence of SCD1 mice are resistant to diet-induced obesity, have decreased liver triglyceride accumulation and show a reduction in post-prandial plasma insulin and glucose levels. This is contemporaneous with an increase in metabolic rate, thermogenesis and insulin sensitivity (Ntambi and Miyazaki 2004, Dobrzyn and Ntambi 2004) resulting in a reduction in body adiposity.

1.6.1.2 Glycerol-3-phosphate acyltransferase (GPAT): EC 2.3.1.15

GPAT catalyses the initial step of triglyceride synthesis (figure 1.5): acylation of glycerol-3-phosphate with long chain fatty acyl–CoA to form 1-acyl-glycerol 3-phosphate (LPA). There are three isoforms of GPAT: mircosomal GPAT ($_{er}$GPAT) and mitochondrial GPAT ($_{mt}$GPAT1 and $_{mt}$GPAT2), present in the endoplasmic reticulum and outer mitochondrial membrane respectively (Lewin *et al* 2003). Activity between the two isoforms differs by sensitivity to sulfhydryl group reactive reagents (e.g. N-ethylmaleimide (NEM)) which inhibits only the $_{er}$GPAT (Linden *et al* 2004). Null mice studies show that $_{mt}$GPAT1 is associated with obesity and dyslipidaemia. Mice lacking the gene have reduced body weight caused by both a reduction in body fat and hepatic triglyceride content (Hammond *et al* 2002).

Figure 1.4: Triglyceride synthesis; (adapted from methods in enzymology; Lipids part C, edited by John Lowenstein, 1981.)

Glycerol 3-Phosphate (G-3-P)

$\downarrow$ **GPAT**

Lysophosphatidic acid (LPA)

$\downarrow$

Phosphatidic acid (PA)

$\downarrow$

Diacylglycerol (DAG)

$\uparrow \downarrow$ **DGAT**

Triacylglycerol (TAG)
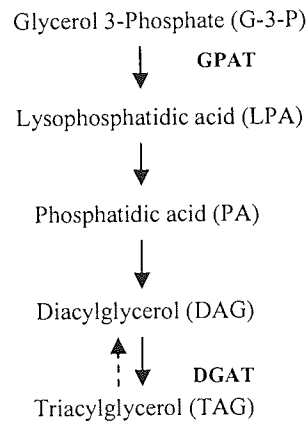
This is consistent with studies showing a general increase in mRNA, protein and activity of $_{mt}$GPAT1 in the liver and adipose tissue with carbohydrate feeding and insulin stimulation, this occurs in the absence of $_{er}$GPAT activity change (Lewin *et al* 2004). The second variant of mitochondrial GPAT is relatively new; $_{mt}$GPAT2 is located with $_{mt}$GPAT1 in the mitochondia but its enzymic properties differ. It is inhibited by NEM and is temperature sensitive in direct contrast to $_{mt}$GPAT1. Further information regarding this variant is outlined in Lewin *et al* (2004).

1.6.1.3 Diacylglycerol acyl-transferase (DGAT): EC 2.3.1.20

DGAT is a microsomal enzyme and is important in the metabolism of glycolipids (Bell and Coleman 1980, Lehner and Kulsis, 1995), providing the terminal step (figure 1.5) in triglyceride synthesis (Chen *et al* 2002). It forms a homotertramer of which the biochemical significance of the formation is still unknown (Cheng 2001).

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In the late 1990s it became apparent that there were two DGAT genes. DGAT1 is expressed in all tissues examined and is in relative high abundance in the small intestines indicating it may have an important role in intestinal fat absorption (Meegalla et al 2002). In addition the activity of DGAT in triglyceride synthesis and energy storage suggests it may be involved in lipoprotein assembly and the regulation of plasma triacylglycerol concentrations (Bell and Coleman 1980; Haagsman and Van Golde, 1982), fat storage in adipocytes (Coleman and Bell, 1976), energy metabolism in muscle (Swanton and Saggerson, 1997). As DGAT is currently the only known enzyme to catalyze the final step in the synthesis of triacylglycerol it is an ideal candidate for therapeutic manipulation. It belongs to the acyl-CoA: cholesterol acyltransferase (ACAT) gene family. In the glycerol phosphate pathway, acyl-CoA is esterified to glycerol-3-phosphate in two consecutive steps to form lysophosphatidic acid and the phosphatidic acid. This is hydrolyzed to form diacylglycerol which is one of the substrates for DGAT. Diacylglycerol is subsequently acylated to form TAG by DGAT.

DGAT2 is a member of the MGAT1 family of genes that are unrelated to that of DGAT1 by sequence homology. It is more hydrophilic than DGAT1, is membrane bound and is found on the endoplasmic reticulum and may be found on the organelle membranes.

1.6.2 Glucocorticoids

Glucocorticoids regulate innumerable homeostatic and metabolic processes and mediate the response to stress (Harris, 2001). Diurnal signals and stress activate the hypothalamic-pituitary-adrenal (HPA) axis, which in turn causes the release of glucocorticoids. In response to neural signals the hypothalamic paraventricular nuclei (PVN) release corticotrophic releasing hormone (CRH) into the median emmenes capillary network, subsequently inducing adrenal corticotrophic hormone (ACTH) release from the anterior pituitary. ACTH stimulates

adrenal corticoid secretion from mainly the zona fasciculata of the adrenal cortex. Glucocorticoids act via 2 intracellular receptors: glucocorticoid receptors (GR) and mineralocorticoid (MR) receptors. In adipose tissue, glucocorticoids act via GR to increase lipolysis and decrease glucose uptake, opposing the action of insulin (Walker 1999). Normally glucocorticoid secretion is regulated by balancing the HPA axis forward drive and the glucocorticoid negative feed-back. A chronic imbalance in control causes either Cushing's syndrome (excess) or Addison's disease (deficiency).

Cushing's syndrome is characterised by high blood concentrations of cortisol and is typically associated with hyperglycaemia, muscle wastage and a redistribution of fat atypical sites (cheeks, upper back and trunk) (Stewart 2005) causing development of reversible central obesity. Interestingly patients with diet/lifestyle-induced "simple" obesity plasma cortisol levels are normal or slightly reduced (Fraser *et al* 1999). Treatment for Cushing's results in almost complete reversal of the clinical symptoms, thus research into the reversal of this altered cortisol state or manipulation of the enzymes involved in the regulation of glucocorticoids could provide possible targets for novel drugs and could potentially ameliorate symptoms associated with the metabolic syndrome. Individuals with excess plasma levels of glucocorticoids exhibit glucose intolerance and insulin resistance (Andrews *et al* 2002, Kerstens and Dullaart 2003) and increased upper body obesity. In addition it has been proposed that relative leptin resistance may also be induced by glucocorticoids (Zakrzewska *et al.* 1997). Targeting the HPA axis to manipulate the cortisol level would induce a wide array of side effects, producing an effect equivalent to symptoms of Addison's disease. Fortunately due to variations in responses to glucocorticoids that are tissue-specific, a local cellular control mechanism such as 11β-HSD1 could be targeted (Walker 1999).

1.6.2.1 11β-HSD [EC 1.1.1.146]

There are two enzymes currently known that convert inactive cortisone to active cortisol in humans. 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2) inter-convert active and inactive glucocorticoids. Therefore, these two enzymes play a major part in the control of glucocorticoids levels presented to the corticosteroid receptors in target tissue cells.

Figure 1.5 Human glucocorticoid conversion.

$$ \text{Cortisone} \underset{\substack{\text{[dehydrogenase]}\\ \text{11B-HSD2}}}{\overset{\substack{\text{11B-HSD1}\\ \text{[oxoreductase]}}}{\rightleftarrows}} \text{Cortisol} $$
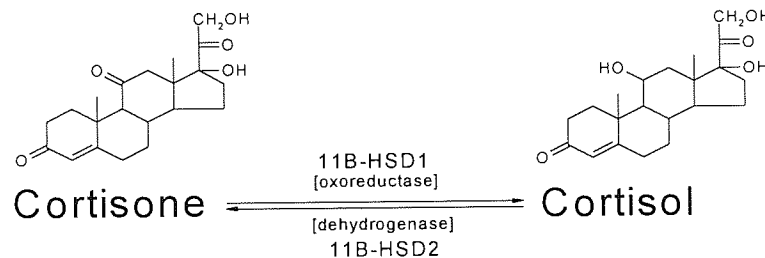
Figure 1.6 Mouse glucocorticoid conversion

$$ \text{11-dehydrocortisone} \underset{\substack{\text{[dehydrogenase]}\\ \text{11B-HSD2}}}{\overset{\substack{\text{11B-HSD1}\\ \text{[oxoreductase]}}}{\rightleftarrows}} \text{corticosterone} $$

11β-HSD1 is a bidirectional enzyme. *In vivo* the reductase activity predominates, however in tissue homogenates only the dehydrogenase activity is observed (Tomlinson *et al* 2002). 11β-HSD1 is generally thought to act as a reductase, converting inactive 11 keto-derivatives in to active glucocorticoids. In knock-out mice lacking the 11-reductase activity, there is an inability to create active glucocorticoids above that of circulating corticosterone levels, therefore preventing negative

feedback and adrenal gland hypertrophy as a compensatory effect. This compensation will result in increased circulating corticosterone levels (Kotelevtsev *at al*. 1997). The hypertrophy and hyperplasia of the adrenal gland was isolated to the cortex due to the compensatory over stimulation of corticosterone. Increased stimulation of corticosterone caused an alteration in the regulation of the HPA axis. Normally the glucocorticoids self regulate predominantly via a negative feedback loop, however this feedback is absent in 11β-HSD knockout mice. Thus renders the mice unable to compensate for 11β-HSD1 activity loss when regulating the HPA axis (Holmes *et al* 2001). The enzymes phospho-enolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are the catalysts for the rate limiting step of gluconeogenesis. The encoding genes are regulated by classical glucocoticoid inducible promoters (Pilkis and Granner 1992) and induced upon fasting. In starvation of wild type mice, there is substantial induction and activity increase of both G6Pase (152%) and PEPCK (296%) in the liver. However, in 11β-HSD1 deficient mice G6Pase is not reduced and there is a reduction of PEPCK stimulation following starvation. It is proposed that as the glucocorticoids cannot be converted by 11β-HSD1, they also cannot regulate the induction of the gluconeogenic genes. PEPCK activity appears less affected due to alternate regulation pathways involving cAMP response elements (Imai et al 1990), seen in 11β-HSD knock out mice (Cole *et al* 1995). In stressed conditions lower glucose levels were noted in 11β-HSD -/- mice on high fat diets even though similar body weight had been gained. Thus, it appears 11β-HSD -/- mice are more resistant to hyperglycaemia under dietary stress or obese situations.

1.6.3 Adiponectin

Adiponectin (Acrp30, GBP28, apM1 and AdipoQ) was discovered independently by four different groups (Fasshauer *et al* 2004, Koerner *et al* 2005). Adiponectin is of the collagen superfamily and secreted by adipocytes (Arita 1999). It is a 3kDa monomer protein consisting of 247 amino acids. The monomeric subunits combine to form oligomeric complexs which can differ in size from trimers up to oligmers in a rose formation (Koerner *et al* 2005) which have recently been shown to use up to 36 subunits. In addition it has been suggested that the number of subunits may be essential for some of its biological effects (Tsao *et al* 2003). Adiponectin is exclusively expressed in mature adipocytes during adipogenesis (Fasshauer 2001); interestingly decreased levels of adiponectin are associated with obesity, insulin resistance, T2DM and cardiovascular disease (Matsuzawa et al 2004). Adiponectin is known for its role in improving insulin sensitivity and is increased in the presence of insulin sensitising agents such as thiasolidinediones (TZDs), thus could provide a novel target for manipulating in obesity induced T2DM. In addition adiponectin has shown potential inhibitory effects on initial factors promoting atherogenesis, thus preventing cardiovascular events. Individuals with increased visceral adiposity have reduced adiponectin levels and increased plasma lipoprotein thus resulting in a highly atherogenic environment (Matsuzawa et al 2004).

1.6.4 G protein coupled receptor 41 (GPR41)

The GPR41 receptor family are activated by medium (GPR40) and short chain fatty acids (SCFA, GPR41-43) (Brown *et al* 2002). Studies show that stimulation of adipocytes with SCFA (*in vitro* and *in vivo*) increases the production of leptin and may serve as one of the control mechanisms regulating leptin production (Xiong *et al* 2003). The exact function and

location of these receptors are yet to be elucidated. In chapter 6 preliminary investigation of GPR41 expression in adipose tissue is explored by RT-PCR.

1.6.5 Tissue necrosis factor α (TNFα)

TNF is a 26K-Da transmembrane pro-hormone that is cleaved proteolytically to form the 17KDa soluble TNFα molecule. Stimulation by TNFα directly alters glucose homeostasis and lipid metabolism, as well as being involved in the development of insulin resistance. The role within lipid metabolism exhibited by TNFα occurs at three biochemical sites; fatty acid uptake, lipogenesis and lipolysis. Each of these is regulated by extracellular stimuli such as insulin, cortisol, chatecholamines, growth hormone, testosterone, free fatty acids (FFA) and cytokines (Ramsay, 1996). Elevated TNFα can induce insulin resistance in obesity (Hotamisligil et al, 1995; Uysal et al, 1997; Hotamisligil et al, 1994) through over expression in adipose tissue and muscle. In obese patients TNFα is elevated and may contribute to elevated basal lipolysis levels in adipocytes (Ramsay, 1996). TNFα stimulated leptin release from adipocytes may account partially for the adipostat mechanism that regulates circulating leptin concentrations with respect to triglyceride stores by post transcriptional mobilization of preformed pools of leptin. While in principle, blockade of TNFα production or action would offer a beneficial improvement of insulin sensitivity, it must be remembered that TNFα has a role in the immune system, and concomitant compromise of this role may be undesirable.

1.6.6 Inflammatory adipokines

Many chemokines produced by adipose tissue has previously been shown to play a role in control of inflammation, therefore by extension research into a possible link between obesity and low grade inflammation via production of adipokines was inevitable. In this section the

adipokines commonly associated with inflammation are briefly outline, however for an in-depth review see Juge-Aubry and Meier's review paper (2005).

1.6.6.1 Resistin, Adipsin and Visfatin

Resistin, adipsin and visfatin are all molecules associated with inflammation and are believed to be a possible link between the inflammatory response associated with obesity. Resistin is a member of the resistin-like molecules (RELMs), also known as 'found in inflammatory zone (FIZZ). Measured levels of resistin in obese subjects have varied within and between research groups.

Adipsin (human complement factor D) is the rate limiting enzyme in the alternative complement activation pathway. It is expressed in adipocytes and monocyte macrophages in humans. Visfatin (or PEBF) is a recently discovered adipokine which is primarily secreted via the visceral depot of adipose tissue. Visfatin binds to and activates the insulin receptor, thereby mimicking the effect of insulin both *in vitro* and *in vivo*.

1.7 Therapeutic intervention

1.7.1 Current therapeutic intervention agents for Diabetes

Type 2 diabetes mellitus (T2DM) is a multi-faceted disorder that can range greatly in severity. The drugs used to treat it are prescribed in conjunction with both dietary and lifestyle alterations. This section will outline the main agents used against diabetes and their main mode of action.

1.7.1.1 Sulfonylureas

The sulfonylureas stimulate insulin secretion, which subsequently reduces hyperglycaemia (Groop, 1994). The effectiveness of treatment with sulfonylureas is dependent on the patient having retained partial β-cell function (Bailey 2000). In addition, sulfonylureas may cause some weight gain. Repaglinide is an insulin releasing agent and was developed from the non-sulfonylurea component of glibenclamide. It works in much the same fashion as the sulfonylureas, but its action is rapid onset and of a short duration (Plosker and Figgitt 2004). It can be combined with metformin to give an additive glucose lowering effect (Bailey 1996).

Table 1.7 Action of interventions for T2DM with permission from Bailey (2002)

Actions	Insulin	Sulfonylureas, repaglinide	Metformin	α-Glucosidase inhibitors	Thiazolidinediones
Effect on glucose	• Hepatic glucose output • Peripheral glucose utilization	• Insulin secretion	Counter insulin resistance	↓ Rate of intestinal carbohydrate digestion	↑ Insulin sensitivity
Fasting plasma glucose	• ↓	•	↓	−	↓ [a]
Postprandial plasma glucose	• ↓	↓	↓	↓	↓ [a]
Plasma insulin	• ↑	↑	− or ↓	− or ↓	− or ↓
Body weight	•	↑	− or ↓	−	− or ↑
Plasma free fatty acids	↓	− or ↓	− or ↓	−	↓
Plasma triglyceride	−	−	− or ↓	−	− or ↓
Plasma total cholesterol	−	−	− or ↓	−	− or ↑
Tolerability	Injections	−	GI [b]	GI [b]	−
Safety risks	Hypo [c]	Hypo [c]	LA [d]	−	? [e]

• Blood-glucose-lowering efficacy is best in combination therapy.
[b] Gastrointestinal disturbances, especially if dosage is increased too rapidly.
[c] Hypoglycaemia (low blood glucose causing neurological disturbances).
[d] Lactic acidosis is a rare risk; it is important that exclusion criteria (e.g. impaired renal or hepatic function and predisposition to hypoxia) are observed.
[e] Safety risks (e.g. hepatotoxicity, fluid retention, haemodilution and anaemia) might vary with different thiazolidinediones.
Symbols: •, increase; ↓, decrease; ↑ ↑, strong increase; ↓ ↓, strong decrease; −, no consistent effect; ?, not fully established.
Abbreviations: GI, gastrointestinal; Hypo, hypoglycaemia; LA, lactic acidosis.

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1.7.1.2 Metformin

Metformin is a biguanide that lowers glucose concentrations by negation of insulin resistance (Bailey 2000) there by reducing the knock on detrimental effects insulin resistance causes.
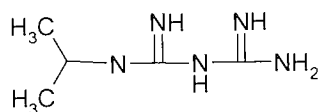
Figure 1.7 Metformin

Biguanides (Metformin and phenformin) mode of action is principally to reduce intestinal absorption of glucose and inhibition of hepatic glucose production (Williams 1994). Metformin reduces hepatic gluconeogenesis by enhancing its suppression by insulin and reducing its stimulation by glucagon (Bailey, 1987; Bailey, 1993; Wollen and Bailey, 1988). Patients on metformin therapy will have increased glucose disposal in all tissues, improved glucose tolerance and decreased fatty acid oxidation (Bailey, 2000). In addition metfomin has been shown to prevent weight gain and in some cases a weight reduction has been observed.

1.7.1.3 Thiazolidinediones (TZD)

TZDs are a relatively new class of oral antidiabetic drugs. They are potent agonists of the perioxisome proliferator-activated receptor γ (PPARγ). Stimulation of these receptors increases transcription of specific insulin sensitive genes such as lipoprotein lipase, FATP, aP2, acyl CoA synthase and GLUT4. PPARγ is abundant in adipose tissue and as a result TZD stimulation increases fatty acid uptake, lipogenesis and glucose intake in adipose tissue (Bailey 2004). This positively facilitates diabetes because there is a concomitant improvement in insulin sensitivity and glucose utilization (especially skeletal muscle) and therefore lowering blood glucose levels (Bailey 2000).

1.7.1.4 Acabose

Acobose is an α-glucosidase inhibitor that causes the reduction in postprandial blood glucose concentration elevations and consequently prevents acute increases in insulin secretion. Acabose slows digestion of complex carbohydrates at the brush border of the small intestine by inhibition of α-glucosidases including amylase, dextrinase, glucoamylase, isomaltase, matase and sucrase. This leads to lower basal glucose concentrations and reduced triglyceride levels (Bailey 2000). Side effects of treatment include abdominal bloating, flatulence and diarrhea.

1.7.1.5 Insulin

Insulin (also see section 1.4.4) is required when there has either been excessive β-cell damage or when drugs, diet and lifestyle alterations have been exhausted and no longer control plasma glucose levels within satisfactory boundaries. Insulin is required by about 5-10% of patients who are already taking one or more oral agents each year (Bailey, 2000). Presently there is no alternative method of administering insulin other than injection although inhalable medication is in the advanced stages of development. It reduces hepatic glucose output and increases glucose uptake into peripheral tissues. The type of insulin (long, short acting or combination) and number of injections is dependent on several factors, including the lifestyle and diet. Flexibility in insulin type is a great advantage and advances are being made each year to accommodate both those with regular and irregular eating patterns.

1.7.2 Current therapeutic intervention agents for obesity

Obesity is a multi-factorial disease and has many potential targets for therapeutic intervention. However, medication is only prescribed if the patient can demonstrate weight loss via

compliance to a reduced calorie diet as all weight related drug therapy is accompanied by diet modification and lifestyle change. The first drug used in the treatment of obesity was thyroid extract in 1893 and was done so on the belief that obese people had a low metabolism. Over the years many different drugs have been used including Dinitrophenol, which uncouples phosphorylation but has serious side effects (neuropathies and cataracts), amphetamine (1937) and digitalis.

More recent work has been carried out into the mechanisms of obesity and thus the targets for drug treatment have become more defined. Agents used for either clinical or research purposes are outlined in table 1.8. This section in with consideration to the previous section will outline the currently available drugs used for weight reduction and their mode of action.

1.7.2.1 Classification

Drugs can be classified into four groups by their mechanism of action;

1. Reduction of food intake [Inhibitors of energy intake]
2. Alteration of metabolism (pre or post absorption nutrient partitioning)
3. Increase of energy expenditure and/or thermogenesis
4. Stimulation of fat mobilisation

Metabolic process/ modulator	Drugs/agents	Mode of action
Pre-absorption nutrient partitioning		
gastric emptying	Threochlorocitric acid	Reduces food intake by increasing stomach volume and signalling early satiety
inhibitors of carbohydrate digestion	Acabose, miglitol, AO128	Amylase inhibitors, α-glucosidase inhibitors and disaccharide inhibitors reduce digestion of starch and disaccharides and thus reduce absorbance. Side effect: flatulence
inhibition of fat digestion or absorption	Cholestyramine, neomycin, perfluorooctyl bromide, Orlistat	Cholestyramine binds to bile acids and disrupts micelle formation decreasing TG digestion. Neomycin reduces fat absorption and perfluorooctyl bromide blocks all absorption of macro nutrients. Orlistat blocks lipase activity decreasing intestinal TG hydrolysis. Side effects: bloating, fatty stool, fat-soluble vitamin deficiency.
use of sweeteners and pseudonutrients	Sweeteners: Saccharin, cyclamates, aspartame,acesulfame-K; Fibres: guar, mucilage, pectin, chitosan Microparticulate protein, Olestra	Sweeteners are unable to be broken down and fibres are non-digestible, bulking agents. Reduce intake of refined sugars and aid satiety respectively. Microparticulate protein has the texture of fat but has reduced energy value. Olestra is a mixture of sucrose esters that cannot be broken down by pancreatic lipase.
Post-absorption nutrient partitioning		
Growth hormone	Growth hormone	Thermogenic- increases lean mass and reduces fat mass
Lipid modulators	Metformin	Enhanced peripheral utilization of glucose, and enhances lipolysis
testosterone	Testosterone	Increased testosterone in females and decreased testosterone levels in males had a detrimental effect, causing the increase in visceral fat.
Inhibition of glucocorticoids	Glucocrticoids	Increased levels of glucocorticoids caused increased fat accumulation as seen in Cushing's syndrome
Inhibition of other steroids	Megestrol acetate (Megace)	Increases food intake possibly via NPY, and increases fat storage

Table 1.8 Action of agents against obesity

The first in this group focuses mainly on appetite suppressants that decrease hunger or increase satiety in order to reduce the amount of food intake (see table 1.8). They would be used in conjunction with a reduced calorie or modified diet to increase success of weight loss. The most prominent drugs (Table 1.9) affecting food intake, via suppression of appetite used in the treatment of obesity include fluoxetine (Michelson *et al*, 1999), phentamine, fenfluramine (Wellman and Maher, 1999), sibutramine (Meridia) (McNeely and Goa, 1998) and more recently metformin. The drugs fluoxetine, phentermine and sibutramine act upon the brain to modulate serotonin and/or noradrenaline concentrations, particularly within the hypothalamus. The potency at which the drug binds with the receptors in the hypothalamus directly correlates with the degree of appetite suppression (Hauger *et al*, 1986). They affect the satiety regions of the brain to reduce hunger and therefore decrease food intake. Patients taking appetite suppressant drugs showed approximately 10% loss of their initial body weight (Campfield and Smith, 1999).

Table 1.9 Mechanism and status of appetite suppressants used to treat obesity (adapted from Campfield *et al.* 1998)

Drug	Target	Mechanism	Status
Fenfluramine	Serotonin neurons	Serotonin re-uptake	Withdrawn
Fluoxetine	Serotonin neurons	Serotonin re-uptake	Approved (but not necessarily recommended)
Phentermine neurons	Noradrenaline re-uptake	Noradrenaline	FDA approval
Fenfluramine/Phentermine (fen/Phen)	Serotonin and noradrenaline neurons	Serotonin re-uptake and release, noradrenaline re-uptake	Approved (Fenfluramine withdrawn). Still used in some countries
Dexfenfluramine (Redux)	Serotonin neurons	Serotonin re-uptake	Withdrawn
Sibutramine (Meridia) neurons	Serotonin neurones, noradrenaline	Serotonin re-uptake, noradrenaline re-uptake inhibitor	FDA Approved
OB protein (leptin)	OB protein	Activate OB-R receptor in brain and reduce food intake	Clinical trials (under investigation for the future)
Inhibitors of fat absorption Orlistat (Xenical)	Pancreatic lipase	Inhibition of fat absorption	Approved
Stimulators of fat mobilisation OB protein (leptin)	OB protein receptor	Mobilisation of fat mass	Approved
Rimonobant	Canabinoid CB_1 receptor	CB_1 receptor antagonist	Phase III trials

However, due to a cluster of reports of heart valve disease in obese patients treated with fenfluramine and phenteramine or dexfenfluramine in the USA, these drugs were removed from the market in 1997 (Campfield, *et al.* 1998, Campfield and Smith, 1999).

1.7.2.2 Sibutramine (Meridia)

Sibutramine (McNeely and Goa, 1998) developed by Knoll pharmaceuticals, is one of the few drugs still on the market. It is a serotonin and noradrenaline (amine) re-uptake inhibitor, which acts on the brain to induce a reduction in food intake. Serotonin suppresses food intake in freely feeding or food deprived rats. The effects of serotonin has been localized to the paraventricular nucleus (PVN) and the ventromedial regions of the hypothalamus (Wilding, 1997). Preventing its re-uptake prolongs feelings of satiety and reduces food intake. The approved appetite suppressants (table 1.9) are mainly derivatives of β-phenethylamine, except mazindol which is a tricyclic compound acting as a noradrenalin reuptake inhibitor.

1.7.2.3 Rimonabant (Acomplia)

Rimonabant is emerging as a multi-faceted agent against obesity. It is an appetite suppressing agent acting antagonistally upon the CB_1 canabinoid receptor (Rinaldi-Carmona et al 1994) initially conceived as an assistant to cessation of smoking and other addictions (Carai et al 2005, Henningfield et al 2005). The endocanabinoid system in the brain causes modification to release of standard neurotransmitters thus via this process of 'retrograde signalling' endocanabinoids can modulate synaptic transmission (Bailey and Day 2006). The CB_1 receptor is located in the hypothalamic region of the brain which is associated with hunger and satiety. Suppression of neurochemicals associated with hunger (see section 1.4.4.1) would lead to an increase in satiety and thus could decrease energy intake. In addition to its effects on the central control of adiposity rimonabant is postulated to have a peripheral effect on adipose tissue triglyceride storage, proliferation and adipokine expression (Bailey and Day 2006, Gary-Bobo et al 2005). Studies in rodents have shown rimonabant causes a reduction in body weight and fat mass in conjunction with an improve lipid profile and increased insulin sensitivity (Gray-Bobo et al 2005). Recently rimonabant treatment has been shown to upregulate adiponectin mRNA expression in rodent models resulting in an increase in insulin sensitivity (Bensaid et al 2003). Investigations into other effects of rimonabant are ongoing and may provide a multi-target attack against increasing adiposity.

1.7.2.4 Classification continued

The second category (see list in 1.6.1.2) can be further sub divided into pre-absorption and post-absorption nutrient partitioning (see table 1.8). The former of these refers to interference with nutrient availability prior to its absorption in the gut. These include inhibitors of gastric emptying, inhibitors of carbohydrate digestion, inhibition of fat digestion or absorption and

use of sweeteners and pseudonutrients (Bray and Ryan 1997). The latter refers to interference of metabolism with those nutrients that are absorbed. This can be achieved by growth hormone, lipid modulators, testosterone, inhibiting action of glucocorticoids and other steroids (Bray and Ryan 1997). The third category relies upon increased energy expenditure via thermogenesis. There are three components of thermogenesis;

1. basal metabolic rate

2. Thermal effect of food

3. voluntary physical activity

Thyroid hormones are thermogenic drugs that increase basal metabolic rate and there by decrease body weight. However, they can also increase protein loss and Ca^{2+} loss from the body (Bray *et al* 1973) and so cannot be used as a treatment for obesity. Growth hormone, androgenic and anabolic steroids also have thermogenic effects, along with some adrenoreceptor agonists nor adrenaline and adrenaline.

The final category represents drugs used to mobilise fat.

1.7.2.5 Orlistat (Xenical)

The most recognised drug for fat mobilisation is orlistat developed by Hoffman-La Roche. It acts on pancreatic lipase molecules to prevent the digestion of dietary fat to fatty acids and monoglycerides (Proietto, 2000). As a result it prevents the absorption of fat into the body. In addition orlistat has unpleasant side effects (diarrhoea), which worsen in the event of deviation from a low fat diet. Some believe that this side effect encourages the patient to adhere to the diet proposed for them.

1.8 Summary

It is greatly acknowledged that a reduction in insulin resistance and improved glycaemic control results when there is a reduction in adiposity in particular visceral adiposity. However weight loss is difficult to achieve by diet and exercise alone and is even harder to maintain. A reduction in weight also has benefits in prevention of other ailments including cardiovascular disease, hypertension and dyslipidaemia (Wing *et al* 1990).

1.9 Aims

The aim of this research programme is to investigate the hypothesis that human visceral and subcutaneous adipose tissue is exhibit subtle differences in their function. The purpose of this project is to firstly identify and then to manipulate potential new therapeutic targets to reduce adiposity, especially in the visceral compartments, and to improve insulin sensitivity.

Chapter 2: Materials

2.1 Materials

All chemicals were standard laboratory reagents and of analytical grade, unless otherwise stated.

2.1.1 Chemicals and Suppliers:

AB gene, Epsom Kent, UK

Assay on demand primers

Master Mix

Applied Biosystems, California, USA

Taqman Gold cDNA kit

Random Hexomers

RNase inhibitors

Assay Designs Ltd., Michigan USA

Cortisol Correlate™-EIA, FPIA (ELISA Kits)

Amersham International Ltd, Bucks, UK

RNA Guard

BDH Chemical Ltd. Pool, UK

Agarose

Fisher Scientific, Loughborough, UK

Chloroform

Copper sulphate

Ethanol

D-Glucose anhydrous

Hydrochloric acid

Magnesium sulphate

Methanol

Methylated spirit

Percholric Acid

Potassium chloride

Potassium dihydrogen orthophosphate

Potassium hydroxide

RNase Away

RNA Bee

Sodium carbonate

Sodium chloride

Sodium dihydrogen orthophosphate

Sodium hydrogen carbonate

Sodium hydroxide

Sucrose

ICN Biomedicals, Hampshire, UK

Phosphate buffered saline tablets (PBS)

Invitrogen, Paisley, Scotland, UK

Antibiotic mix (Penicillin, streptomycin and Gentamycin)

Bicinchoninic Acid Kit for Protein Determination

Bovine foetal calf serum (FCS)

Dulbecco's Modified Eagle Medium (DMEM)

Dulbecco's Modified Eagle Medium F12 (DMEM:F12)

Glutamate

Penicillin-Streptomycin

Trypsin/EDTA

Trizol reagent

Qiagen Ltd, Sussex, UK

RNeasy mini column kit

Roche Applied Sciences

XTT assay kit

BrdU assay kit

Sigma-Aldrich Co. Ltd, Dorset, UK

Adenosine 5'-triphosphate (ATP)

Amphotericin

Bovine serum albumin (BSA)

Calcium chloride

Collagenase type II

Cortisol

Cortisone

Diethyl pyrocarbonate (DEPC)

Dexamethsone

2-Deoxy-D-Glucose

Ethyl acetate

Dimethyl sulphoxide (DMSO)

(Ethylenedinitrilo)tetraacetic acid (EDTA)

Ephinephrine (adrenaline)

L-Glutamine

Glycerol kinase

Insulin

3-Isobutyl-1-methylxanthine (IBMX)

Isoproterenol

Lactate dehydrogenase

Magnesium chloride

-Nicotinamide adenine dinucleotide-reduced form (NADH)

Norephinephrine (noradrenaline)

Oil red O

Potassium chloride

Pyruvate kinase

Sodium bicarbonate

Triethanolamine

Trypan blue

2.1.2 Analytical Equipment

ABI sequence detector (Taqman 7700) – Applied biosystems, California, USA

ABI sequence detector (Taqman 7900) – Applied biosystems, California, USA

Computer Equipment – Dell, UK

Multiscan EX plate reader – Thermo Electron Corporation, UK

MSE Mistral 3000 – Fisher scientific, UK

MSE Microcentrifuge – Fisher scientific, UK

Nanodrop spectrophotometer ND-1000 - LabTech International, UK

Spectrophotometer – Ultraspec LKB Bichrom UK

Student Camera – Scicam Videolabs Inc USA

TL-100 Ultracentrifuge – Beckman USA

Chapter 3: Methods

3.1 Animal tissue

Lean or obese (*ob/ob*) mice were killed by cervical dislocation and the adipose tissue was immediately removed. The animals were prepared in surgically sterile conditions. The experiments were approved by the Bioethical Committee and Aston University. They were performed with adherence to good scientific practice for the experimental procedures concerned. The experiments were performed under authority of licences issued to Aston University by the UK Home Office Scientific Procedures Act. Animal tissue was weighed and either used fresh (held at bout 4°C for <30min in psychological buffer) or frozen at -80°C or below.

3.2 Human Tissue

Abdominal subcutaneous and visceral adipose tissue biopsies were acquired from adult patients undergoing elective surgery at City Hospital (Birmingham, UK). All surgery was performed under general anaesthesia and all patients were preoperatively fasted for >6h. City Hospital Research Ethics Committee approval was obtained, and all patients involved gave their informed consent. Tissue was weighed and either used fresh (held for <1h at about 4°C in physiological buffer) or frozen in liquid nitrogen. There were 56 Patients, aged between 23-76 years of which 80% were female and 20% were male. Samples from both depots (Subcutaneous and Visceral) were taken from the same patient in approximately 90% of cases and were match paired. Lean patients were catagorised as BMI 20-26 kg/m^2, overweight and obese patients were catagorised as BMI 28-33 kg/m^2.

3.3 Chapter 4 protocols

3.3.1 Isolation of animal adipocytes

Mouse adipose tissue the parametrial and epididymal fat pads were removed, cleaned in Krebs Ringer buffer (Krebs Ringer buffer (NaCl, KCl, NaHCO$_3$, MgSO$_4$ 7H$_2$O, KH$_2$PO$_4$, Gas (5%CO$_2$, 95% O$_2$) 20min, stored at 4°C. Add 1.27mM CaCl$_2$ and 5mM Glucose to working volume) and weighed. Sample size had a total weight of approximately 2g. The samples were minced into small pieces in a bijou containing 5ml culture media containing 1mg/ml collagenase and incubated in a slow shaking water bath (37°C) for1h. After incubation the sample was transferred to a 15ml falcon tube, the bijou was washed twice with 15ml Krebs Ringer. The mixture was centrifuged (3000rpm/800g for 7min at room temperature (MSE Mistral 3,000, Fisher Scientific, UK) between washing steps. After centrifugation cells were aspirated and resuspended in the appropriate media (see sections 3.3.2).

3.3.2 Isolation of Mature human adipocytes

Human adipose tissue (0.5-1.5g) was minced into small pieces and placed in a bijou containing 1ml culture media containing 3mg/ml collagenase and incubated in a slow shaking water bath (37°C) for1h. After incubation the sample was transferred to a 15ml falcon tube, the bijou was washed twice with 15ml Krebs Ringer. The mixture was centrifuged (3000rpm for 7min at room temperature (MSE Mistral 3,000, Fisher Scientific, UK) between washing steps. After centrifugation cells were aspirated and resuspended in the appropriate media (see sections 3.4, 3.5).

3.3.3 Pre-adipocytes isolation

Adipose tissue was extracted as in section 3.1 and 3.2. As in the mature human adipocyte isolation procedure (3.3.2) the samples were minced into small pieces in a falcon tube

containing 1ml culture media containing 3mg/ml collagenase and incubated in a shaking water bath at 37°C for 1h. Post incubation the cell suspension was transferred into 10ml culture media and was centrifuged (2000 rpm, 5min). After centrifugation the supernatant was aspirated and discarded. The pellet was re-suspended in 5ml red blood cell lysis buffer and left to stand at room temperature for 5min. The suspension was then centrifuged (2000 rpm, 5min), again the supernatant was discarded. The pellet was re-suspended in 2ml warmed culture media and a cell count was performed. Cells were further suspended in culture media to approximately 1×10^5 cells per ml to seed T75cm^3 flasks.

3.3.4 Human cell culture

3.3.4.1 Pre-adipocyte cell culture

Cells were isolate as in 3.3.3 and were seeded into T75cm^3 flasks at 1×10^5 cells. Cells were suspended in culture medium (20ml) and left to adhere for 4-6 days. Upon satisfactory adherence (>5% by eye) media was changed and cells were left to grow for 7-14 days. For cell growth studies cells were seeded into 12 well plates at 1×10^4 cells and allowed to adhere for 3-5 days, upon adherence media was changed. At this point media was removed from 4 wells and the adhered cells in each well were treated with 200μl trypan blue (4%) for 3 min at 37°C. All cells in each well were then counted under a microscope to determine the adherence percentage. Subsequent cell counts were taken every 3rd day until day 9 by the same method, post day 9 cells were treated with trypsin and removed. Post day 9 cell counts were determined using the standard haeomcytometric method outlined in section 3.5.1. Pre-adipocyte growth curves are shown in chapter 4.

3.3.4.2 Mature adipocyte culture

The tissue was removed as outlined in section 3.2 and isolated as in section 3.3.2. The aspirate was counted using a modified hemocytometric method (see 3.5.2) and re-suspended in a 50ml falcon tube with warmed culture media (DMEM:12, 20% FCS, 100u/ml penicillin, 10mg/ml streptomycin, 25µg/ml amphotericin B, 10mg/ml Gentamycin). The resulting tri-phased suspension was used to seed culture plates. The top phase comprised of lipid and mature adipocytes, the middle phase mature and intermediate adipocytes and the pellet of pre-adipocytes and other cells including red blood cells. The lipid was carefully aspireated and discarded, the remaining supernatant was aspirated and mixed and a modified hemocytometric cell count was taken. For the subsequent seeding of plates cells were used at approximately 1 x 10^4 cells per ml. Cell counts were performed on day 0 and every second day via a computerised method outlined in section 3.5. a growth chart was plotted (see figure 4.6).

3.3.5 Cell count

Three methods of cell count were used. The standard haemocytometer method (3.5.1) for adherent cell lines, a modified haemocytometer method (3.5.2) and a computerised cell count method (3.5.3).

3.3.5.1 Standard Haemocytometer cell count

Cell counts were performed on a standard haemocytometer (Neubauer, Weber, UK) in the presence of a cover slip and results recorded in terms of number of cells per ml.

3.3.5.2 Modified haemocytometer cell count

Cells counts were performed on a standard haemocytometer in the absence of a coverslip. Floating cell cultures required the removal of liquid via capillary action to allow focusing of cells and counting grid. Results were recorded in terms of number of cells per ml.

3.3.5.3 Computer based cell count

By means of a computer linked camera (Scicam, Videolabs Inc., USA) photographs were obtained of floating adipocytes. Under the same magnification, further photographs were taken of a graticule. Using a computer illustrating programme (Adobe illustrator 10) a grid of 100 μm^2 with 20 μm^2 intervals was created from the photograph of the graticule. This grid was used as a computerised haemocytometer by overlaying it with the photographs of the cells. The cells were counted in four 20μm^2 grid blocks, from 3 different photographs of the same plate. The mean value was calculated, from which the number of cells per plate were computed. Consequently, graphical representation of the cells growth curve was formulated (see section 4.8.2).

3.3.6 Cell proliferation assays

3.3.6.1 XTT assay

The assay is performed using flat bottomed 96 well microplates. Cells were seeded at 10^3 cells per well for pre-adipocyte assays and over a density of 10^4-10^6 cells per well for mature adipocyte assays. Pre-adipocytes were allowed to adhere for 3days. Media was changed and cells were left to grow for a further 2 days. Mature adipocytes were analysed 2hr after incubation.

XTT labelling reagent and electron coupling reagent were combined. This mixture was added to each well (50μl) containing cells in 100μl culture media. Cells were incubated at 37°C in a humidified atmosphere for 2h or 21hr (pre-adipocytes). Absorbance was read at 450nm with a reference wavelength of 650nm.

Controls tested included media only, XTT assay mixture only, media with cells and media with XTT assay mixture to determine any cross reactivity. No cross reaction was apparent (data not shown)

3.3.6.2 BrdU Assay

The assay is performed using flat bottomed 96 well microplates. Cells were seeded at 10^3 cells per well for pre-adipocyte assays. Pre-adipocytes were allowed to adhere for 3days. Media was changed and cells were left to grow for a further 2 days. BrdU labelling solution (10μl) was added to cells in 100μl culture media. Cells were re-incubated for additional 4 h at 37°C. Solution was aspirated and 200μl/well "FixDenat" was added, cells were then incubated for 30 min at room temperature. After incubation FixDenat solution was thoroughly removed by flicking off and tapping. Anti-BrdU-POD working solution was added and cells were

incubated for approximately 90 min at room temperature. The antibody conjugate was removed by flicking off and rinse wells three times (300μl) and tapped dry. Substrate solution (100μl) was added and cell were incubated at room temperature until colour development was sufficient for photometric detection (approx. 5-30 min). The reaction was stopped using sulphuric acid, shaken for 1min at 300rpm and read immediately at 450nm with a reference wavelength of 690nm.

3.3.6 Lipolysis assay

Cells were isolated as in section 3.3.1 (animal) and 3.3.2 (human). The aspirated adipocytes were resuspended in Krebs-BSA to a concentration of approximately 5.5×10^5 (counted using modified haemocytometric method (see 3.5.2)) cells and added to a series of samples, including controls for the experiment. The controls in the experiment consisted of a negative control (Krebs-BSA buffer only), and a base line glycerol control (Cells only). Adipocytes (900μl) were added to test compounds (100μl: isoproteronol, BRL37344). Samples were gassed (5% CO_2 95% O_2) and incubated for 2h at 37°C in the slow shaking water bath. After incubation, 0.5ml of the medium was added to 0.5ml of 10% perchloric acid (1.7M) to stop the reaction. After centrifuging at 13000rpm (11600g) for 5min, the supernatant was collected and the pH for each sample was adjusted to pH 7.0 by the addition of 40% potassium hydroxide (7.1M). The amount of glycerol released by the adipocytes was then measured using the method described below (3.6.1)

3.3.6.1 Measuring Lipolytic Activity

The concentration of glycerol released was determined enzymatically by the method of Wieland, 1974. A 200µl sample of medium was added to 830µl spectrophotometer buffer. The buffer consisted of Magnesium Sulphate (0.0493g), Triethanolamine (1.5g), PEP (0.0183g), ATP (0.0609g), NADH (0.0177g) made up to 100mls with distilled water. The pH was adjusted to 7.4 with hydrochloric acid or potassium hydroxide. Finally, lactate dehydrogenase (73µl) and pyruvate kinase (53µl) was added to the reaction mixture.

Glycerokinase was diluted 1:4 with distilled water and 10µl was added to the assay mixture. The samples were shaken and immediately absorbance read at 340nm using a spectrophotometer (Ultraspec®K LKB Biochrom, UK). Samples were allowed to stand at 37°C and then a second reading was taken after 15min. The difference between the two values was calculated.

Glycerol is converted to glycerophosphate by ATP and glycerokinase (GK). The oxidation of glycerophosphate to the NAD-dependent lactate dehydrogenase (LDH) serves as the indicator reaction (see below).

Principle of Method

$$\text{Glycerol} + \text{ATP} \underset{\longrightarrow}{\overset{\text{GK}}{\longleftarrow}} \text{L-(-)-Glycerol-3-P} + \text{ADP}$$

$$\text{L-(-)-Glycerol-3-P} + \text{NAD}^{+} \underset{\longrightarrow}{\overset{\text{LDH}}{\longleftarrow}} \text{DAP} + \text{NADH} + \text{H}^{+}$$
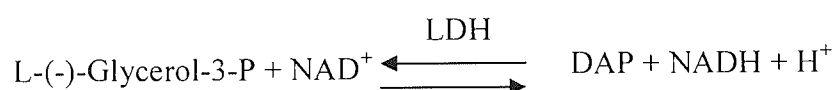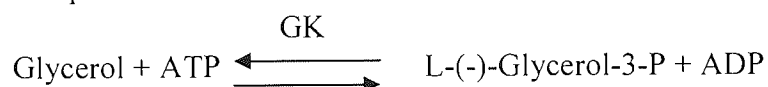
The formation of NADH, as measured by the changes of extinction at 340nm is proportional to the amount of glycerol present. To calculate the concentration of glycerol (mM) in each sample, the following equation was used.

$$\frac{(\text{change in absorbance}) \times 1.61^{*}}{0.078542598^{**}} = \text{mM [Glycerol]}$$

Using the equation, the lipolytic activity of putative agents was expressed as mM glycerol released/10^{5} adipocytes/2h.

* 1.61 is obtained for the extinction co-efficient of NADH (6.22)

** Dilution factor

3.3.7 RNA extraction and quantification

3.3.7.1 Precautions

RNA is extremely sensitive to RNAse activity, which will cause rapid degradation of the sample. RNAse activity is frequently brought about by contaminants on untreated glass and plastic ware and by contamination from the operator and the tissue specimen. Thus the extraction procedure involved extensive precautions to avoid contaminant RNAse activity. Gloves, glassware, pestle and mortar and the immediate work area were decontaminated by washing with 'RNAse Away' (Molecular Bio-Products, San Diego) prior to all experimentation to remove contaminating RNA. In addition RNA/DNA-free plastic ware was utilised were possible, and solutions were prepared diethyl pyrocarbonate (DEPC) treated water. RNA Bee (Biogenesis, Poole Dorset), a reagent containing inhibitors of RNAse activity was used to prevent degredation by contaminating and innate RNAse.

3.3.7.2 RNA extraction

The extraction procedure was conducted in accordance with all local safety regulations and COSHH requirements. Fresh tissue was snap frozen in liquid nitrogen and pre-frozen tissue was used immediately after removal from liquid nitrogen storage. A decontaminated pestle and mortar (70% IMS and 'RNAse Away') were utilised to macerate the tissue into a powder. The extraction procedure was based on the method of (Chomczynski and Mackey, 1995; Reaven, 1994) with minor modifications.

The tissue (approx 800-1100mg) was transferred in a darkened area to a 50ml polypropylene tube (wrapped in foil to keep dark) and 4ml RNA Bee was added per 100mg of tissue and thoroughly mixed. Chloroform (0.2ml) was added per 2ml of homogenate and shaken

vigorously manually for approximately 15 seconds. The mixture was left to stand on ice for 5 min prior to centrifugation for 20min at 4°C at 15,000rpm (TL-100 ultracentrifuge, Beckman, USA). The aqueous upper phase was carefully transferred to a fresh tube, isopropanol was added at a volume equal to half that of the original volume of RNA Bee and the mixture was left to precipitate for >2h at 4°C. The precipitate was collected by centrifugation for 15min at 4°C at 10,000rpm, and the supernatant removed. The tube was inverted and allowed to drain completely. The pellet was washed in 2ml 75% ethanol/DEPC and centrifuged as before (15min at 4°C at 10,000rpm). The supernatant was removed and the tube was inverted to drain any remaining fluid. The pellet was finally re-suspended in 0.5ml 1mM EDTA/DEPC and the concentration of RNA was determined spectrophotometrically.

3.3.7.3 Spectrophotometrical analysis

The concentration of RNA recovered was quantified spectophotometrically: A 10μl sample of the RNA extract was transferred to microfuge tubes containing 1ml DEPC water, to give a 1:100 dilution. The solution was transferred to a quartz cuvette and the absorbance determined at 260nm and 280nm using DEPC water as a blank (Ultraspec 1000E, Pharmacia biotech, UK). Pure RNA has a spectrophometric absorbance ratio (A_{260}/A_{280}) that is greater than 1.6 and should ideally be 1.8-2.0. The concentration of RNA was calculated using the equation:

Concentration (mg/ml) = [A_{260} x dilution factor]/20

$$= [A260 \text{ x } (1:100)]/20$$

All tissue extractions for RNA reported in this thesis met the requirement of purity A260/A280 ratio (1.6-2.0).

3.3.8 Protein quantification

3.3.8.1 Tissue preparation

Cells were isolated as in section 3.3.2 and resuspended in Krebs Ringer with 1% SDS buffer at 5.5×10^5 cells per ml. Cell suspension (900µl) was dispensed into Microfuge tubes (6 x 1.5ml) and were homogenized (electric homogenizer) for 30s then microfuged (13000rpm, 2min). Supernatant was aspirated and dispenced into a fresh microfuge tube, pellet was resuspended in Krebs Ringer with 1% SDS buffer. These suspensions were used in the protein analysis.

3.3.8.2 Protein analysis (BCA Assay)

Standard or unknown samples (25µl) were dispensed into a 96 well microplate. A working reagent prepared by mixing BCA reagent A (Pierce) to BCA reagent B (copper sulphate 4%) in the ratio of 50:1 was added to each well (200µl) and samples were mixed thoroughly on a plate shaker (30s). The plate was covered and left to incubate (37°C, 30min). The plate was then cooled to room temperature and the absorbance was measured at 590 nm on a plate reader.

The mean absorbance value of both standards and unknowns were determined and corrected against the blank. Standard error within the triplicates and across the samples was determined.

3.4 Chapter 5 protocols

3.4.1 Isolation of total RNA from adipose tissue

Utilising the triazol method combined with RNeasy columns. Approximately 200mg of frozen tissue sample (-80^0C) per 2ml triazol solution was added to a cooled round bottomed tube (4^0C, starsted), homogenised (full speed, $\approx$30s) (polytron homogeniser) then returned to ice.

The homogenised tissue was incubated at room temperature for 5 min then centrifuged (10 000rpm, 10min, 4^0C). After centrifugation excess fat separates as a top layer, intracellular components in the bottom layer and the remaining lysed cell material as a pellet. The bottom layer was transferred to a fresh tube and the fat and pellet are discarded. Cloroform (400µl/200mg original tissue) was combined with the transferred sample, capped, vigorously manually shaken (15s), incubated at room temperature for 2-3min and finally centrifuged (10 000rpm, 15min, 4^0C). The aqueous phase separates into the top layer; this phase was transferred to a fresh tube ($\approx$ 600µl/ 200mg original tissue). To this an equal volume of ethanol (70%) was added and the sample vortexed. The sample was loaded onto an RNeasy mini column (2ml), centrifuging between loading sample and discarding the effluent. At this point the sample may be DNase treated. RW1 buffer (350µl) was added to the column, centrifuged and the effluent discarded. DNase 1 (80µl) working solution (1:8 ratio of DNase stock:RDD buffer) was added to the column and left to incubated at room temperature for 15min. Rw1 buffer (350µl) was subsequently added to the column and centrifuged and the effluent was discarded. The column was washed using 500µl RPE buffer and eluted by centrifugation (microfuge, 1min, 3000rpm), this step was repeated with a 2min centrifugation. Changing the collecting tube the column was dried by a further 1min centrifugation without loading any fluid. Finally the RNA was eluted in 30µl RNase free water. The 30µl eluant was reloaded onto the column and collected. To each sample, 1µl of RNA guard was added to prevent any degradation of sample. Samples are visualised on an agarose gel (10% of sample was loaded) and quantified by NanoDrop. RNA was used as a template for cDNA.

3.4.2 Making cDNA

3.4.2.1 DNase treatment

RNA sample not DNase treat on the column have to undergo a separate process of treatment as follows. Untreated RNA samples (3.9.1) were DNase treated using a DNase free kit (Ambion inc). Samples were added to the Turbo kit mix and incubated (37°C, 20min). Inactivation slurry (10µl) was added for 2-3 min at room temperature. The sample is microfuged (13000 rpm 3min). Treated RNA (1µg) is used to make cDNA. The alternative method is described above, and is an "on the column" method.

3.4.2.2 Converting to cDNA

Using a master mix kit (Taqman Gold, applied biosystems), the samples are converted to cDNA. For each RNA sample (1µg, 10µl) is added to 10 x Taqman reverse transcriptase (RT) buffer (10µl), 25mM $MgCl_2$ (22µl), Multiscribe RT (50 U/µl, 2.5µl), DEPC water (28.5µl), Random Hexomers (50µM, 5µl), RNase inhibitors (20U/ml, 2µl). A set of controls are run with the absence of RT for each RNA sample. Samples are prepared in a 96 well PCR plate and run on a Taqman 7700 machine for one cycle (10min 25°C, 30min 48°C, 5min 95°C). Samples can then be stored at 4°C for 1 month or -80°C for longer periods.

3.4.2.3 RT-PCR "Taqman"

Using a 384 well PCR plates, cDNA samples are plated out and assayed against genes of interest. Each well contains cDNA sample (each sample dilute 1:40, 2µl diuent used), 2 x Abgene mastermix (5µl), 20 x assay on demand primers* (0.5ul) and double distilled water (8µl). Standard curves are derived using a pool of all the tested cDNA (10µl). A serial dilution

of the pooled sample (1:5 -> 1:320) was used A sticky lid is attached and the samples centrifuged (1min, 800rpm). The plates are run on a Taqman 7900 for 40 cycles. Data was analysed against the standard curve enabling CT values to be converted to concentration. Housekeepers were analysed by GeNorm (see chapter 5).

*assay on demand primers included housekeepers (β-Glucuronidase and Ribosomal protein) and DGAT1, DGAT2, 11β-HSD1, SCD, Adiponectin and leptin. Other primers and probes used were generated by Abgene, these include GPR41

3.5 Chapter 6 protocols

3.5.1 Sample preparation

Cells were isolated as in section 3.3.2. The cells were counted using the modified haemocytometric method (section 3.5.2) and resuspended at approximately 1×10^6 cells per ml. Cell suspension (800µl) was added to 100µl test agent within the concentration range 1×10^{-4}-1×10^{-8} and 100µl cortisone (1µM). Samples were incubated at 37°C for 2h. Upon completion all samples were extracted via the addition of ethyl acetate (2.5ml), vortexed and allowed to stand for 1 min. The uppermost ethyl acetate layer was transferred to a fresh glass vial and evaporated at 40°C under nitrogen gas. The sample was resuspended in phosphate buffer (100µl, pH 7.4). Samples were analysed for cortisol content via an ELISA assay procedure as described below (section 3.10.2), 10µl of sample was added to 90µl of kit buffer for analysis.

3.5.2 Cortisol assay kit (Assay designs inc.)

Assay Procedure

Standard diluent (100 µL, Assay Buffer), standards and samples (100µl) were dispensed into the appropriate wells as denoted by the plate lay out. A further 50 µL of assay buffer was dispensed into the NSB wells. Conjugate (50µl) was dispensed into all wells, except the Total Activity (TA) and Blank wells. Finally, antibody (50µl) was dispensed into each well, except the Blank, TA and NSB wells. The plate was subsequently covered and incubated at room temperature on a plate shaker for 2 hours at ~500 rpm. After incubation the plate was emptied and washed (400µl) three times. After the final wash, the wells were aspirated and firmly tapped on a lint free paper towel to remove any remaining wash buffer. Blue Conjugate (5µl)

was added to the TA wells and pNpp Substrate solution (200μl) was added to every well. A further incubation at room temperature for 1 hour was administered without shaking.

The reaction was stopped after incubation via the addition of Stop Solution (50 μl) to every well. The plate was read immediately at 405nm and corrected at 590nm, the value obtained for the blanks were averaged and subtracted from all the readings. The unknown values were determined from the standard curve.

Chapter 4: Characterisation of adipocytes

4.1 Introduction

White adipose tissue expansion takes place rapidly after birth via hypertrophy and hyperplasia of adipocytes (Gregoire et al 1998). Fat cell acquisition from precursor cells occurs throughout the life span. Compilation of current knowledge outlining the proliferation, expansion and differentiation of the adipocyte cell development is shown in figure 4.1. Most of this information has been determined using adipocyte cell models including cell lines (e.g. 3T3-L1) and rodent adipose tissue.

Figure 4. 1: Adipocyte development
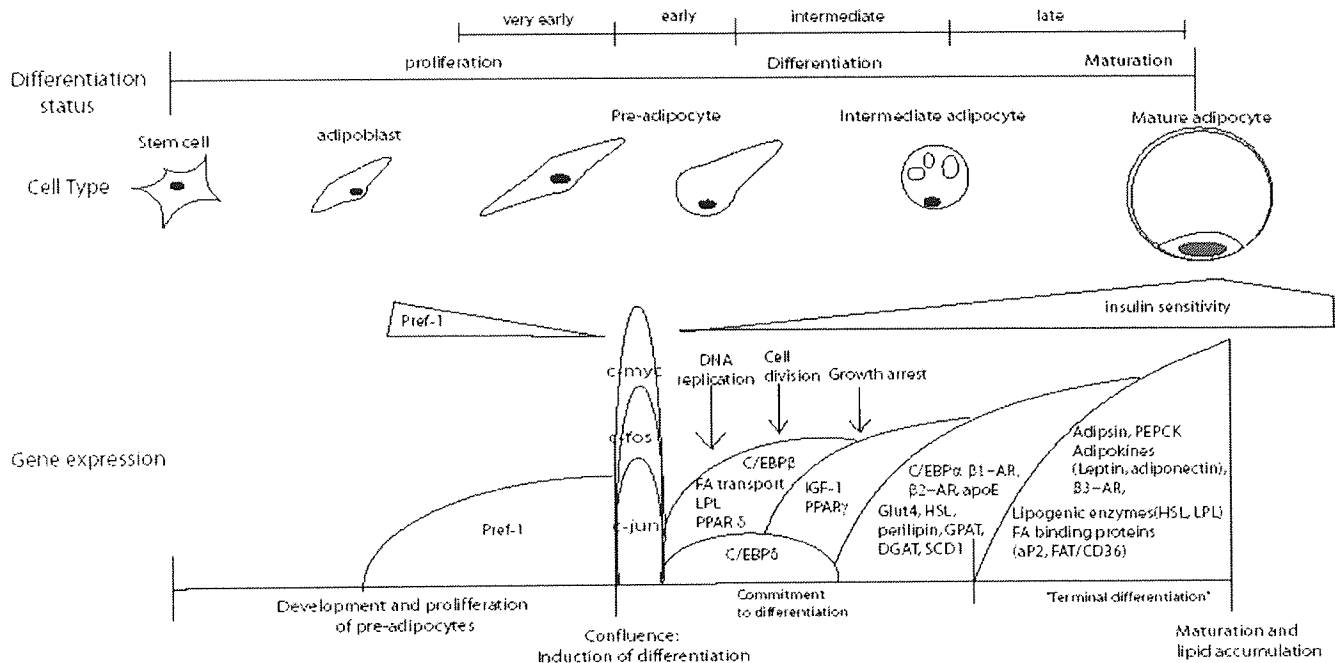
Figure 4.1: Adipocyte development: created from Ailhaud and Hauner 1998, Gregoire et al 1998, Ntambi and Kim 2000.
Pref-1:preadipocyte factor-1; FA:fatty acid; LPL:lipoprotein lipase; C/EBP:CCAAT/enhancer binding protein; IGF:insulin-like growth factor-1; PPAR:proliferator-activater receptor-1; AR:adrenergic receptor; HSL:hormone sensitive lipase; GPAT:glycerol-3-phosphate acyl transferase; DGAT:diacylglycerol acyl transferase; SCD:stearoyl-CoA desaturase; PEPCK:phosphenolpyruvate carboxykinase.

The 3T3-L1 cell line is a stable model of murine adipocytes that has been manipulated to undertake preliminary studies to understand adipocyte function and determine suitable agents which control adipose metabolism. Several murine 3T3-L1 pre-adipocyte cell lines were established from mouse fibroblasts and cloned by Green and Kehinde (1974, 1975 and 1976). After differentiation the 3T3-L1 cell line forms rounded cells containing large droplets of lipid and can be maintained at confluence for 2-4 weeks in fresh medium (Rubin et al 1978). This compares with the initial stages of mature human adipocyte development (see chapter 1.4). Upon cell confluence differentiation may be induced and accelerated by treating the cells with isobutymethyl xanthine (IBMX), dexamethasone and insulin (Frost and Lane 1985). In addition to the inducing chemicals listed above it is known that cortisol is responsible for *in vivo* (cortisol in humans, corticosterone in mice) differentiation and lipid accumulation of human adipocytes (see chapter 5) and can independently initiate differentiation (Paulmyer Lacroix *et al* 2003). The 3T3-L1 cell line has been extensively utilized to study adipocyte behaviour and has been successfully characterised as a model for the study of glucose uptake (Reed *et al* 1981) as well as lipogenesis and lipolysis. Isolation of human pre-adipocytes provides a less stable but more relevant model for human obesity. In addition, differentiated 3T3 cell lines are characterised by multiple fat droplets rather than one large droplet as seen in mature human adipocytes, and produce only 1-2% of the leptin of mature cells (Flier 1997). There may be other functional differences between 3T3 cell lines and primary isolated cells yet to be determined, thus in the following programme of study has concentrated on isolated mature and pre-adipocytes as a source of cells. Furthermore investigation has focused primarily on human adipose tissue as a cell source due to the physical differences observed between rodent and human samples (see 4.2). Using the extensive knowledge of 3T3-L1

culture systems combined with current understanding of primary adipocyte culture techniques this chapter characterises the isolation and aspects of human adipocyte behaviour *in vitro*.

4.2 Adipocyte isolation

Isolation and digestion methods used to separate adipocytes from adipose tissue extracted from human visceral and subcutaneous adipose tissue are described in chapter 3. Isolation results in four distinct fractions, two liquid phases and two essentially solid phases. When observed (figure 4.2) under a microscope Phase 1 material contained predominantly lipid accumulated from cell lysis, but also contained a small number of mature adipocytes. Phase 2 contained the remaining unlysed, non-collagenased tissue matter and trapped isolated mature adipocytes. Phase 3 contained the majority of the mature adipocytes. Phase 4 (pellet) contained the lysed cell matter and the vascular-stromal fraction including the pre-adipocytes (figure 4.2). Therefore it was determined that cells intended for pre-adipocyte culture were to be isolated from phase 4, and cells intended for mature adipocyte culture were to be taken from phase 2 and 3. Mature cells isolated in this manner have also been used in subsequent studies to determine characterisation of isolated cells (chapter 4), and potential therapeutic control of glucocorticoid action (chapter 6); whilst whole tissue was utilized in the extraction of RNA for chapter 5.

Figure 4. 2: Human isolated adipocytes
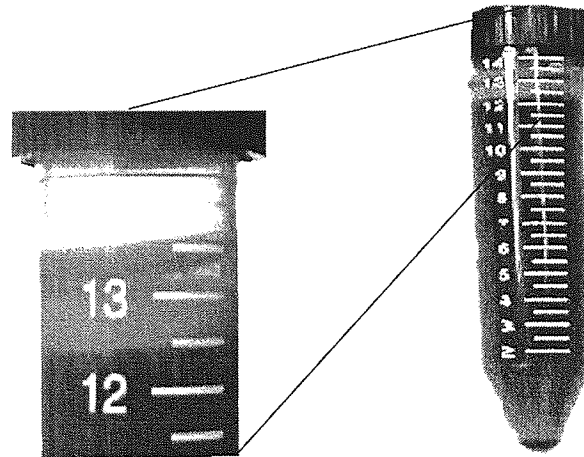
4.3 Pre-adipocyte results

4.3.1 Pre-adipocytes (stromal vascular cells) cell growth

Human pre-adipocytes were isolated (phase 4), washed and re-suspended in supplemented DMEM:F12 as proposed by Zhang (2000) outlined in chapter 3. Initial adherence was slow taking 7-10 days for adequate attachment estimated to be up to 5% of seeded cells. Growth to confluence was equally lengthy requiring between 10-15 days. Upon passage adherence was far greater (≥95%) and subsequent growth to confluence was much faster (3-5 days).

4.3.2 Photographs of cell growth

Digital photographs of pre adipocytes were taken *in situ* (without removal of cells) over the period of growth and differentiation (Figure 4.3a-f). Trypan blue staining was carried out (data not shown*) to validate viability and Oil red O staining was performed to confirm fatty acid uptake (data not shown*). Assays to confirm cell growth and differentiation were performed: XTT (figure 4.4) and BrdU (figure 4.5) assays were performed as outlined in chapter 3 on primary isolated pre-adipocytes during the growth phase (days3-5) after passage 1. Results are shown as absorbance (450nm) compared with a blank (see chapter 3). In addition cell proliferation was determined using manual cell counts every 2-3 days and presented using graphical illustration (figure 4.6). All data pertain to cells isolated from visceral tissue. Cells isolated from subcutaneous tissue had little or no adherence and thus could not be investigated.

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* These data were not shown as resolution of the image was not sufficient in low light to depict colour contrast, although this colour could readily be seen under a microscope. Photographs taken of mature isolated cells can be used as an example as they are much bigger and therefore colour distinction can be shown using digital photographic equipment.

Figure 4. 3: Photographic depiction of pre-adipocyte cell growth
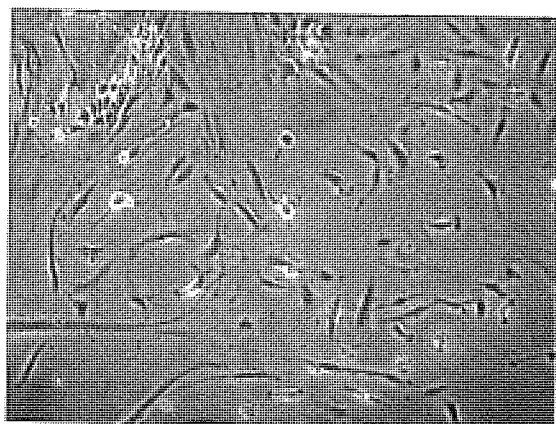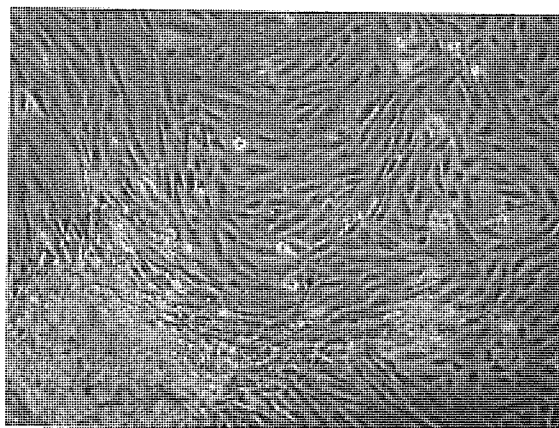
a   b

Visceral pre-adipocytes day 11 before passage: cells in figure 4.3a located peripheral to the cluster formation in figure 4.3b.
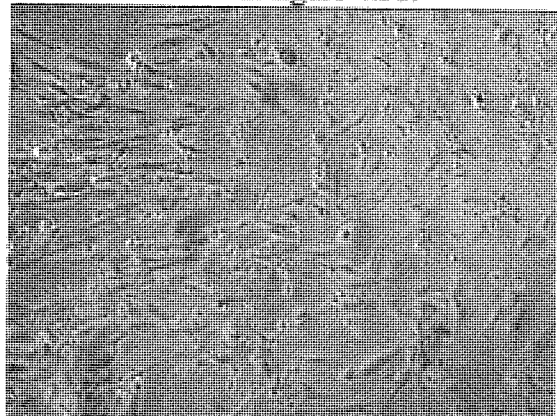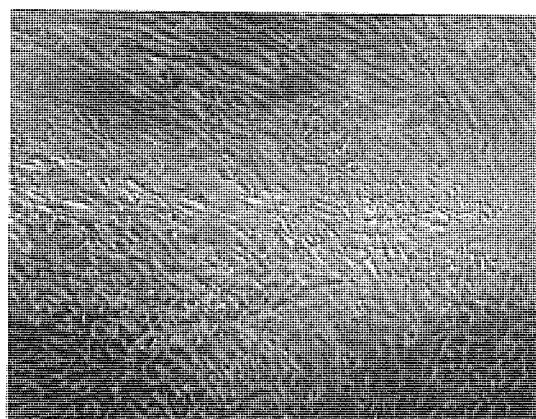
c   d

Visceral pre-adipocytes day 3 after passage (c) and day 5 after passage (d) : post confluence on day 5 growth media was exchanged for differentiation media.
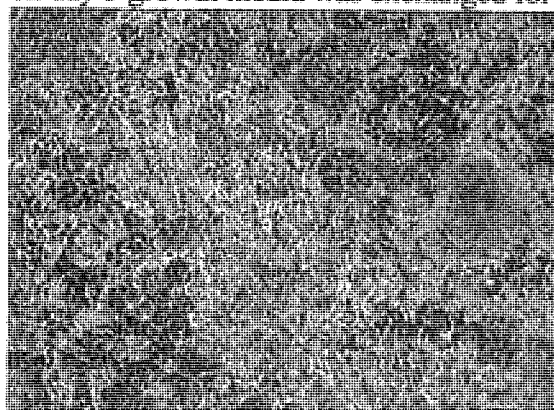
e   f
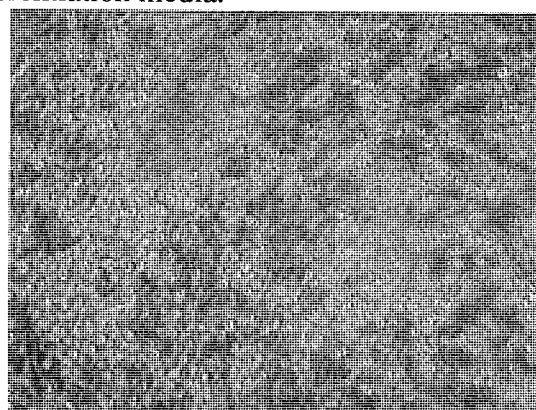
Visceral pre-adipocytes day 3 after differentiation (e) and 4 after differentiation (f), showing signs of maturation and formation of spherical shape and lipid droplet formation.

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Figure 4. 4: XTT cell proliferation assay

Primary pre-adipocyte cell proliferation using the XTT assay: day 4 after passage 1, seeded at 10^3 cells

* P<0.001 versus blank
θ P<0.001 versus 2h
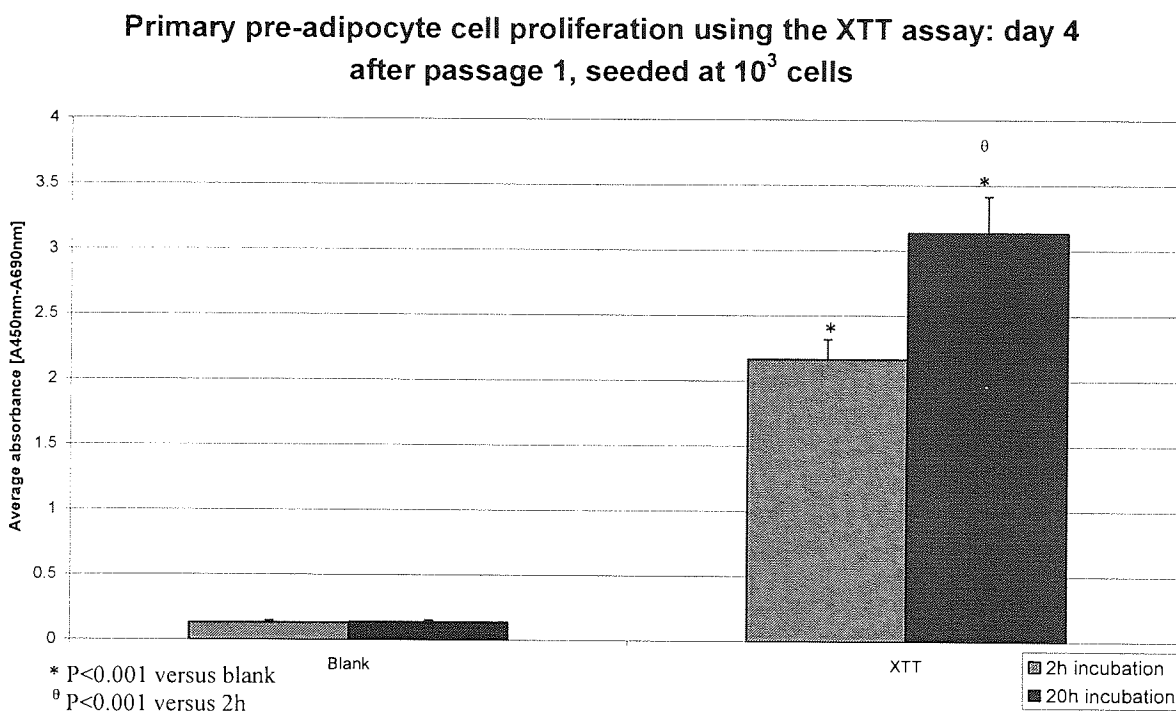
Figure 4. 5: BrdU colourometric assay of cell proliferation

Cell proliferation of primary visceral pre-adipocytes seeded at 10^3 cells using the BRDU colorometric assay

* P<0.001 versus blank
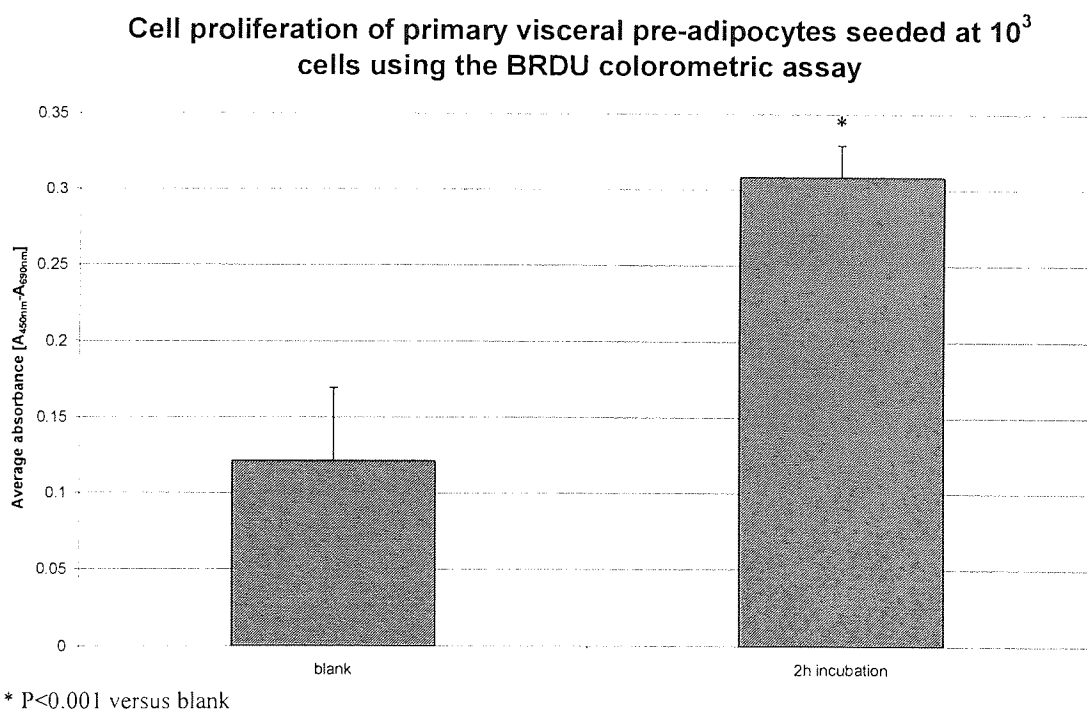
Figure 4. 6: Cell growth: graphical illustration

a) Pre-adipocyte cell growth over 14 days using haemocytometric cell counting techniques
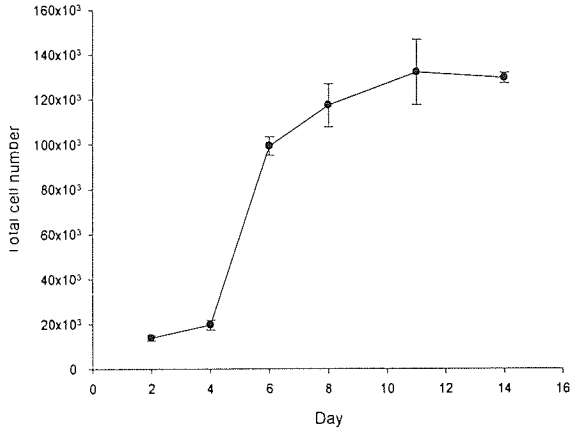
b) Pre-adipocyte cell growth over 14 days using a computerised cell counting techniques
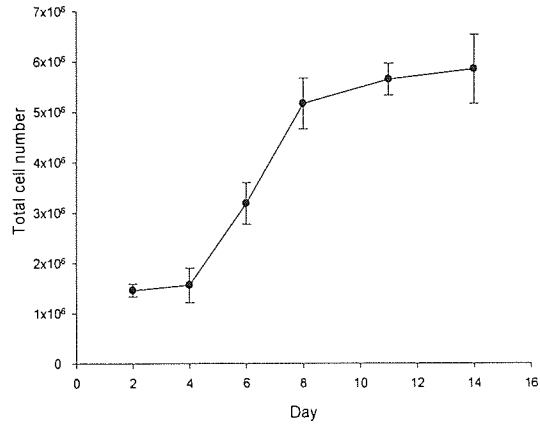
c) Cell count using haemocytometric and computerized counting techniques adjusted by a factor of 0.02*
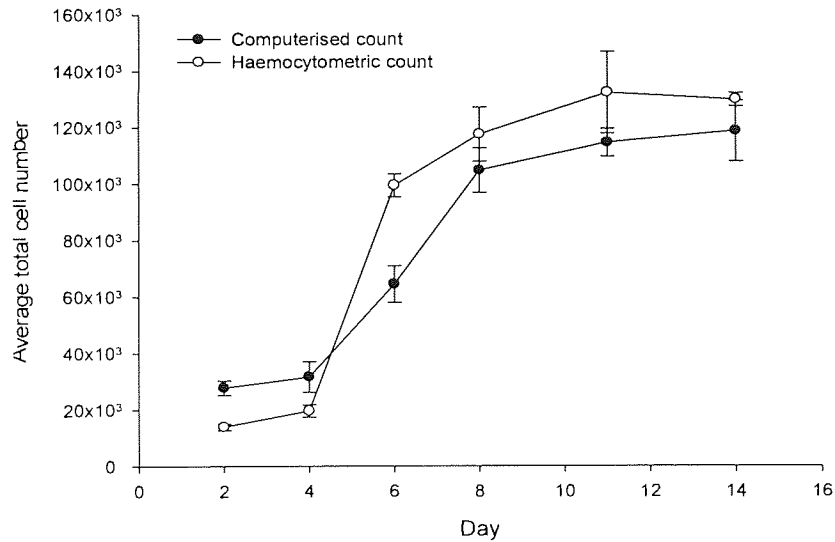
* The adjustment was performed using the calculated average of the ratio difference between cell number derived using each method per individual cell count. The average ratio was calculated to be 0.020409. The variance between the data range was 7.5×10^{-5}. As the variation between the data range was relatively stable an average was taken and used as a factor adjustment to enable comparability.

Nb: There was substantial clumping of cells in haemocytometric counts and thus increased cell number may have been determined through lack of clarity.

4.4 Mature adipocyte results

4.4.1 Mature adipocyte background

The content of a mature adipocyte is approximately 90% lipid (Frubeck *et al* 2002) with the cell oraganelles, nucleus and cytoplasm being pushed to form a thin rim. Adipose tissue can expand via hypertrophy and hyperplasia of cells (Hirsch and Batchelor 1976) and it is usually the hypertrophic cell that is associated with insulin resistance. The size of a mature adipocyte has an extensive range (5-200 μm) and as will be illustrated below has the capacity to divide (see section 4.3.2.1c). After the 'terminal' stage of differentiation mature adipocytes have previously been assumed to lack proliferative ability (Prins and O'Rahilly 1997, Stewart and Tomlinson 2002). However, Zhang et al (2000) employing ceiling culture technique has provided photographic evidence depicting budding of daughter cells. Corroboration of this finding was discovered using our free floating technique shown in section 4.9

4.4.2 Isolated human mature adipocytes

Cells were isolated (chapter 3), selected (section 4.2) and seeded into plates. They were subsequently photographed to enable sizing and counting (section 4.7) or stained with either oil red O or sudan III to validate cell type (section 4.8 and 4.9). Counted wells were initially seeded at 10^5 cells and maintained in culture. Photographs were taken every 2-3 days and computerised cell counts were taken, illustrated in figures 4.10 and 4.11.

Figure 4. 7: Photographs of isolated mature adipocytes: subcutaneous and visceral

Adipocytes were isolated from human samples as outlined in section 3.7.3 and photographed using a video stream microscope camera.
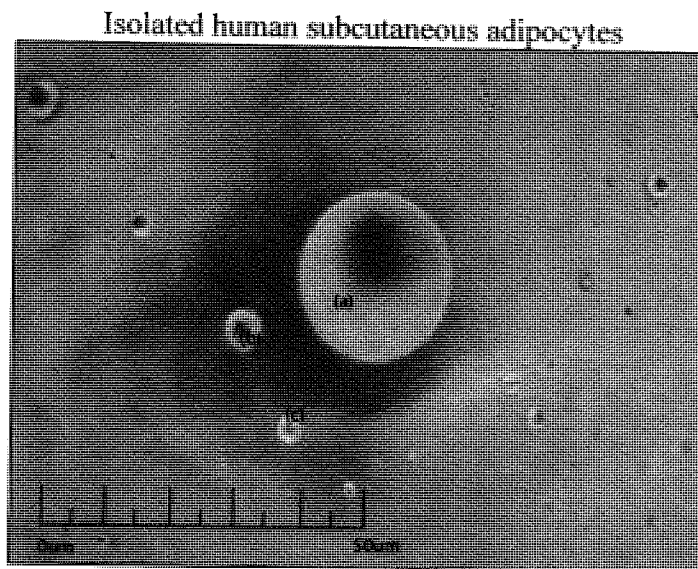
Figure 4.7a. Isolated human subcutaneous adipocytes from human sample ID 232400.
(a) $25\mu m$ x $26\mu m$, (b) $6\mu m$ x $7\mu m$, (c) $4\mu m$ x $5\mu m$
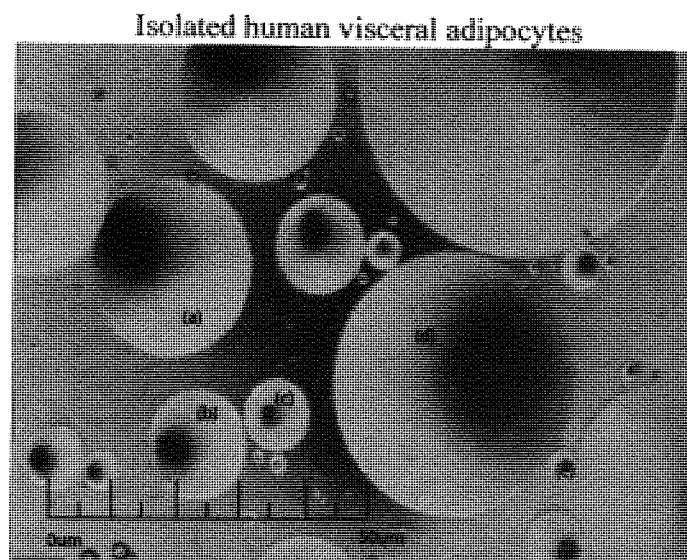
Figure 4.7b. Isolated human visceral adipocytes from human sample ID 232400. (a) $26\mu m$ x $28\mu m$, (b) $15\mu m$ x $17\mu m$, (c) $11\mu m$ x $11\mu m$, (d) $37\mu m$ x $40\mu m$, (e) $5\mu m$ x $6\mu m$

Figure 4. 8: Stained isolated mature adipocytes:

Subcutaneous human isolated mature adipocytes: a and b treated with Oil red O (2h), b) magnified at x8, c) treated with Sudan III (2h) magnified x10.
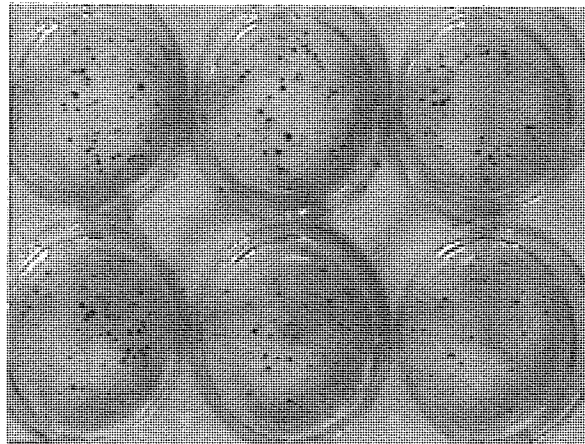
a) View of whole plate
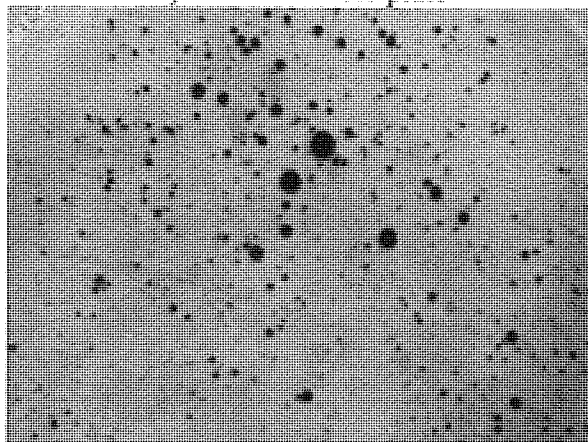
b)Single well with Oil red O
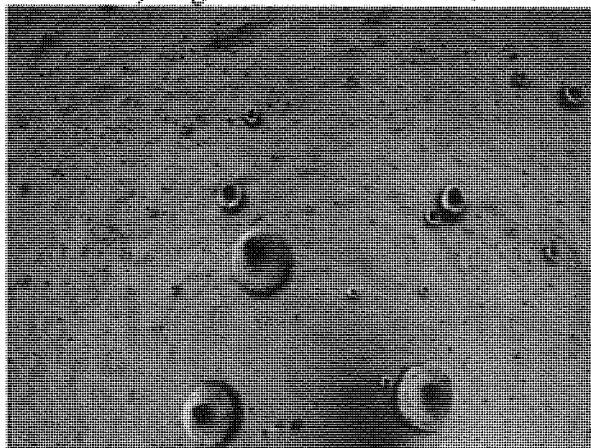
c) single well with Sudan III

Figure 4. 9: Stained isolated mature adipocytes:

Visceral human isolated mature adipocytes treated with Oil red O(2h), b and c magnified at x20.
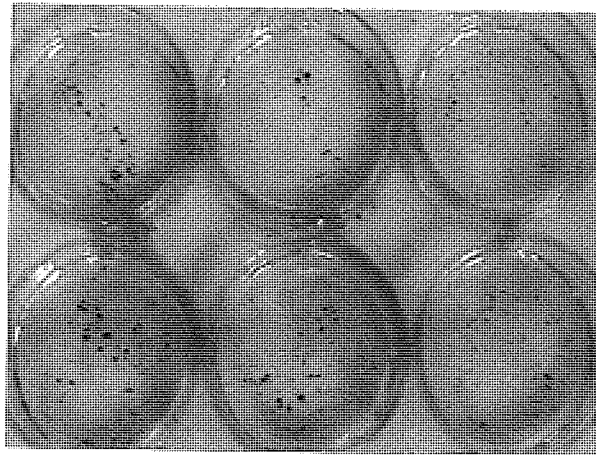
a) View of whole plate
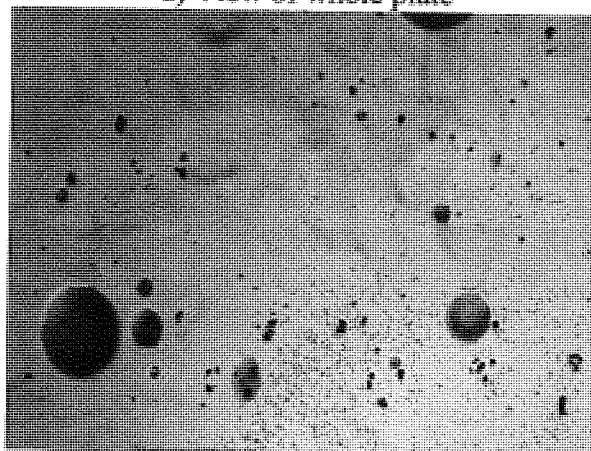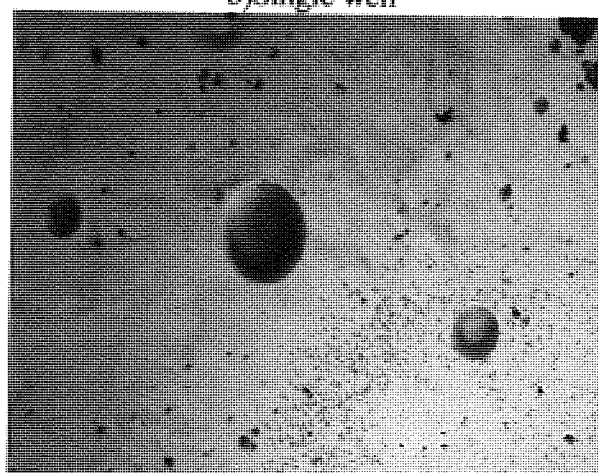
b)Single well

c) single well

4.4.3 Division of mature human adipocytes

Previously unrecognised; the identification of mature cell division is emerging as an important feature of adipose cell continuity and survival (see section 4.5). The images below have been taken *in vitro* of isolated cells in free floating culture. Figure 4.11 depicts graphical illustration of cell growth showing an increase in cell number over time. The presence of cell division is supported by XTT assay results incubated for 20-23h showing incremental increases in absorption only at the highest tested density (approx 10^6 cells per well) of cells (see figure 4.12).

Figure 4. 10: Mature adipocyte cell division

Cell division: Late prophase
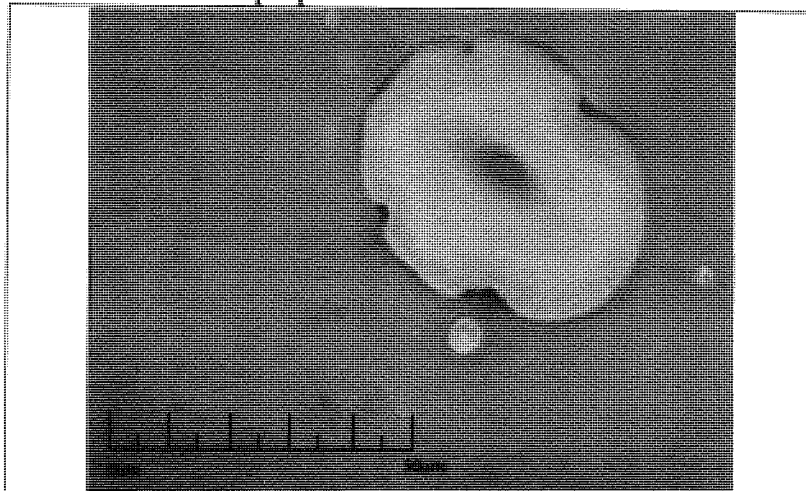
Figure 4.10a. Isolated mature adipocyte from human subcutaneous tissue. Size 42μm x 52μm

Cell division: Anaphase
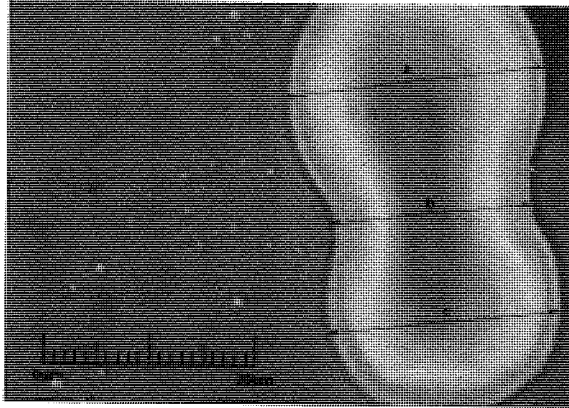
Figure 4.10b Isolated human adipocyte. (a) 25μm, (b) 18μm (c) 22μm. At higher magnification (not clearly in focus for photography) shows an indication of spindle formation around the cytoplasmic periphery of the cell

Cell division: Early Telophase
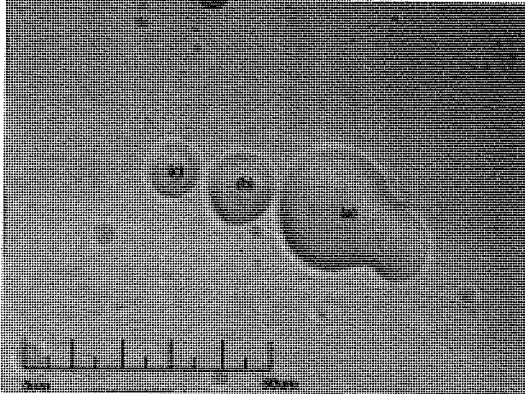
Figure 4.10c Isolated subcutaneous human adipocytes. (a) 42μm x 25μm, (b) 14μm x 14μm, (c) 10μm x 11μm
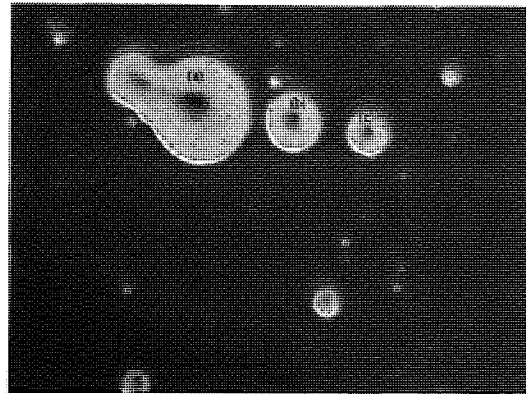
Figure 4.10d Isolated subcutaneous human adipocytes as seen in figure 2a, with an alternate light filter enabling observation of the nuclei

Cell division
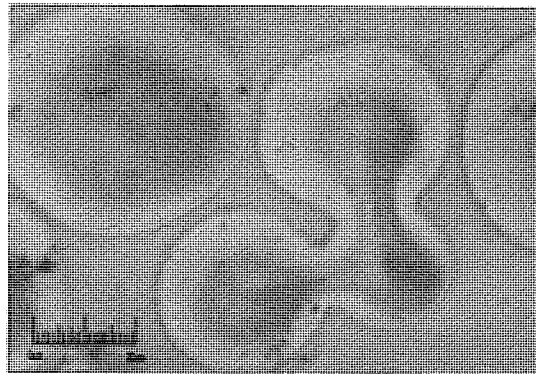
Figure 4.10e Isolated human adipocyte showing budding daughter cell

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Figure 4. 11: Growth curves

Human isolated adipocytes were grown as described in section 3.7.3, and their growth was monitored. Using a computerised cell counting method (3.8) the cells were counted every other day and growth rate was determined.
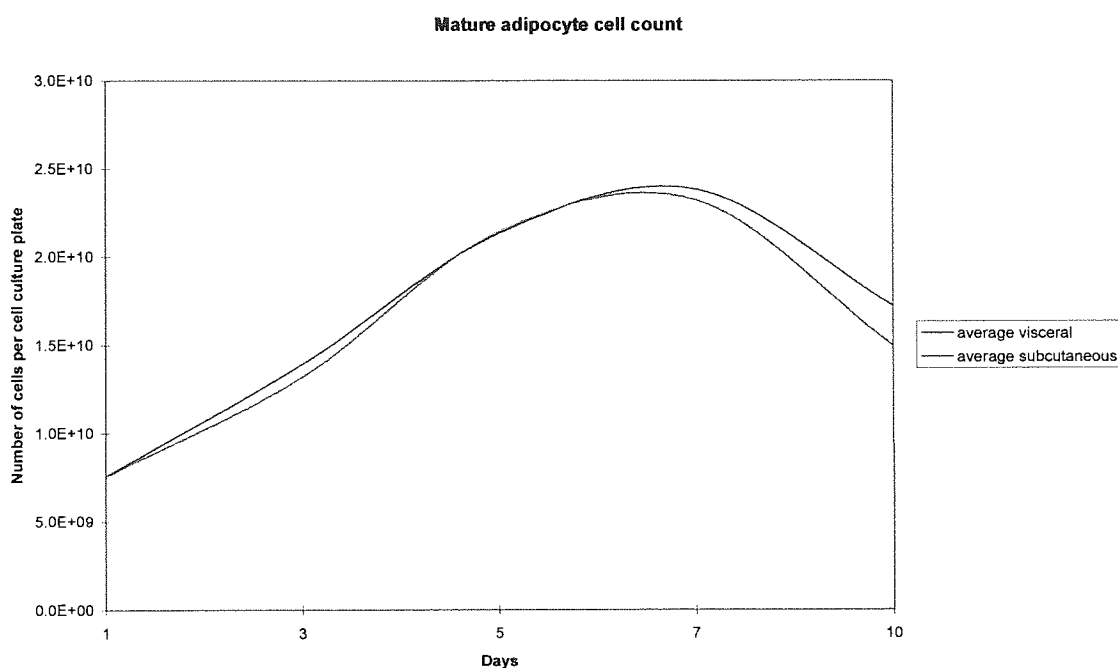
Figure 4.11: Mature adipocyte cell proliferation (y-axis numerical notation e.g. 5.0E+09 is equivalent to 5×10^9).

It is apparent that cell number shows an initial linear increase from the time of plating to approximately 5days. The number of cells begins to plateau after 5 days and subsequently decreases after 7 days. Therefore, for long-term culture studies experimentation should be conducted between days 5 and 7 to obtain stability of cell number which will ensure maximal accuracy and uniformity of expression of results. The decrease in cell number after 7 days was due to an apparent apoptotic effect. The changes in cell number may account for some

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inconsistencies in the literature with regard to the production and release of cytokines and other products of adipocytes in culture (Fain et al 2004, Koerner et al 2005)

Figure 4. 12: Mature adipocyte: XTT assay
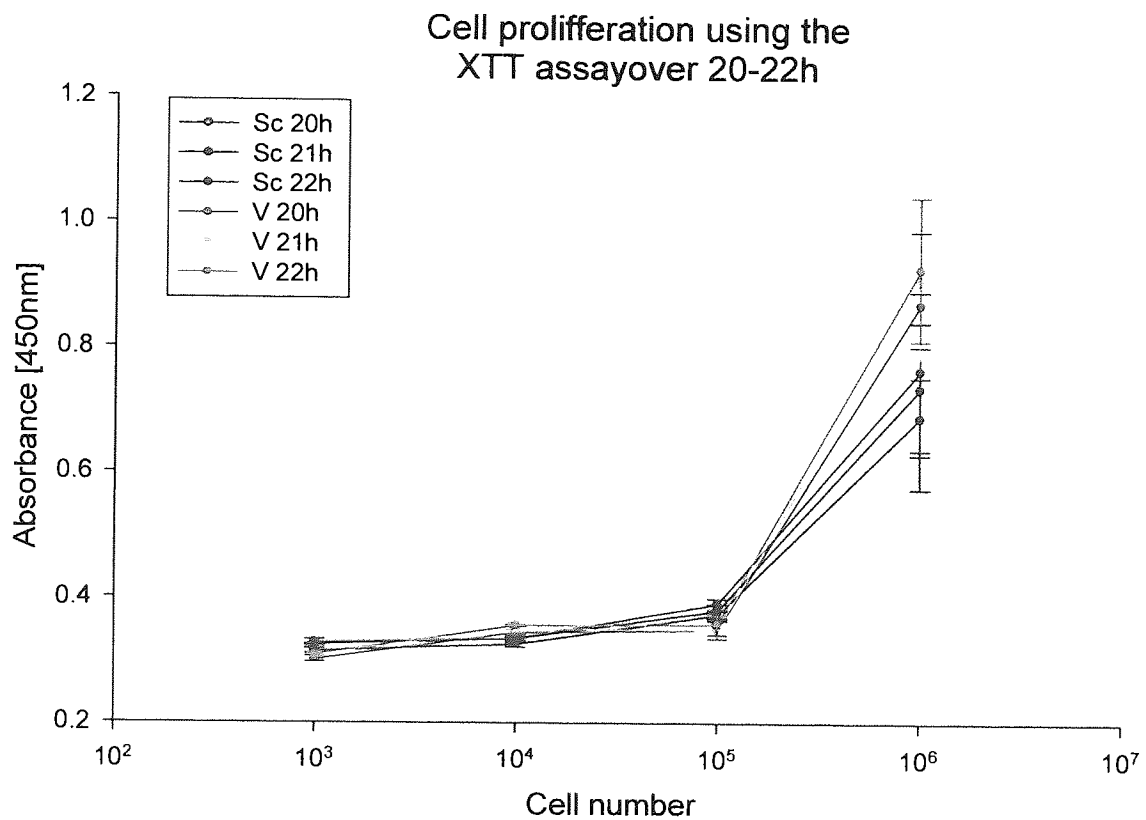
4.4.4 Protein analysis

Protein analysis (see figure 4.13) was determined to verify uniformity in cell dispensing between samples. Over several samples (n=16) the variation between depot specific data denoted by the standard error (visceral: s.e. = 17.04, subcutaneous: s.e. = 5.78). The depot variation is <10% of total protein (visceral: 6.003%, subcutaneous 2.636%). There is a statistical significant difference in cell number between subcutaneous and visceral depots using the t-test (p<0.0001).

Figure 4. 13: Mature adipocyte protein extraction analysis

Concentration of protein in 10^6 cells

*P<0.001 versus subcutaneous
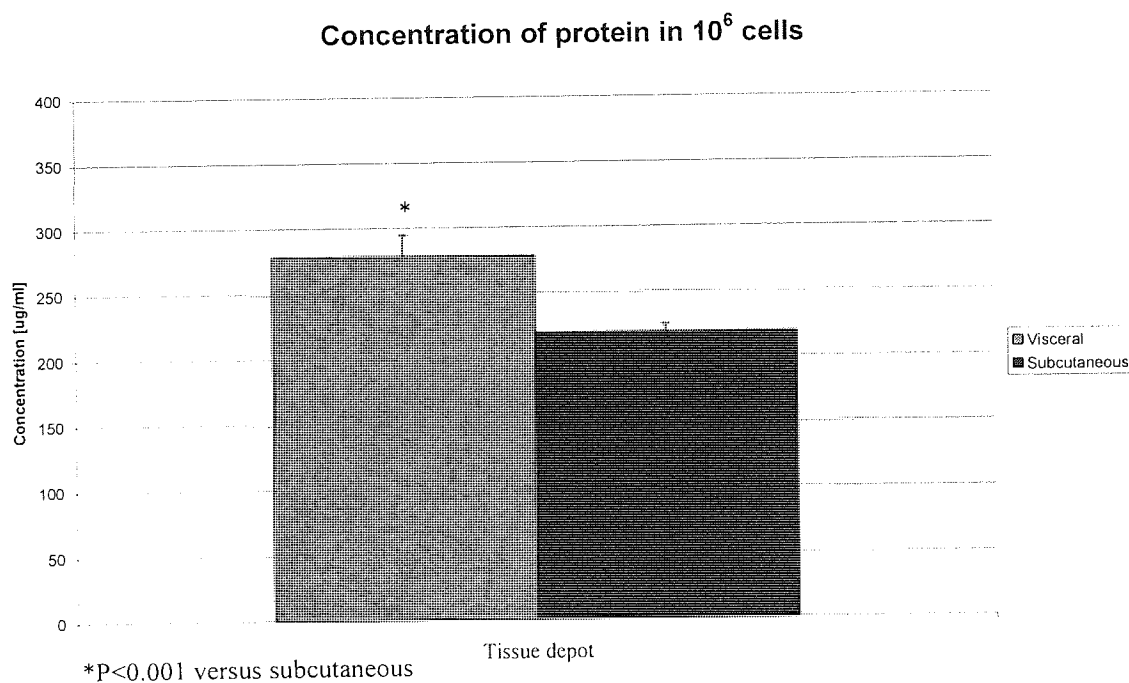
4.4.5 Lipolysis

Lipolysis is the stepwise breakdown of triglyceride into fatty acids and glycerol primarily to provide a source of energy during periods of low glucose (Arner 2005). Upon liberation via HSL enzymic breakdown; fatty acids can be re-esterified, undergo β-oxidation or be released, albumin-bound, into the circulation for use by other tissues (Albright 1998). In addition to providing an energy source, fatty acids elicit a multitude of responses, for example: fatty acids can influence insulin action and glucose metabolism (Arner 2005, Frayn 2002). Studies suggest that increased exposure to free fatty acids is correlated with a decrease in insulin sensitivity (DeFronzo 1997, 2004). In addition fatty acids display regulatory effects on insulin production by the pancreas and can alter gene function as transcription factors in various tissues (Arner 2005). Figure 4.14 illustrates the ability of isolated mature adipocytes to undertake lipolysis measured by release of glycerol. This provided an indication of cell viability and functionality in the present studies.

Figure 4.14: lipolytic activity

Lypolytic activity of isolated mature human adipocytes in the presence of increasing concentrations of isoproterenol
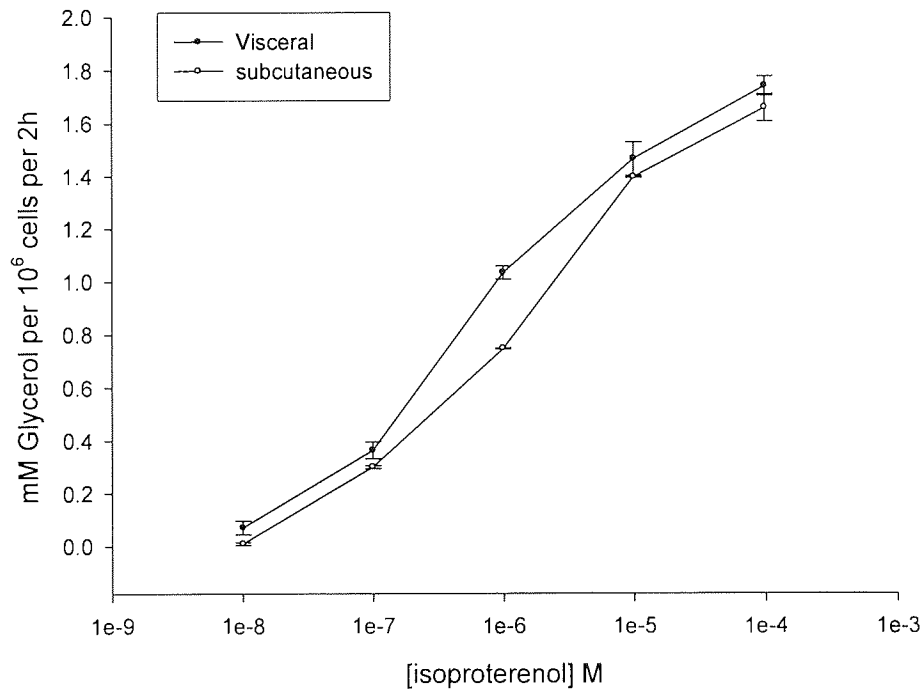
Figure 4.14: Values at each concentration of isoproterenol was significantly different ($p<0.05$, Student's t test) from each other concentration for the same type of adipocytes (visceral or subcutaneous). Visceral fat showed the higher rate of lipolysis at 10^{-6}M isoproterenol ($p<0.05$, Student's t test); This case of isoproterenol is approximately equivalent to the EC50, indicating increased sensitivity of visceral fat to lipolytic effect of isoproterenol.

4.5 Discussion

Historically it has been suggested that fat cell mass is relatively stable in adults. Most changes occur slowly as total adipose mass adjusts to an imbalance of energy intake versus expenditure and most of the changes in cell size with only a minor contribution of changes in adipocyte number (Bailey 1978, Gregoire 2001). With the emergence of evidence for the origin and differentiation of adult adipocytes from pre-adipocytes, attention focused on the possibility that the adipose mass represented a dynamic equilibrium in which some large mature adult adipocytes undergo apoptosis and are replaced by differentiation from the pool of pre-adipocytes in each adipose depot (Ashwell 1978, Ashwell and Meade 1981). Nevertheless there have been few attempts to quantitate this process, largely hampered by difficulties in the isolation and long-term culture of primary pre-adipocytes and mature adipocytes especially from humans. Moreover, possible differences between gender (Anderson *et al*) and different adipose tissue depots with regard to lipogenesis and lipolysis, (which have received considerable recent attention) have not been matched by similar attention to cell turn-over. The present section of this study was undertaken to fill some of the gaps in the understanding of adipocyte development.

A great deal of discussion has arisen in the field of obesity regarding the importance of depot specific adipose tissue function (Bakker *et al* 2004, Wajchenberg 2000, Arner 1997). There are clearly important structural and time-dependent functional disparities relating to the differentiation of visceral and subcutaneous adipocytes. Particularly evident from the present study was the relatively high percentage adherence of human visceral pre-adipocytes (>1%) to the basal surface of the tissue culture plate, whereas human subcutaneous pre-adipocytes showed low percentage adherence (<0.01%). Possible reasons for this could be due to cell

properties such as cytoskeletal adherence molecules, proportion of pre-adipocytes susceptible to induction into early differentiation and environment of extraction depot (Gregoire *et al* 1998). When compared with mature adipocytes adhesion pre-adipocytes have greater percentage adhesion in both depots (Eguchi *et al* 2005). The lack of attachment of mature cells may in part be as a result of their shape, the spherical form provides a smaller surface area to size ratio for adhesion, additionally lipolytic rate may impinge on membrane dynamics reducing membrane turnover rate. Expression quantity, function and specificity of adhesion molecules in isolated pre and mature human adipocytes has yet to be fully determined which may elucidate reasons for poor adhesion *in vitro* in both subcutaneous pre-adipocytes and mature adipocytes (Eguchi *et al* 2005, Gregoire *et al* 1998).

Photographic illustration (figure 4.3), cell proliferation assays (figure 4.4 and 4.5) and growth curves (figure 4.6) depict consistent growth of visceral pre adipocytes. Further investigative comparison between paired differentiated pre-adipocytes and isolated mature adipocytes would be an interesting path for future work in order to discover whether there is any significant difference in proliferation between tissue depots. Moreover, co-culture technique could be investigated to emulate the cohabitation environment *in vivo*, this methodology may provide important insight into the probable interaction of adhesion molecules release by pre-adipocytes with mature cells, the molecular triggers involved in differentiation of pre-adipocytes to mature cells and the relative rate of mature cell division in the presence of a pre-adipocyte pool; all this information is currently lacking from the body of knowledge.

Comparison of gene expression of these cell subtypes has previously been unavailable due to the relatively small quantity of pre-adipocytes and isolation techniques. However, using these methods, isolation and cell growth would enable these studies to be carried out. Relative gene and adipokine differences between human pre-adipocytes and mature adipocytes from the

same participant within and across the two tissue depots (subcutaneous and visceral) could be studiedfurther to reveal further specificity and action of current adipokines and potential targets for therapeutic intervention.

It has been almost universally noted that mature adipocytes do not have the capacity to divide although the evidence for this is by no means rigorous, and relies mainly on failure to observe dividing cells in early histology studies of human rodent fat (Bailey 1978, Arner 1997). The "terminal" phase differentiation, marked by the increase in stored lipid to a monolocular lipid droplet and increase sensitivity to insulin (Gregoire *et al* 1998) was assumed to be the end stage of adipocyte development. Studies regarding changes in cell number (hyperplasia of adipocytes) previously suggested that significant division will only occur in early childhood, and this has primarily been attributed to differentiation of the small pre-adipocyte pool (Gregoire *et al* 1998). In this study mature adipocytes were isolated and photographed (figure 4.7) to determine size, stained with either oil red O or sudan III to confirm the presence of unilocular stored lipid (4.8 and 4.9) and lipolysis was induced to substantiate function (figure 4.14). Total protein concentration was determined in order to evaluate variation in cell number between aliquots of samples. Analysis showed there was only a low variation between aliquots (<7% of total). Interestingly, a lower level of protein was determined for subcutaneous when compared with visceral aliquots. This may be due to increased solubilisation of membrane protein in the liberated triglyceride or due to an increase cell size versus cell number. Further mathematical analysis of the lipolysis data showed that differences between depots was >10% and therefore excludes variation due to a variation in cell number. During the study it was discovered that the quantity of cells was increasing: thus a method of counting the free floating cells was devised (see section 3.3.5). Cell growth charts were plotted (figure 4.11)

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confirming an increase in cell number from carefully isolated cells to reduce and prevent potential pre-adipocyte contamination. Indeed cultures were thoroughly scrutinised at all times to exclude presence of morphologically identifiable pre-adipocytes. Further verification of this phenomenon was captured by photographs (e.g. figure 4.10) which clearly depict cell division. Unfortunately the whole process, which appears to be relatively slow, has yet to be captured to video due to the floating nature of the culture technique keeping the cells in constant motion. As a consequence they often drift out of the field of vision. The cell division process has been discovered independently by Zhang *et al* (2000) who used a ceiling culture technique also providing photographic affirmation of this event. Quantification of cell proliferation was also determined by XTT assay (figure 4.12). Interestingly at lower concentrations no significant difference in cell number is seen over the 24h incubation period. However, seeding at a "critical mass" (10^6 cells) there is a substantial increase in cell proliferation which is notably higher in visceral cells when compared with subcutaneous. This increase in cell number is incremental over the 3 time periods assessed. This observation may alter our understanding of adipocyte development beyond the current perception. The ability of these cells to divide and the consideration of a "critical cell number" prior to division of mature cells may provide another mechanism to ensure the presence of smaller more insulin sensitive cells to enable further fatty acid uptake. The present data support an increased proliferation of mature cells in the visceral depot, thereby backing an earlier theory suggesting visceral cells have a greater capacity to remain small and insulin sensitive. It must be noted that division of mature adipocytes occurred in relatively low levels of fatty acids (derived from FCS supplement of the media). Future investigation focused on mature adipocyte division could examine the effects of varying the concentrations of fatty acids (chain length and saturation) to represent current dietary and physiological proportions in lean and obese individuals.

Chapter 5: Genes involved in the sensing and control of adiposity

5.1 Introduction

Since the discovery in recent years that adipose tissue is an endocrine organ there has been a dramatic increase in the number of identified genes and potential therapeutic targets is this tissue. Investigation of genes involved in the sensing and control of adipose tissue deposition and density will improve understanding of potential new drug targets and enable production of pharmaceutical intervention products of greater efficacy and potency for the ultimate pursuit of decreasing obesity, associated T2DM and other-related disorders.

The following chapter will provide background information on the genes of interest and detail the results obtained by Taqman RT-PCR from visceral and subcutaneous fat depots of male and female participants who were either lean or obese. The findings will be evaluated with regard to obesity.

5.2 Background

5.2.1 Stearoyl-CoA desaturase (SCD or Δ9 desaturase)

SCD is located in the endoplasmic reticulum and catalyses the biosynthesis of monounsaturated fatty acids (MUFA) from saturated fatty acids in the presence of NADH (Dobrzyn and Ntambi 2005a). This rate limiting enzyme primarily catalyses the formation of oleate (18:1) and palmitoleate (16:1) which are the major monounsaturated fatty acids of membrane phospholipids, triglycerides, wax esters and cholesterol esters (Ntambi and Miyazaki 2004). The proposed mechanism of action has been comprehensively described by Ntambi and Miyazaki (2004) and is beyond the scope of this thesis.

See figure 5.4

```
                              LCE
     Palmitate  ──────────────────────────►   Sterate
     (C16:0)                                    (C18:0)
        │         ◄──────  SCD  ──────►            │
        ▼                                          ▼
     Palmitoleate ────────────────────────────  Oleate
     (C16:1n-7)          │    │    │    │       (C18:1n-9)
                         ▼    ▼    ▼    ▼
                        CE   PL   TG   WE
```

See figure 5.2
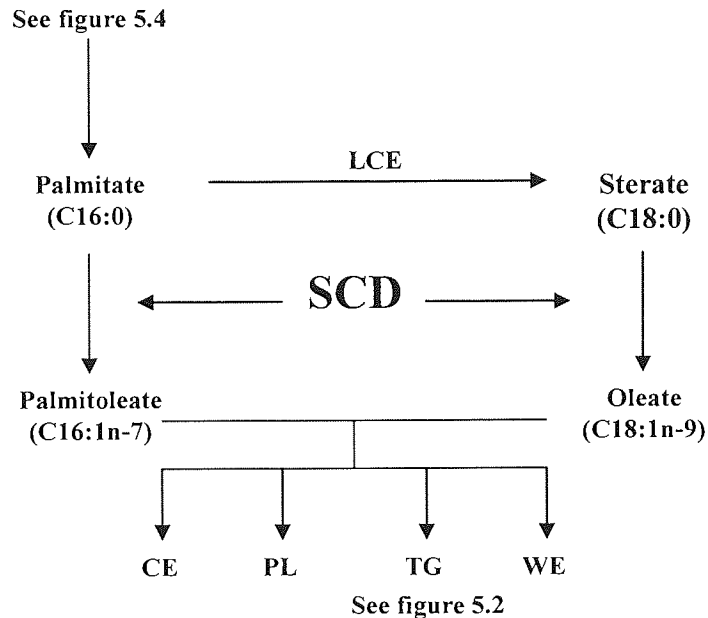
Figure 5. 1: Role of SCD1 in lipid synthesis

CE: Cholesterol ester; TG: Triglyceride; WE: Wax esters; PL: Phospholipids; SCD: Stearoyl-CoA desaturase; LCE: Long chain elongase. Modified from Ntambi and Miyazaki (2004).

MUFA synthesised by SCD are the major substrates for acyl-CoA: cholesterol transferase (ACAT) and DGAT (Described in section 5.2.3 and chapter 1). Animal studies on the mutated SCD1 gene have elevated interest in both gene and product as potential targets for pharmaceutical manipulation or intervention. Mice deficient in SCD1 have increased energy expenditure, reduced adiposity, increased insulin sensitivity and are resistant to diet-induced obesity (Dobrzyn and Ntambi 2005b). SCD contributes to adipocyte differentiation by providing the building blocks required for the triglyceride component of mature adipocytes. In addition it was discovered to be a key signalling molecule involved in the response to leptin stimulation (Cohen *et al* 2002). Leptin has been shown to repress SCD1 gene expression and SCD hepatic enzyme activity (Cohen *et al* 2002).

Further evidence of SCD's pivotal role in the path to obesity comes from animal studies. Namely mice on a high carbohydrate diet have increased hepatic SCD1 gene expression and increased expression of other lipogenic genes via the sterol regulatory element binding protein (SREBP-1) dependent mechanism. This ultimately results in the increase of MUFA and hepatic triglycerides. However, in the absence of a functional SCD1 gene this process is nullified and the animal is unable to build up triglycerides, even though their dietary intake is 25% greater than wild type. On a high fat diet, similar resistance to weight gain is observed. Thus the absence of the gene appears to confer protection against diet induced weight gained from triglyceride formation and obesity (Drobrzyn and Ntambi 2005b).

Knocking out SCD1 gene provides therapeutically desirable effects with regard to obesity; in the mouse model of SCD deficiency there is a reduced level of circulating triglyceride in the VLDL and LDL particles in comparison to wild type. In humans it has been proposed that SCD acts as a "check point" for metabolism of cholesterol, triglyceride and lipoproteins, and thus would provide a novel target for treatment of hypertriglyceridaemia and obesity (Ntambi and Miyazoki 2004).

However, SCD1 expression has a critical role enabling formation of Oleoyl-CoA which enables ACAT-mediated cholesterol esterification, DGAT-mediated triglyceride synthesis, and wax synthase-mediated wax ester synthesis (Ntambi and Miyazoki 2004). These processes allow the homeostatic maintenance of the skin and eyeball. In addition the increase in free cholesterol is known to result in cell death (Ntambi and Miyazoki 2004). One may speculate that an increase in free cholesterol may also increase the risk of cardiovascular disease especially in the presence of obesity.

Therefore, given the evidence outline here and by Ntambi and colleagues, attenuation of gene expression or enzyme activity would be preferable to complete evisceration of expression or

enzyme action. SCD remains a viable and scientifically attractive therapeutic target, and pharmaceutical intervention prior to severe obesity would potentially reduce probable side effects.

5.2.2 GPAT: Glycerol-3-Phospahate Acyltransferase

The synthesis of TG and other glyceropholipids commences with the acetlylation by glycerol-3-phosphate (G-3-P) by GPAT to form lysophosphatidic acid (LPA) (Hammond *et al* 2002). There are two isoforms of GPAT in mammalian cells, encoded by different genes. Mitochondrial (mt) associated GPAT and microsomal (m) associated GPAT which is present in the endoplasmic reticulum. An increase in specific activity of mtGPAT has been shown to up-regulate TG synthesis. It may also direct the fatty acid flux towards glycolipid synthesis in preference to β-oxidation. It has been suggested that carnitine palmitoyl-transferase 1 (CPT-1) which is also located on the outer mitochondrial membrane and competes for acyl coenzymes against mtGPAT. These enzymes, CPT-1 and mtGPAT, are reciprocally regulated by AMP-activated kinase. AMP kinase blocks synthesis of malonyl-CoA via inhibition of GPAT. Malonyl-CoA is the natural inhibitor of CPT-1 thus when mtGPAT is inhibited CPT-1 is activated and there is a subsequent expected increase in fatty acid oxidation (Hammond *et al* 2002). This process occurs naturally when energy stores are decreased. AMP-K is up-regulated resulting in an increase in β-oxidation of fatty acids. This potential target for manipulation is supported by animal studies. In mtGPAT deficient mice there was a reduction in weight and fat deposition when compared with wild type. In addition there was a corresponding decrease in the insulin level and a subtle increase in insulin sensitivity (Hammond *et al* 2002).

The G-3-P pathway enzymes have been shown to be more responsive than enzymes in the mono-acyl glycerol pathway (MG) in obesity, therefore would be better targets for manipulation. Experiments have demonstrated a rise in activity of all G-3-P enzymes in adipose tissue and intestine of mice with an obese phenotype (Jamdar and CaO 1994)
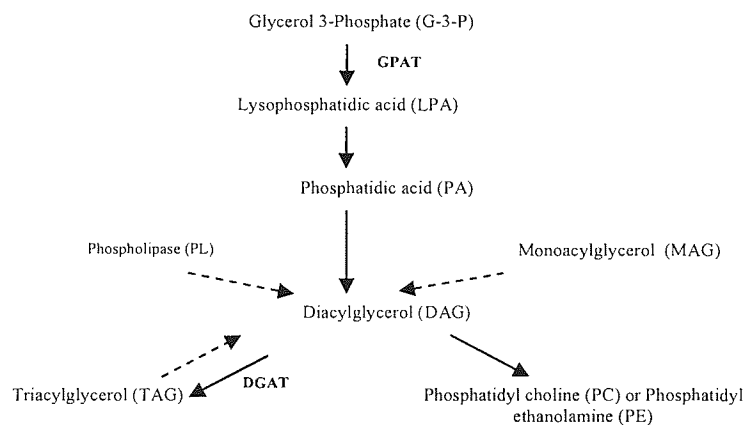
Figure 5. 2:Triglyceride synthesis

5.2.3 Diacylglycerol transferase (DGAT): EC 2.3.1.20

There are two DGAT enzymes which have little gene homology. Differentiation between the activity of DGAT1 and DGAT2 is accomplished as a result of its sensitivity to $MgCl_2$ (Cases *et al* 2001).

5.2.3.1 DGAT1

DGAT1 is a microsomal enzyme required for the catalysis of the terminal and only dedicated step in mammalian triglyceride sysnthesis (Chen *et al* 2002).
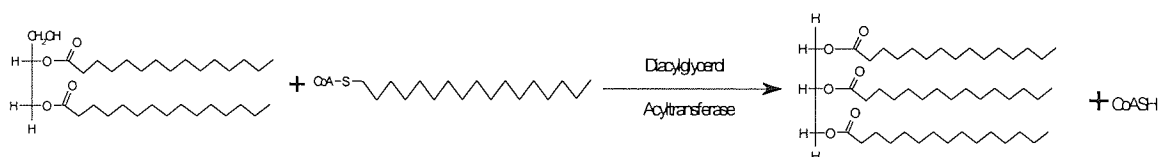
Figure 5. 3: Triglyceride synthesis by DGAT
Adapted from methods in enzymology; Lipids part C, vol. 71 p521, edited by John Lowenstein, 1981

In addition DGAT1 has an important role in the production of glycolipids (Lehner and Kulsis 1996). In animal studies DGAT1 deficiency did not alter expression of key signalling molecules in the hypothalamic leptin pathway, nor did it alter energy intake or energy expenditure in these mice (Chen *et al*, 2002). In addition DGAT1 knock out mice (DGAT1$^-$/-) exhibit normal plasma triglyceride levels. However, DGAT1 deficient mice are resistant to diet-induced obesity due to increased energy expenditure, partly facilitated by increased physical activity. The increase in energy expenditure was independent of changes in ambient temperature and DGAT 1 deficient mice appear to have acquired constitutive activation of thermogenesis resulting in unrestrained heat dissipation to the environment (Chen *et al*, 2002). These animals also showed increased glucose disposal after a glucose load and increased sensitivity to both leptin and insulin: conditions which are associated with reduced levels of

tissue triglycerides with the exception of adipose tissue (Chen *et al*, 2001; Chen *et al*, 2002). Chen *et al*. (2002) demonstrated that leptin sensitivity is enhanced in the presence of peripheral leptin infusion; however there is no observed enhancement of energy expenditure after intracerebroventricular leptin infusions. This suggests that DGAT deficiency has a specific local effect on leptin sensitivity independent of leptin sensitivity in the brain. Recent studies have shown a strong correlation between tissue (liver and muscle) triglyceride content and insulin resistance (Boden, 2001; Boden *et al*, 2001; Chen and Farese, 2002). Hence reduced triglyceride content of the tissues increases their sensitivity to insulin. A decrease in adipocyte size was also observed in the DGAT1 knock out mice, possibly due in part to an increase in physical activity. It is conceivable that increased activity of the mice caused an increase in muscle contractions which subsequently caused an enhancement in muscle tissue sensitivity to insulin. DGAT1 deficiency appears to increase activation of the leptin pathway and leptin has been shown to enhance insulin action (Bailey *et al*, 2000). Increased leptin sensitivity in DGAT1 knock out mice seems to have an effect on energy expenditure independent of the effects on food intake. Moreover, DGAT1 deficiency appears to protect against obesity in animal models of obesity with an intact leptin gene, but there is no effect of DGAT1 deficiency in *ob/ob* mice which do not produce active leptin (Chen *et al* 2001).

Prolonged fatty acid exposure has been found to cause lipotoxicity of the pancreatic β-cells and impair insulin secretion. Prolonged exposure to fatty acid has been associated with increased triglyceride accumulation in islet cells (Zhou *et al*, 1996), a phenomenon which Kelpe *et al* (2002) determined to be dependent on the presence of elevated glucose levels. This TG accumulation may also be due to HSL-deficiency as seen in mice with impaired insulin secretion (Roduit *et al*, 2001).

Kelpe *et al* (2002) showed that DGAT1 over-expression in rat islet cells reduced secretion of insulin in response to glucose after prior prolonged elevated glucose concentrations. Two mechanisms to explain the effects of FA on insulin have been proposed

1) Operation of a local glucose-fatty acid cycle in the mitochondria

2) The accumulation of long chain CoA in the cytosol.

The former proposal suggests that an increase in mitochondrial FA oxidation inhibits glucose oxidation through the differential selection of metabolic fuel based on proportional availability within tissues (Randle *et al*, 1963). The latter proposal is focused on malonyl-CoA/LC-CoA and postulates that metabolism of glucose generates malonyl CoA, which inhibits carnitine palmitoyl transferase 1 (CPT1). A reduction in CPT1 would then reduce the transport of LC-CoA into the mitochondria resulting in reduced mitochondrial oxidation of fatty acids. Reduced CPT1 activity also leads to accumulation of fatty acyl-CoA in the cytosol (Prentki *et al*, 1992). Chronically raised cytosolic LC-CoA can then act as a signal to uncouple secretion by β-cells (Prentki and Corkey, 1996).

Kelpe (2002) demonstrated that in cases of prolonged glucose elevation accompanied by an increase in TG synthesis impairs glucose-induced insulin secretion. Thus the presence of increased FA esterification in high glucose concentrations is probably a key mechanism of lipotoxicity (Unger 2003, Unger 2001).

Acetyl- CoA is a precursor for LC-FA (Wakil *et al*, 1983). It is derived from glycolysis and is transferred to the cytosol via an ATP-citrate lyase (ACL) for fatty acid synthesis.

Acetyl-CoA

Acetyl-CoA Carboxylase
(ACC)

Malonyl-CoA

Acetyl-CoA

NADPH

Fatty acid synthase
(FAS)

Palmitate
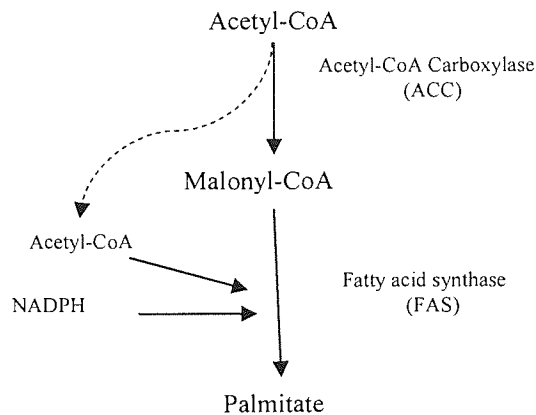
Figure 5. 4: Malonyl-CoA Pathway

NADPH is produced by reactions catalyzed by the enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme, all of which are regulated to some extent by glucose and insulin (Towle *et al*, 1997; Girard *et al*, 1997).

Triglyceride is synthesized primarily in the small intestine, liver and adipocytes. In the intestine, dietary fat is converted to free fatty acids via pancreatic lipase. These are absorbed by the intestinal epithelial cells. These long chain fatty acids are re-esterified to triglycerides and packaged into chylomicrons, instigated by LPL. Apo-lipoprotein (CII) on chylomicrons activates lipoprotein lipase (LPL). Then free fatty acids are released and are taken up by various tissues for utilisation or lipid storage. Alternatively fatty acids are obtained by exogenous synthesis from dietary carbohydrate that is not used for energy production. This takes place in the liver, where the fatty acids are further esterified into triglycerides and associated into VLDL lipoprotein particles. The VLDL particles are absorbed by target tissues. Adipose tissues obtain fatty acids from chylomicrons or VLDL and re-esterified into triglycerides to be stored (Frayn 2003 chapter 4).

5.2.3.2 DGAT2

The second DGAT may account for some of the residual DGAT activity in knock out mice, apparent at low concentrations of $MgCl_2$. DGAT2 mRNA expression was increased 3 fold in white adipose tissue (WAT) of leptin-deficient *ob/ob* mice, which was not observed in high fat feeding or $A^{y/a}$ mice where DGAT deficiency confers protection against insulin resistance and obesity. This increase in DGAT2 may therefore be a compensatory effect for DGAT 1 in the absence of leptin.

DGAT activity is enhanced in the presence of glucose due to increased DGAT1 mRNA levels, whereas insulin elevates DGAT activity via a specific increase in expression of DGAT2 mRNA (Meegalla *et al* 2002). Therefore carbohydrate and insulin appear to stimulate TG synthesis through independent mechanisms, acting in an additive fashion. It was noted that carbohydrate is needed to maintain high DGAT activity levels in adipocytes. In carbohydrate starvation DGAT 1 appears to take precedence over DGAT2.

5.2.4 Adiponectin

The adiponectin (ACRP30, AdipoQ) gene encodes for a 244 amino acid polypeptide (Haluzik *et al* 2004). It is expressed in white and possibly brown adipose tissue. Adiponectin expression decreases with an increase in adiposity, glucocorticoids, β-adrenergic agonists and TNFα. Adiponectin increases with a decrease in adiposity, exposure to cold, adenalectomy and IGF-1 (Fasshauer *et al* 2002). Insulin is also a regulator of adiponectin gene expression but has different effects dependent on duration and dose (Haluzik *et al* 2004). Adiponectin is the most abundant adipose tissue protein product (Stefan and Stumvoll 2002). It has been shown to decrease with obesity relative to lean individuals (Arita *et al* 1999, Yang *et al* 2001) and appears to be higher in females than males (Arita *et al* 1999). The decrease in adiponectin

associated with increased adiposity appears to be inherently linked to insulin resistance, and the administration of the insulin sensitizers thiazolidinediones (pioglitazone and rosiglitazone) which activate the PPARγ receptors may improve insulin sensitivity in part by up-regulating adiponectin (Haluzik *et al* 2004). Yamauchi *et al* (2003) describe two adiponectin receptors: AdipoR1 and AdipoR2. The former is located in muscle and is activated by the globular domain of adiponectin; the latter is only activated when presented with the full length ligand (Miner 2004).

5.2.5 Leptin

Leptin is a single-chain polypeptide hormone, 16KDa in size comprising of 167 amino acids encoded for by the Ob-gene. Leptin is the natural ligand for the leptin receptor, of which there are several isoforms. The active receptor is the long-form (Ob-Rb) which has an extracellular hormone-binding region and an intracellular signalling domain. The short forms of the receptor within the choroid plexus my be involved in leptin transport across the blood-brain barrier and enable the hormone to activate the Ob-Rb receptor within the hypothalamus (Frayn 2003). Leptin provides a signal for lipostasis: the account of leptin is approximately proportional to the adipose tissue mass. Leptin in conjunction with insulin acts within hypothalamic nuclei to reduce neuropeptide Y signalling (NPY) and POMC pathways (Stanley *et al* 2005, Konturek *et al* 2005). In the presence of low levels of leptin there is an increase in the NPY pathway stimulation, which in turn increases hunger and decreases satiety (Frayn, 2003). Levels of leptin mRNA expression correlate significantly with body fat mass (Van Gaal *et al* 1999).

5.2.6 11β-HSD1

11β-HSD1 is one of the enzymes known that convert inactive cortisone to active cortisol in humans. Local level control of cortisone activation in adipocytes may play an important role in the manifestation of obesity. Further research into the application of 11β-HSD1 within the study of obesity is outline in chapter 1 and chapter 6 and shall not be repeated here.

5.2.7 GPR41

The relatively little known about GPR41 is outlined in section 1.5.1.4 and will not be repeated here.

It is unclear at present if there are differences in gene expression levels encoding for protein signalling molecules between gender, adiposity or tissue depots. Therefore expression of obesity related genes (adiponectin, leptin, SCD, GPAT, DGAT1, DGAT2, 11β-HSD1 and GPr41) genes have been investigated in the present study in human lean and obese and paired visceral and subcutaneous tissue from male and female subjects.

5.3 Method

5.3.1 Tissue analysis

Tissues were extracted from human participants (lean patients: BMI 20-26 kg/m^2 overweight and obese patients BMI 28-33 kg/m^2; Age range 27-78 years; 7 male; 11 female) undergoing elective surgery as outlined in section 3.1. Tissues were frozen at -80°C after being cleaned in Krebs-Ringer buffer and weighed. Upon use tissues were maintained in their frozen state on dry ice and immediately transferred into an appropriate volume of tiazol in preparation for RNA extraction (see chapter 3.4.1). After mechanical homogenisation the required volume of homogenate was transferred to a fresh centrifuge tube and the remaining homogenate was frozen at -80°C for later use. RNA was extracted as described in chapter 3.7 and the resultant RNA was measured via nanodrop technology (see chapter 2 and 3.7). RNA was then converted to cDNA for use in the Taqman assay (see chapter 3.7). An example of a RT-PCR amplification plot is shown in figure 5.4 (also see appendix t chapter 5). The best performing two house keepers were chosen from a pool of 8 tested genes (HPRT, Cyclophilin, β-Tubulin, Ubiquitin C, Ribosomal protein, Transferin receptor, Phosphoglycerate kinase, β-Glucaronidase). These housekeeper normalisation factors (see 5.3.2) were used to normalise the gene of interest (GOI) results.

5.3.2 Data analysis

House keepers utilised were derived using the computer freeware package 'GeNorm' (http://medgen.ugent.be/~jvdesomp/genorm/). The most stable reference (housekeeping) genes were determined from the set of candidate reference genes (listed in 5.3.1), resulting in a gene expression normalization factor being calculated for each sample. Normalisation factors are

based on the geometric mean of the house keeping gene data for each sample (**http://genomebiology.com/2002/3/7/research/0034/**). Thus the normalization factor derived for each sample was subsequently used to normalize the results obtained from the GOI. These data were plotted for each paired sample from both depots, for all GOI. However, as the assay products for each gene vary no direct quantitative comparison can be made between genes. Data are presented as values (mean of triplicate values) for each gene in each adipose depot (subcutaneous and visceral) of each subject studied. SEM was not included as values would be too small to be observed on the graph. Tissue of each participant has been depicted graphically in a paired fashion. Data were analysed by three way analysis of variance (3-way ANOVA). 3-way ANOVA was determined using factors of gender (male/female), depot (subcutaneous/visceral), and adiposity (lean/obese). Post hoc analysis was performed using the Holm-Sidak test in the sigma stat 3.0 statistical analysis software; a pair-wise comparison of multiple differences of means correcting for interaction between subgroups in order to corroborate previous ANOVA statistics (SPSS inc. Chicago IL, http://www.systat.com/products/SigmaStat/).

5.4 Results

5.4.1 Introduction

There have been a plethora of genes relatively recently discovered that inscribe molecules which play an important role in the metabolic regulation of adiposity, either directly (such as leptin) or indirectly within the central and peripheral signalling cascade. However, comparatively little information has been documented regarding the difference in expression between tissue depots, gender and adiposity. This project examined these differences in a pilot study using laproscopically excised tissue which reduces cell damage upon removal from the patient. The following results pertain to 8 genes: SCD1, GPAT, DGAT1, DGAT2, 11β-HSD1, Leptin, adiponectin and GPR41. The first four genes encode for important regulators of fatty acid synthesis and as such provide novel targets for therapeutic intervention both individually and as a group. The opportunity to reduce 11β-HSD1 would locally reduce cortisol production and adipocyte differentiation. Up-regulation of leptin should increase satiety. Leptin acts directly upon the brain to increase satiety. Finally GPR41 provides a novel target within the leptin signalling cascade previously determined to be absent in adipocytes. All genes were normalised (see method) and interpreted graphically and statistically.

5.4.2 Taqman amplification plot
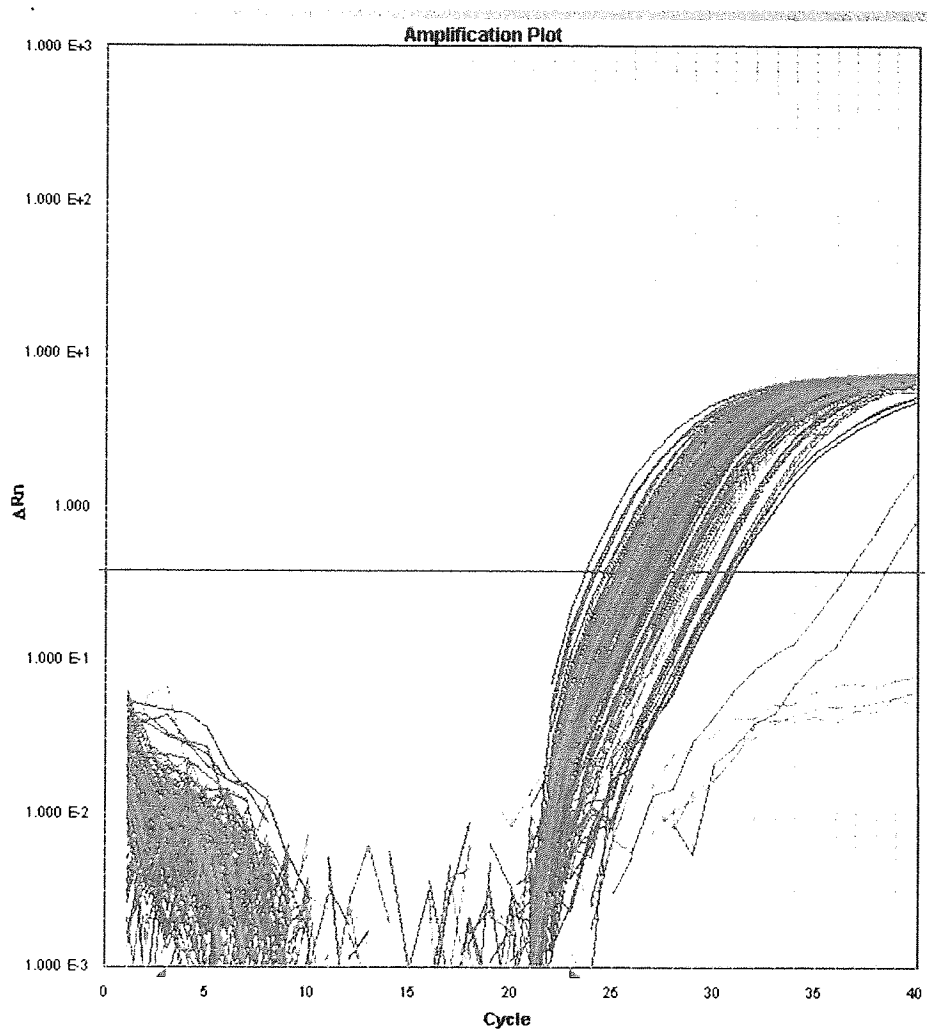
Figure 5. 5: Amplification Plot of house keeper gene; ribosomal protein.

(For all amplification plots see appendix to current chapter).

5.4.3 Stearoyl-CoA Desaturase (SCD)

Expression of SCD in human adipose tissue

Figure 5.6 Normalised SCD1 expression in (a) lean males, (b) obese males, (c) lean females, (d) obese females. Data are expressed as normalised gene expression, normalised against ribosomal protein and β-glucuronidase (see method 5.3.2). All samples were measured in triplicate and a mean was taken after normalisation.

SCD1 expression showed that there was no significant difference between genders, or depots (P=0.165, P=0.193 respectively). There was a significant difference between levels of adiposity (P<0.001) allowing for possible interactive differences from other the factors. Further statistical evidence was achieved using the Holm-Sidak comparison procedure substantiating that there was a statistically significant difference within adiposity (P<0.0016).

5.4.4 Glucose-3phosphate acyl-transferase (GPAT)

Expression of GPAT in human adipose tissue

Figure 5.7 Normalised GPAT expression in (a) lean males, (b) obese males, (c) lean females, (d) obese females. Data are expressed as normalised gene expression, normalised against ribosomal protein and β-glucuronidase (see method 5.3.2). All samples were measured in triplicate and a mean was taken after normalisation.

GPAT expression showed that there was no significant difference between genders, depots, or adiposity (P=0.367, P=0.176, P=0.182 respectively). In addition there was no interaction between any of the factors evaluated (all P>0.1).

5.4.5 Diacyl glycerol acyl-transferase 1 (DGAT1)

Expression of DGAT1 in human adipose tissue

Figure 5.8 Normalised DGAT1 expression in (a) lean males, (b) obese males, (c) lean females, (d) obese females. Data are expressed as normalised gene expression, normalised against ribosomal protein and β-glucuronidase (see method 5.3.2). All samples were measured in triplicate and a mean was taken after normalisation.

DGAT1 expression showed that there was significant difference between adiposity after allowing for affects of difference in gender and depot (P=0.02). There was significant difference between depots allowing for gender and adiposity (P<0.001). The effect of difference in gender, depot and adiposity are not dependent on each other. It should be noted that using the Holm-Sidak comparison procedure statistical difference seen using ANOVA were seen within for adiposity (P=0.019), but not within depot (P=0.617).

5.4.6 Diacylglycerol acyl-transferase 2

Expression of DGAT2 in human adipose tissue

a   Male Lean

b   Male Obese

c   Female lean

d Female obese
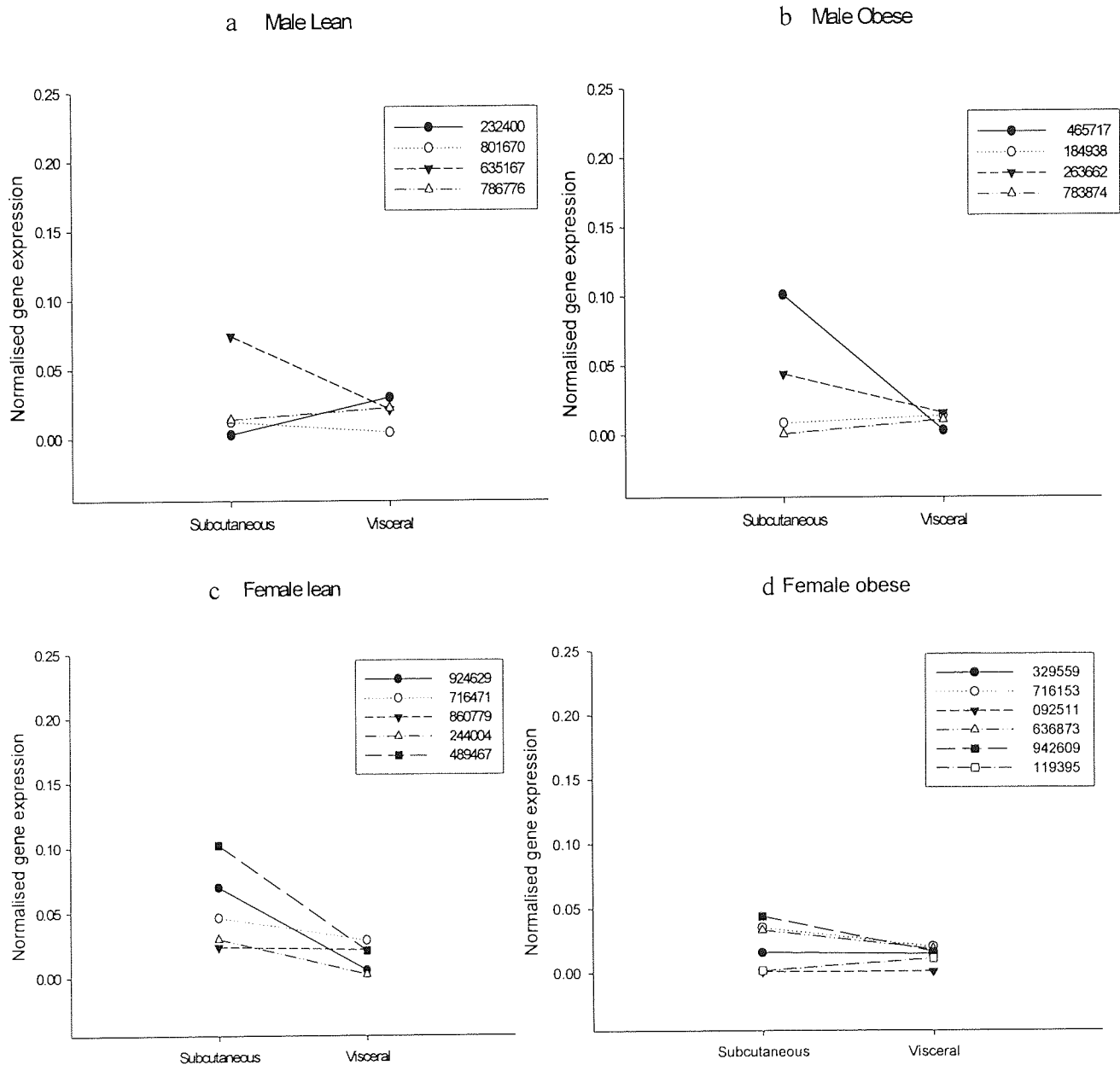
Figure 5.9 Normalised DGAT2 expression in (a) lean males, (b) obese males, (c) lean females, (d) obese females. Data are expressed as normalised gene expression, normalised against ribosomal protein and β-glucuronidase (see method 5.3.2). All samples were measured in triplicate and a mean was taken after normalisation.

DGAT2 expression showed that there was a significant difference associated with adiposity (P=0.005), gender (P=0.002) and depot (P=0.002) after allowing for effects of difference in the alternative factors (i.e. independently of each of the other variables). The effects of difference for the interaction of gender with adiposity and adiposity with depot are not statistically significant (P=0.091 and P=0.309 respectively). There is significant interaction between gender and depot (P=0.049). Under the Holm-Sidak comparison procedure differences in gender (P=0.002) and adiposity (P=0.0047) are significant, however there is no statistical significance seen within depot (P=0.0913). Interactions between the factors are outlined below.

Interactions: Adiposity within male (P<0.001), gender within obese (P<0.001), depot within male (P=0.011), depot within female (P=0.009), gender within subcutaneous depot (P=0.024), gender within visceral depot (P=0.013), depot within lean (P=0.011), depot within obese (P=0.009), adiposity within subcutaneous (P=0.009) and adiposity within visceral (P=0.013) are all statistically significant thus the data are interdependent.

5.4.7 11-β-hydroxysteroid dehydrogenase 1

Expression of 11β-HSD1 in human adipose tissue
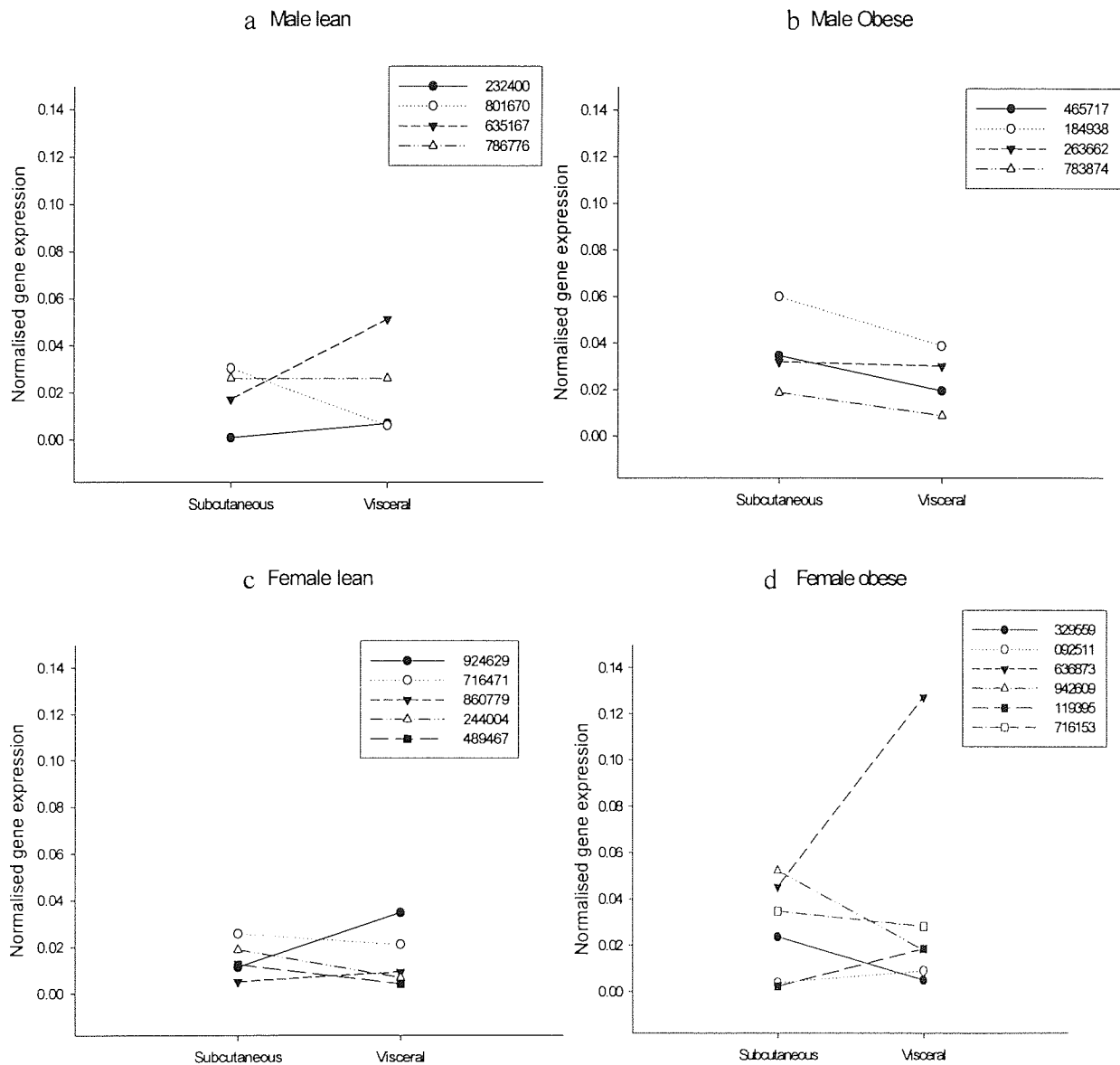
Figure 5.10 Normalised 11β-HSD1 expression in (a) lean males, (b) obese males, (c) lean females, (d) obese females. Data are expressed as normalised gene expression, normalised against ribosomal protein and β-glucuronidase (see method 5.3.2). All samples were measured in triplicate and a mean was taken after normalisation.

There was no significant difference of 11β-HSD1 expression associated with adiposity (P=0.051), or gender (P=0.298). However, there was a significant difference in depot (P=0.03) after allowing for effects of difference in the alternative factors. The effects of difference with the interaction of gender with adiposity and adiposity with depot are not statistically significant (P=0.247, P=0.198 and P=0.078 respectively). Using the Holm-Sidak comparison procedure there was no statistical significance of difference seen within depot (P=0.247).

5.4.8 Leptin

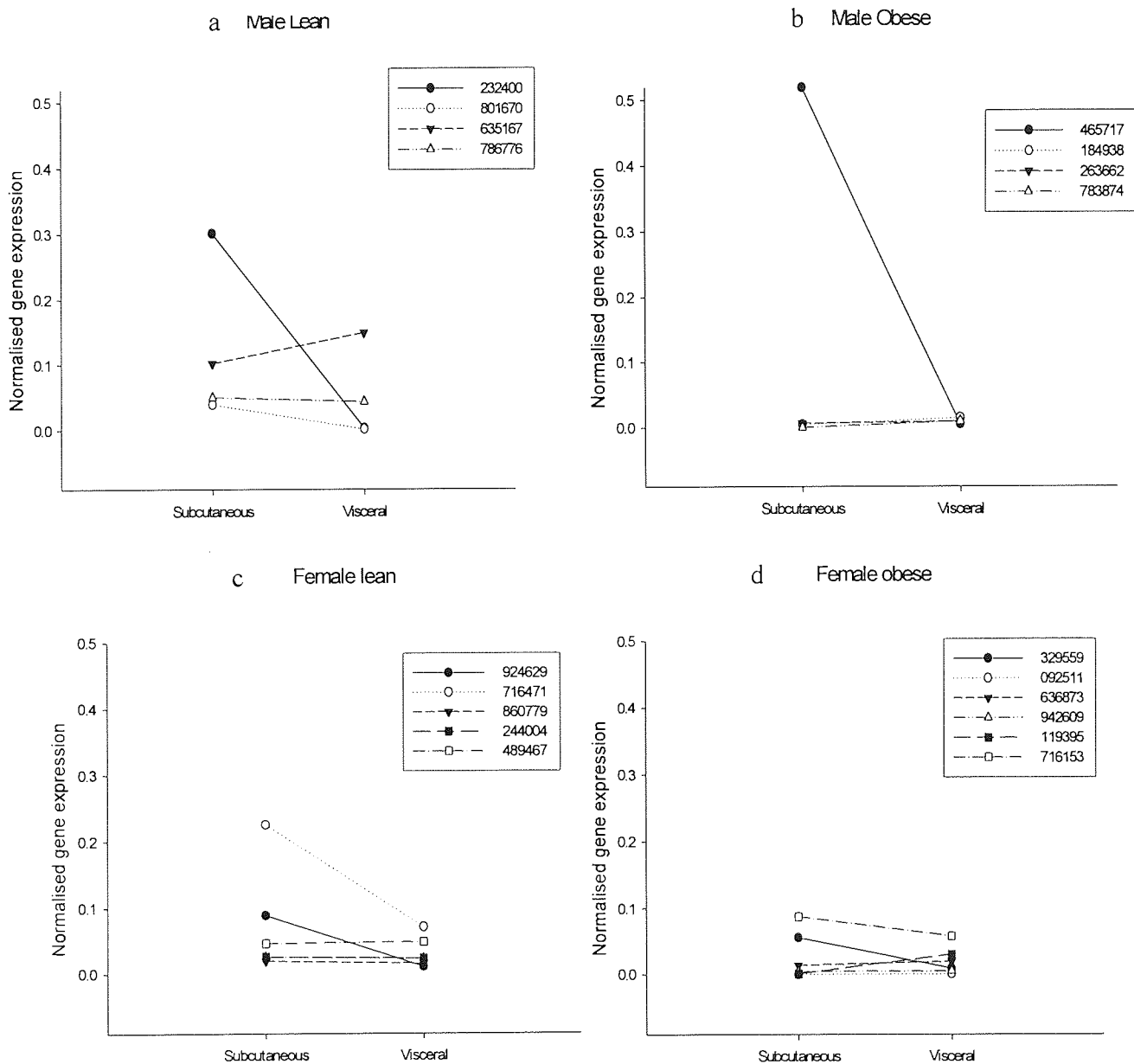# Expression of Leptin in human adipose tissue
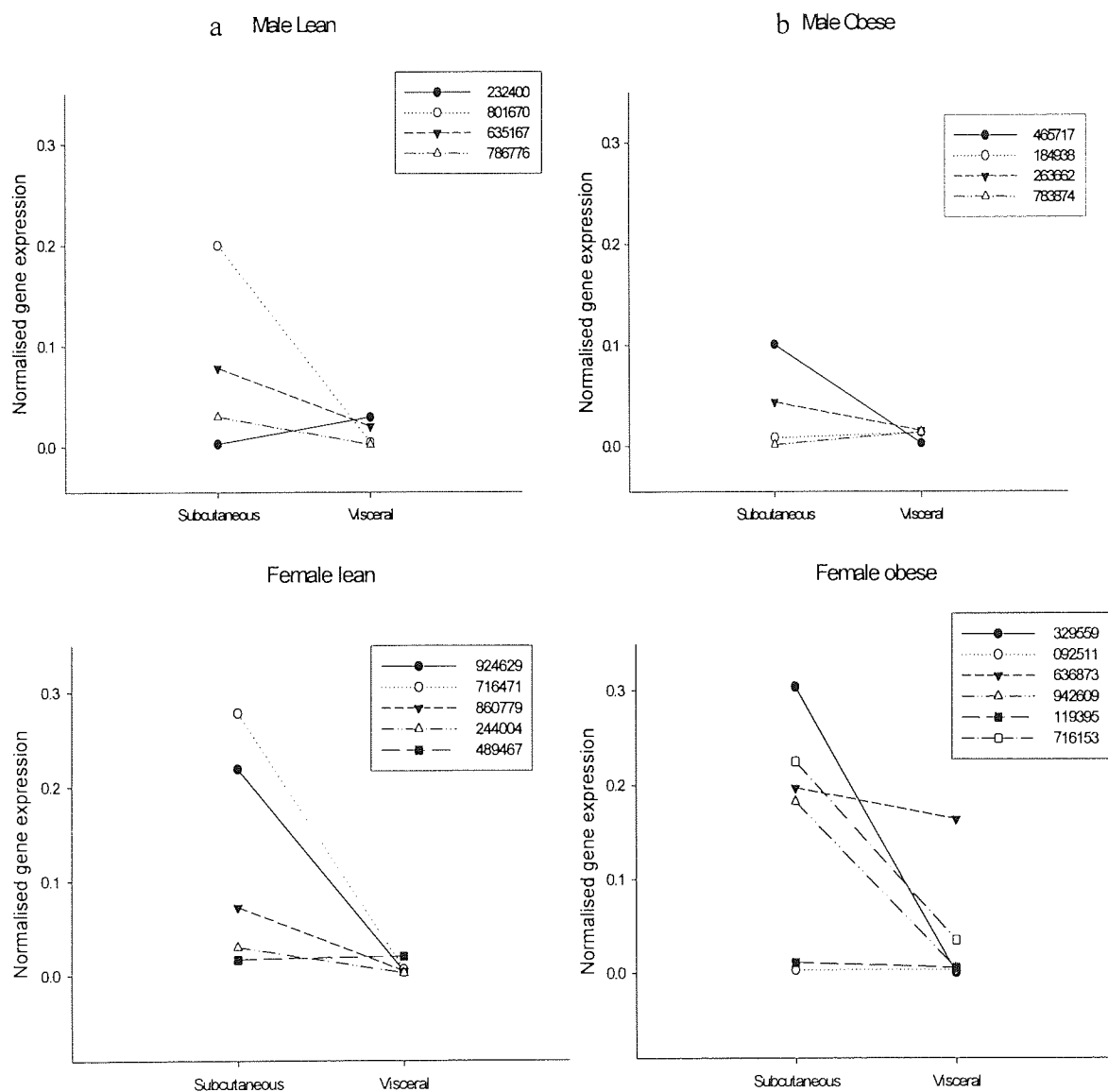
Figure 5.11 Normalised Leptin expression in (a) lean males, (b) obese males, (c) lean females, (d) obese females. Data are expressed as normalised gene expression, normalised against ribosomal protein and β-glucuronidase (see method 5.3.2). All samples were measured in triplicate and a mean was taken after normalisation.

Leptin gene expression showed no significant difference between adiposity (P=0.981). However, there were statistically significant differences in gender (p=0.05) and depot (p=0.001) after allowing for effects of difference in the alternative factors. The effects of difference with the interaction of gender and adiposity and adiposity and depot are not statistically significant. By use of the Holm-Sidak comparison procedure the difference in gender (P=0.049) was significant, however there was no statistical significance seen within depot (P=0.438).

5.4.9 Adiponectin

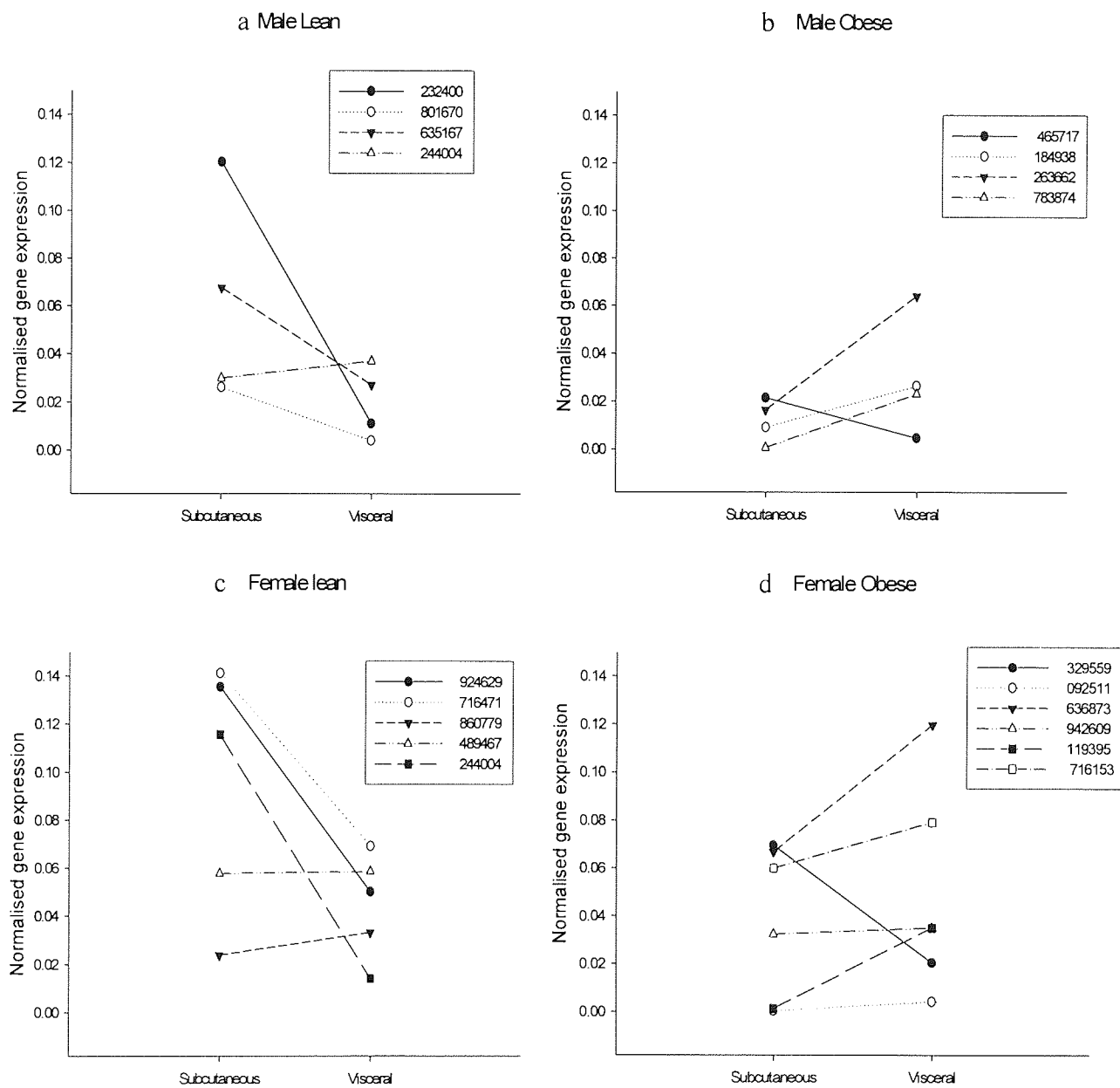# Expression of Adiponectin in human adipose tissue
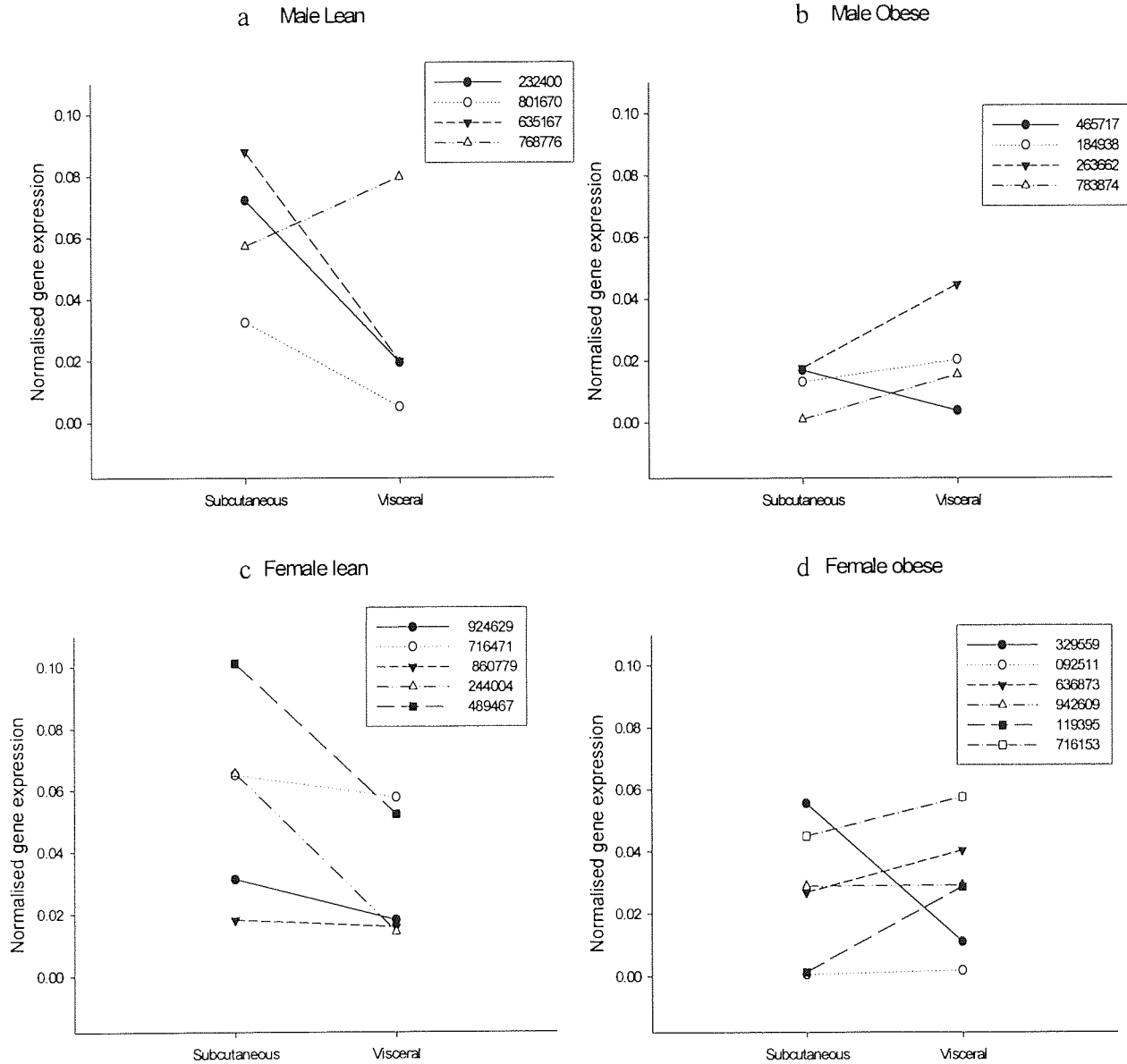
Figure 5.12 Normalised Adiponectin expression in (a) lean males, (b) obese males, (c) lean females, (d) obese females. Data are expressed as normalised gene expression, normalised against ribosomal protein and β-glucuronidase (see method 5.3.2). All samples were measured in triplicate and a mean was taken after normalisation.

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Adiponectin expression showed that there was no statistically significant difference between adiposity (P=0.136), however observation of the subcutaneous depot data illustrate a difference "by eye". Obese subcutaneous and lean visceral data show a decrease in adiponectin gene expression. There is no statistical significance within gender (p=0.263) and depot (p=0.150) after allowing for effects of difference in the alternative factors. The effects of difference with the interaction of gender and adiposity and adiposity and depot are not statistically significant.

5.4.10 G protein-coupled receptor 41

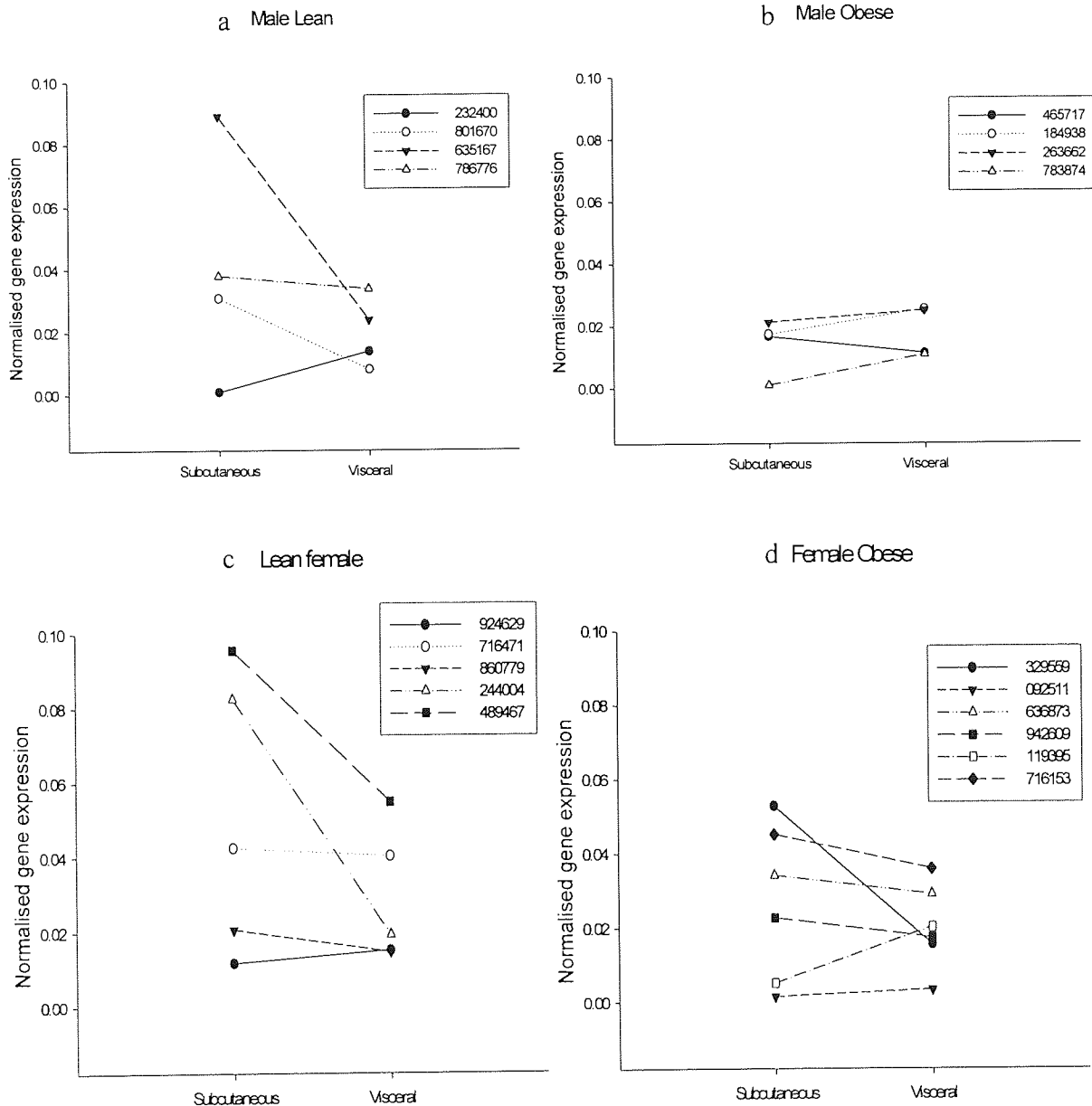Expression of GPr41 in human adipose tissue
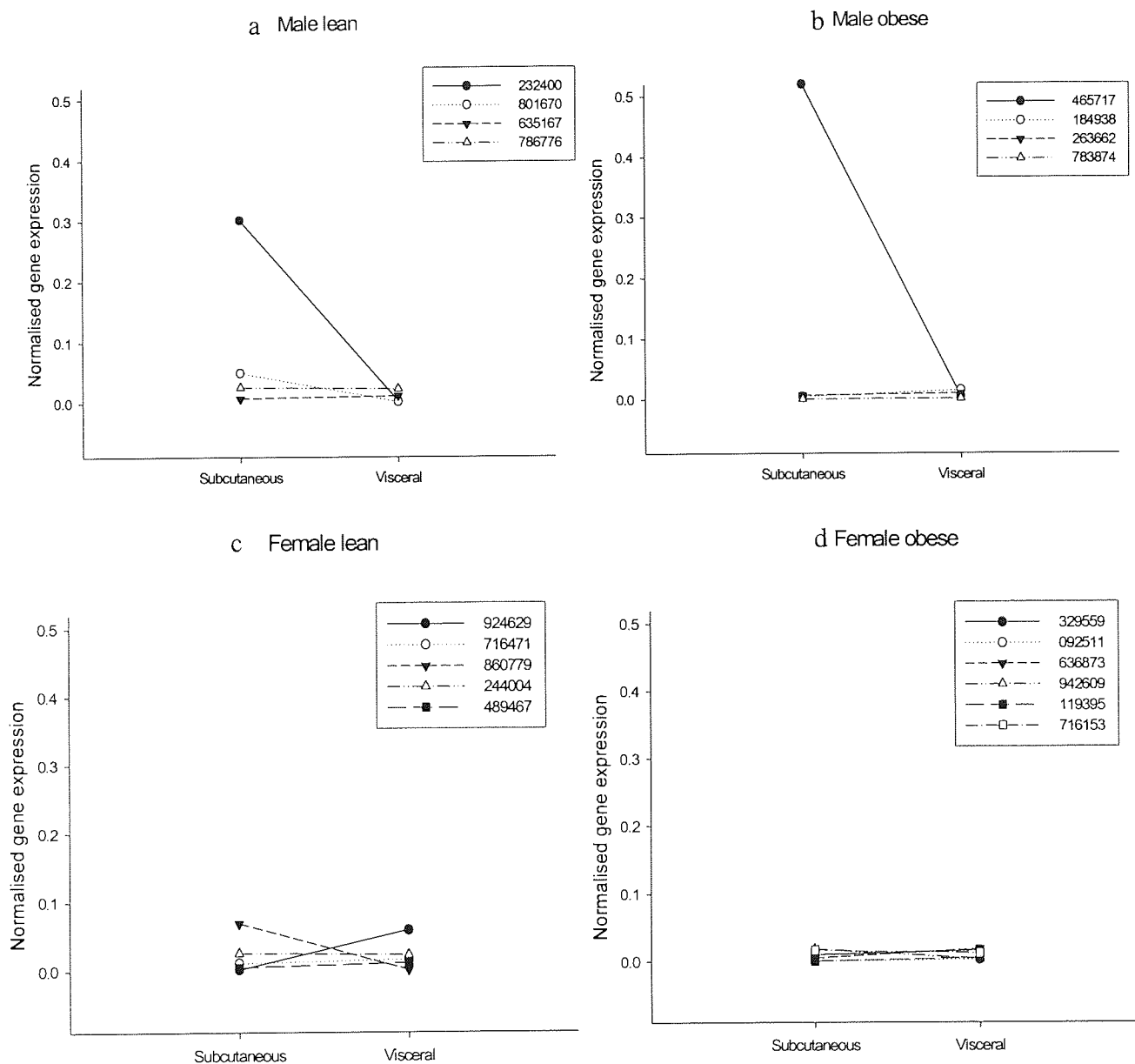
Figure 5.13 Normalised GPR41 expression in (a) lean males, (b) obese males, (c) lean females, (d) obese females. Data are expressed as normalised gene expression, normalised against ribosomal protein and β-glucuronidase (see method 5.3.2). All samples were measured in triplicate and a mean was taken after normalisation.

GPR41 expression showed that there was no significant difference between adiposity (P=0.969), gender (p=0.120) or depot (p=0.113) after allowing for effects of difference in the alternative factors. The effects of difference with the interaction of gender and adiposity and adiposity and depot are not statistically significant.

5.5 Discussion

The genetic determinants involved in the sensing and subsequent control of adipose tissue deposition and maintenance are of great interest due to the massive recent increase in the incidence of obesity. Discovery of the ob gene and its protein leptin lead to an increased interest in the possible role of adipocyte hormones and cytokines in energy balance and satiety signalling. These adipose tissue derived proteins and molecular signals are collectively known as the adipocytokines (adipokines), each singularly deemed an adipokine (Fain *et al* 2004, Trayhurn 2005). In this chapter attention is drawn to the differences in gene expression between subcutaneous and visceral adipose depots and between adipose tissue from male and female participants.

The present study includes consideration of selected adipokine genes such as SCD1, GPAT, DGAT1 and DGAT2 which are involved in triglyceride synthesis, adiponectin and leptin which are involved in hunger and satiety, 11β-HSD1 involved in the localized control of cortisol and GPR41 a g-protein coupled receptor.

Overall expression of genes involved in triglyceride synthesis showed lower expression in visceral depots when compared with subcutaneous with the exception of GPAT in female participants. In male participants there was no statistically significant difference between genders, adipose depots or adiposity levels in SCD1 expression. However, in two participants (one lean and one obese) SCD1 expression was particularly raised in the subcutaneous depot, as extremes are usually rare these results may be unique to this cohort. In females statistical analysis revealed that SCD1 expression levels differed between lean and obese participants,

showing reduced expression in the obese. Increasing SCD1 expression may be a mechanism to promote depot specific storage of fat. Therefore one would expect SCD1 to be increased in the subcutaneous depot compared with the visceral as these data show. Moreover, after significant gain of fat stored in the subcutaneous depot a corresponding decrease in SCD1 expression may occur to prevent further gain. Additional support for this theory is gained from animal studies where SCD1 deficient mice have shown resistance to diet induce obesity, increased energy expenditure and increase sensitivity to insulin (Dobrzyn and Ntambi 2005). One may expect that obese individuals would initially have increased expression of SCD1 thereby promoting weight gain. However in this study the majority of data showed a decrease in gene expression in obese compared with lean tissue suggesting a decrease of SCD1 expression with increase adiposity; this would preclude the use of SCD1 inhibitors as a therapeutic mechanism of reducing adiposity in the obese. In addition animal studies have shown that SCD1 deficiency can directly or indirectly signal the partitioning of fatty acids into the oxidation process as opposed to synthesis (Dobrzyn and Ntambi 2005) the weight loss observed in these animals was attributed in part to this mechanism. It is believed leptin which has been found to specifically repress SCD1 gene expression (Dobrzyn and Ntambi 2005b) may be the trigger for promoting partitioning into the fatty acid oxidation pathway (Dobrzyn and Ntambi 2004). In *ob/ob* mice the absence of leptin results in an increase in SCD1 expression which is concomitant with an increase in weight, when *ob/ob* mice are interbred with *ab^j/ab^j* mice (mutant SCD1 gene) the resulted in double mutant off-spring have a reduced body weight compared with their *ob/ob* littermates (Cohen *et al* 2002, Ntambi and Miyazaki 2004). The literature suggests that when the SCD1 gene is interrupted diet induced obesity is prevented and thus disruption of the SCD1 gene prior to the onset of obesity could provide a novel therapeutic target in patients who are susceptible to obesity. There is no information to show

that the disruption of SCD1 gene expression after the onset of obesity has the same beneficial outcome as that seen in ab^j/ab^j - ob/ob mice. In addition it should be noted that disruption of the SCD1 gene does cause side effects due to the prominent role of SCD1 in the formation of membrane lipid, wax esters, wax diesters and sebum (Sundberg et al 2000). In the absence of SCD1 the ab^j/ab^j mice are smaller, have progressive alopecia, dry skin and are photophobic (Sundberg et al 2000), therefore any therapeutic manipulation of this gene may also cause side effects in humans.

Although GPAT expression showed no statistical difference between any of the factors examined there was a trend towards lower expression in visceral than subcutaneous depots in females. Conversely, 75% of males showed higher GPAT expression in visceral depots. Since GPAT catalyses the initial step of triglyceride synthesis (Hammond et al 2002) higher expression in subcutaneous depots of females and higher expression in visceral depots of males is consistent with current evidence that women are more susceptible to weight gain in the subcutaneous depots, especially the lower abdomen, whereas men are more prone to visceral adipose deposition (Albright 1998, Arner 1997). The mechanisms responsible for the up-regulation in regional expression of GPAT has yet to be fully elucidated, however studies in the literature suggest regulation occurs at several levels, transcriptional regulation dependent on feeding status, hormonal regulation (insulin and glucagon), and by the transcription factor SREBP-1 may all play a role (Lewin et al 2001).

Mean values for DGAT1 expression were consistently higher in lean than obese participants (P=0.02) and generally slightly higher in visceral than subcutaneous depots (P<0.001). In the same pathways as GPAT, DGAT is the terminal catalytic enzyme in triglyceride synthesis

(Chen *et al* 2002). Thus, for the same reasons advanced for GPAT, it is anticipated that females have greater expression of DGAT1 in subcutaneous tissue, and males have greater expression in the visceral depot. The present data do at least partly support the interpretation considered for GPAT. Females generally exhibited higher DGAT1 expression than males in the subcutaneous tissue depot, but there was greater expression of DGAT1 in the subcutaneous depot in lean than obese participants of each gender. This suggests that females may have an increased capability of storing excess subcutaneous fat and as a consequence could have a higher fat accumulation threshold before insulin resistance become apparent. Gender differences in the prevalence of diabetes appear to have varied over the last century and in Europids there is presently a small excess of T2DM in men (Gale and Gillespie 2001): a gender distinction has not emerged within T1DM. In addition post-menopausal woman show a disproportionate prevalence for T2DM (Fitzgerald *et al* 1961) and there are substantial evidence in the literature indicating that loss of ovarian function post-menopause or by oophrectomy is associated with an increase in adiposity (Lenzen and Bailey 1984). However studies have yet to discriminate between subcutaneous and visceral deposition. This may be a protective mechanism to enable increased energy storage in preparation for, or as a consequence of, childbearing and would be a potential explanation for the higher DGAT1 expression levels in females.

DGAT2 expression showed a statistically significant difference between all factors studied (adiposity, gender and depot). There is higher expression of DGAT2 in subcutaneous than visceral depots, and in lean than obese. All interaction statistics indicate that the factors are dependent upon each other and thus they may not occur independently. Since DGAT2 is also involved in the terminal step in triglyceride synthesis (Chen *et al* 2002apr) the expression data

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are consistent with DGAT1 expression where subcutaneous expression was greater than visceral. Although this was a less consistent finding with DGAT2, it generally supports the concept of greater susceptibility to excess fat storage in subcutaneous depots particularly in the lean females. Interestingly in a review by Miles and Jensen (2005) it is controversially noted that the visceral depot of fat contributes less than 5% of the FFA to the systemic pool, even though the visceral depot is reported to show increased lipolysis and fatty acid turnover (Frayn 2002, Bjorntorp 2000). These apparently opposing statements can both be supported by the data in this study assuming there is a natural balance between lipolytic and lipogenic enzyme expression is maintained in the body.

To summarize so far; triglyceride synthesis controlled by SCD1, GPAT and DGAT1 and 2 may be influenced through the levels of RNA production expression of their respective genes. The present data indicate that tissue from lean participant's appear to have greater levels of gene expression in the subcutaneous depot which is not in accordance with the majority of the studies and theories in the literature which suggests that fatty acid turnover is greater in the visceral depot (Frayn 2002). However, further examination into lipolytic enzyme expression could redress this issue. If lipolytic enzyme expressions are in balance with the lipogenic enzyme expressions in visceral depots this may substantiate the studies, suggesting recycling of fatty acids in these depots. In addition subcutaneous depots would have increased lipogenic enzyme expression over lipolytic in order to promote storage and fatty acid stability. Therefore, subcutaneous adipose tissue primarily promotes storage of excess fatty acids, not utilization. This could be further investigated by comparing the ability to cycle fatty acids by visceral and subcutaneous tissue. Teleologically, this cycling of fatty acids could ensure a pool of free fatty acids for use especially by liver during periods of low glucose. Such a cyclical

process could also prevent excess toxicity via recycling local fatty acids, especially during periods of high glucose availability. Accordingly, One could postulate that western diets unbalance these two systems (lipogenic and lipolytic) promoting an excessive increase in visceral tissue. Furthermore rebalancing the systems using medical intervention may lead to an increase in insulin resistance if a low glucose diet is not strictly adhered to.

The activation of cortisone to cortisol can be performed in adipocytes by the enzyme 11β-HSD1 (further detail in chapter 1.6.2 and chapter 6). This enables local management and coordination of adipocyte proliferation and differentiation (Bujalska *et al* 2002). Expression levels of 11β-HSD1 showed a statistically significant difference between depots independent of other factors tested. The overall lower expression of 11β-HSD1 in visceral than subcutaneous depots in males particularly in the obese males, suggests that the subcutaneous pre-adipocyte pool is likely to be differentiated more readily than the visceral pool, thereby increasing the capacity for fat storage in the subcutaneous depot and developing a pool of pre-adipocytes in the visceral depot. This is consistent with earlier conclusions drawn from the triglyceride synthesis enzyme expression data showing mostly greater expression in the subcutaneous depot.

An alternative interpretation of the data is consistent with the recent studies on the mechanism of action of antidiabetic thiazolidinediones such as pioglitazone and rosiglitazone. These agents are PPARγ agonists that also promote the differentiation of pre-adipocytes to mature adipocytes (Krentz and Bailey 2005). The newly formed small adipocytes are mainly found in subcutaneous depots (Bailey 2005). It has been discovered that treatment with a PPARγ agonist results in reduced expression of 11β–HSD1 in visceral but not subcutaneous tissue (Laplante *et al* 2003). Moreover there has been a preliminary data suggesting that the PPAR

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response element exists in a regulatory upstream position of the 11β-HSD1 gene, raising the possibility that PPARγ agonists could initiate subcutaneous adipogenesis, at least in part, by up-regulation of 11β-HSD1 (Berger *et al* 2001).

Other data showed conflicting results with up to 50% of individuals having a lower expression level of 11β-HSD1 in visceral than subcutaneous fat. The reverse trend with a lower expression level in subcutaneous when compared with the visceral depot was evident in the remaining participants. Notably however, there was a marginally higher expression level in the subcutaneous depot of obese participants compared with the same depot in lean participants; this difference is not seen in the visceral depot. This design enables up-regulation of 11β-HSD1 in only the subcutaneous tissue and may be a key pathway involved with directing FFA into the subcutaneous tissue to protect the visceral component responsible for providing energy in periods of low glucose availability. As 11β-HSD1 increases the level of local cortisol responsible for adipocyte differentiation one might anticipate a higher level of expression in obese participants compared with their lean counter-parts, especially in the visceral depot of males and the subcutaneous depot of females, to conform to the gender specific fat distribution pattern (Albright 1998). However, this was not seen in the present data and interestingly one female participant showed a significantly higher expression in the visceral depot compared with the subcutaneous depot which in turn had a comparatively high expression level. Further investigation to resolve these perplexing data may concentrate on whether the abdominal adipose deposition is primarily visceral or subcutaneous, and an additional study into the effects of sex hormones on expression of local adipocyte 11β-HSD1 may lend support to the theory regarding control of fat deposition. In addition, proliferation of pre-adipocytes occurs at a low concentration or in the absence of cortisol (Draper and Stewart 2005): this may substantiate finding lower expression of 11β-HSD1 in visceral depots.

Interestingly in chapter 4 it was noted that there were more viable visceral pre-adipocytes isolated from this tissue depot than subcutaneous. In addition in chapter 6 the investigation into therapeutic inhibition of 11β-HSD1 (Tomlinson 2005, Alberts *et al* 2003) entertains the possibility that inhibition of 11β-HSD1 may result in an increased pool of pre-adipocytes, thereby pre-disposing to obesity upon cessation of treatment. The local balance of active cortisol to relatively inactive cortisone appears to be tightly controlled (Draper and Stewart 2005, Stewart and Krozowski 1999); marginally lower levels of expression of 11β-HSD1 in the visceral depot indicate that there is a local mechanism of protection against insulin resistance by maintaining a constant manufacture of small insulin sensitive cells. However this is precariously balanced and long term over production of pre-adipocytes may be detrimental. In obese patients 11β-HSD1 expression shows an overall marginal increase when compared to lean, this is most noteworthy in the subcutaneous depot, thus again suggesting a protection from free fatty acid toxicity via promoting fat storage in subcutaneous depots whilst retaining the ability to produce smaller insulin sensitive cells in the visceral depot.

Expression of leptin was higher in subcutaneous depots in comparison to visceral in over 70% of the participants. Positive statistical differences were calculated between genders and depots. These differences can be seen in the illustration (figure 5.11). Female participants showed the greatest differences in leptin gene expression between subcutaneous and visceral depots expression (being higher in subcutaneous than visceral), whilst males showed a similar trend in data but displayed lower levels of leptin gene expression in comparison to females. These results are supported by the literature (Kershaw and Flier 2004). Leptin is an adipokine responsible for relaying long term adiposity status to the brain and a short term satiety signal

(Koerner *et al* 2005). It has been implied in the literature that tissue from the visceral depot is metabolically more significant than that of subcutaneous origin, however adipokine concentration within specific fat depots is being investigated (Arner 1997, Kershaw and flier 2004). Collectively these results support the hypothesis that suggests subcutaneous adipose tissue has a significant role in adipocyte regulation and may be an important signalling component in the regulation of long term energy storage and utilisation.

It has been well documented that visceral tissue has a greater capacity for fatty acid turnover than subcutaneous (Frayn *et al* 2003, Arner 2001), this prominent role and the correlation of visceral tissue mass with metabolic syndrome has focused the attention of the therapeutic intervention research. The emerging role of subcutaneous tissue as the long term storage unit for fatty acid and provider of systemic adipokine signals may provide novel targets for manipulation in the search for therapeutic intervention of fat distribution and appetite control.

The mode of action and role of leptin has been extensively researched. It regulates feeding by receptor activation in the brain resulting in increased release of NPY and a decrease in satiety (Wilding 2002). The present data suggest that the leptin signal is primarily provided by the subcutaneous tissue. The literature also suggests there is a direct peripheral role for leptin in part through interactions with other hormones including insulin (Koerner *et al* 2005). The physiological significance of leptin in the overall signalling of energy stores is considered further in chapter 1.4.4.

Although adiponectin was discovered soon after the discovery of leptin, its biological repertoire appears to be more diverse and has not yet been completely explored; at the same time there is much information regarding the correlation of adiponectin and obesity-related disorders (Matsuzawa 2005, Juge-Aubry *et al* 2005, Haluzik 2005). The most investigated role

of adiponectin is connected to insulin. It is increased by insulin-sensitizing agents such as thiazolidinediones (TZDs) and can directly and indirectly influence insulin sensitivity (Fasshauer *et al* 2004). In addition to this function adiponectin has a powerful anti-inflammatory role, thus correlating with the notion that increased adiposity contributes to a low-grade inflammatory response (Rajala and Scherer 2003). It is important to bear in mind that adiponectin levels in the circulation are inversely related to the amount of adipose tissue. Data from this thesis showed that adiponectin expression levels did not vary between tested parameters. Nevertheless, graphical representation depicted higher expression levels in the subcutaneous depot when compared with the visceral depot in lean participants (figure 5.12). Obese patients show conflicting data, with only 25% of participants following the previously described trend and the remaining 75% showing the reverse trend. Interestingly, in a comparison of the data there is an overall lower expression of adiponectin in the obese subcutaneous depot than in the lean. Adiponectin is known as the protective adipokine (Koerner *et al* 2005) as it is inversely correlated with pathogenesis of obesity-related disorders. As expected adiponectin expression is higher in subcutaneous depots (Fisher *et al*), however these data do not show the sexual dimorphism found plasma concentration data (Bottner *et al* 2004). Intriguingly, visceral levels of adiponectin do not appear to decrease with adiposity, but there is a marked decrease in expression level in subcutaneous depots. Therefore by extension the negative correlation of adiponectin levels and insulin sensitivity noted in the literature (Kabuta *et al* 2002, Haluzik *et al* 2004) must be due to the decrease in production by the subcutaneous tissue. Assuming this to be true a decrease in adiponectin expression level may in part be due to the small increased localised glucocorticoid known to reduce adiponectin mRNA in rodent models (Haluzik *et al* 2004). Furthermore, the increased size of adipocyte and increased fatty acid exposure of the subcutaneous tissue may result in the onset

of insulin resistance of these cells which would be concomitant with an increase to insulin exposure. Insulin is known to decrease adiponectin levels in humans and rodents *in vitro* and *in vivo* (Fasshauer and Paschke 2003) and thus may play an important part in the decreased expression of adiponectin seen in these data. In addition these data showed that male adiponectin expression was more severely altered than female, and differences in lean individuals were greater than in obese individuals. These results support an earlier conclusion suggesting that females have a greater capacity to store fat it would appear they can do this with lessened detrimental side effects to key adipokines. To postulate further, the data appear to illustrate a fundamental role of the visceral tissue component to compensate for the loss in adiponectin expression by the subcutaneous depot.

GPR41 is a little known G-protein coupled receptor activated by short-chained fatty acids that has been shown to stimulate leptin (Xiong *et al* 2004). Expression of GRP41 showed no statistically significant differences between the tested factors. Graphical representation, with the exception of two participants showed no starkly apparent differences. Although in the minority, tissue from two male patients showed significantly greater expression of GRP41 in the subcutaneous depot. Further investigation would be required to determine if this would be seen in a significant proportion of the population. Assuming further investigation shows increased incidence of greater GRP41 expression in subcutaneous tissue compared with visceral it may prove to be an additional mechanism by which leptin is increased in the subcutaneous depot. In addition to this role it has been suggested that GPR41 may play a role in lipolysis, however this has yet to be demonstrated (Brown *et al* 2002). Should this role latter become apparent one would expect there to be an increase in lipolysis in the subcutaneous

depot. Again this may be a protective mechanism to decrease cell size via lipolysis and promote futile fatty acid cycling in obesity.

In conclusion it would appear that although the visceral tissue depot has been the most focused upon due to its strong association with insulin resistance and accompanying cardiovascular risk; the subcutaneous tissue depot may play a critical role in "adiposensing", energy balance and by extension weight control. Even though in its absence the body can provide compensatory mechanisms to maintain function it would appear that the subcutaneous depot may provide many of the signals previously attributed to the visceral tissue component and thus provide a plethora of novel targets for therapeutic intervention.

Chapter 6: 11 β-HSD1

6.1 Introduction

6.1.1 Background

Glucocorticoid levels correlate positively with idiopathic obesity (Wake 2004). This is strongly suggests an association between raised glucocorticoids and increased adipocity. The importance of glucocorticoid levels as a cause of obesity is supported by evidence from a range of disease states wherein the homeostatic balance of glucocorticoids has been disturbed (Cushings disease, apparent mineralocorticoid excess and Addison's disease) and changes in adipose deposition are also observed. Glucocorticoids exert extensive local metabolic effects within the adipocyte including adipogenic differentiation, metabolic influences and regulation of gene expression (Engeli 2003). In addition glucocorticoids exercise functional antagonistic effects upon insulin action and can alter β-cell secretory metabolites, decrease insulin dependent glucose uptake, increase lipolysis and increase heptatic gluconeogenesis (Stulnig and Waldhausl 2004).

6.1.2 Glucocorticoids

6.1.2.1 Background

Glucocorticoids are produced under hypothalamic-pituitary-adrenal axis control in the adrenal cortex and play a vital role in the homeostatic regulation of salt and water metabolism, blood pressure and immune function as well as metabolism (Andrews and Walker 1999).

6.1.2.2 Glucocorticoid receptors

There are two major receptor types activated by glucocorticoids in man, type 1 and type 2 corticosteriod receptors; known as mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) respectively. The GR receptors are more widely distributed but have a lower affinity than the MR, thus the GR receptors are said to act as high-capacity, low-affinity receptors; by contrast MR are low-capacity, high-affinity receptors (Stewart 1999). Occupancy of the receptors exhibits a circadian pattern with the lower affinity receptor being occupied during the circadian peak occurring in the morning. However, recent studies have revealed specific tissue control via localised activation of glucocorticoids which are elaborated later in this chapter.

6.1.2.3 Cortisol

Cortisol is the principal active glucocorticoid in man, mediating its effect at a tissue level via interaction with the intracellular glucocorticoid receptors (GR), which results in translocation of the receptors to the nucleus to form a ligand-dependent *trans*activator complex. This complex can form either a homodimer to glucocorticoid response elements (GREs) or form a protein-protein interaction with other transcriptional regulators (Rabbitt *et al* 2002).

Cortisol is essential for the differentiation of human fat cells from pre-adipocytes devoid of fat stores to mature adipocytes with a singular fat droplet store, whilst preventing pre-adipocyte proliferation (Tomlinson 2004). However circulating levels of cortisol are normal in the majority of patients with simple obesity (Stewart and Tomlinson 2002) suggesting there might be local activation of cortisone for use by the adipocyte.

6.1.2.4 11β-HSD1

The enzyme responsible for the localised activation of cortisone to cortisol in adipose tissue is 11 beta hydroxysteroid dehydrogenase type 1 (11β-HSD1). Located on chromosome 1 at q32.2 it is >30kb in length and encodes for a 34kd protein (Stewart 2003). It is a bidirectional enzyme with a reductase activity that predominates *in vivo* (Tomlinson and Stewart 2002). It is abundant in glucocorticoid target tissues including the liver, adipose tissue and central nervous system (Li 2004) physically located in the endoplasmic reticulum (Paulmyer-Lacroix 2003). It was cloned in 1989 (Agarwal *et al*, 1989) and later was discovered to have an NADPH dependent reductase roll *in vivo* (Bujalska *et al* 1997). 11β-HSD1 is transcriptionally regulated with numerous transcription binding sites on its promoter region (Williams *et al* 2000); these include many proteins that are found to be altered in obesity such as AP2, CEPB, PPARs and GH (Tomlinson *et al* 2001). In addition 11β-HSD1 may also be regulated post-transcriptionally (Wake 2004). Most commonly in rodents, 11β-HSD1 exerts a reduction of cortisone to corticosterone in liver but an increase in adipocyte tissue (Sandeep *et al* 2005). Clearly, localised tissue regulation of glucocorticoid activation is important in the development and control of adipose tissue. Stewart and Tomlinson (2002) hypothesised that the direction of overall activity of 11β-HSD1 may be a significant factor underpinning the accumulation of excess visceral fat in people with central adipocity.

6.1.3 Inhibition of 11β-HSD1

6.1.3.1 Natural

Selective inhibition of localise adipose tissue cortisone activation should theoretically assist in reducing visceral fat deposition. Localised inhibition of 11β-HSD1 has been the focus of much research in the race to find a pharmaceutical control for the spiralling obesity epidemic. Obesity is associated with increased insulin resistance (see chapter 1 section 1.3.7). Insulin is a

major inhibitor of 11β-HSD1 expression (Andrews and Reynolds, 1999) and thus an increase in insulin resistance could initially decrease local activation of cortisol due to concomitant hyperinsuinaemia followed by a subsequent increase local activation of cortisol due to an increase in localised adipose tissue insulin resistance resulting in decreased inhibition of 11β-HSD1 by insulin (Reynolds and Walker 2002). Glucocorticoids increase Glut 4 expression in adipose tissue; however translocation of glut 4 to the cell surface in response to insulin is inhibited by the presence of active glucocorticoid (Andrews 1999). Further to this insulin sensitivity is decreased in circumstances of increased 11β-HSD1 as assessed by the HOMA index (Engeli 2004).

6.1.3.2 Pharmaceutical

Glycyrrhetinic acid, the main metabolite of glycyrrhizic acid is a potent inhibitor of glucocorticoid action via inhibition of 11β-HSD (Li 2004). However, the over consumption of products containing glycyrrhizic acid can lead to hypertension and other electrolyte disturbances (Ploeger 2001), this is because glycyrrhizic acid shows non-selective inhibition of 11β-HSD and as such its effects are widespread, and include weight loss (Li 2004). Inhibition of 11β-HSD1 in 3T3 cells by flavanone and monohydroxylated flavinoids, 2'-hydroxy and 4'-hydroxyflavanone was effective with a low IC50 and showed no cytotoxic effects (Schweizer et al 2003). The first groups of compounds shown to suppress selectively or totally inhibit activity of 11β-HSD1 are arylsulfonamidothiazoles, which improved hepatic insulin sensitivity and glucose uptake in mice, and carbenoxolone which comprise of the succinyl ester of glycyrrhetinic acid and have shown similar non-selective effects (Sandeep et al 2005). Recently several novel compounds have been evaluated for potential pharmaceutical development. Those which have received greatest attention are those in development by

Biovitrum (e.g BVT.3498). BVT.3498 is a selective inhibitor of 11β-HSD1 and has shown to reduce tissue cortisol levels, improve glucose uptake without the risk of hypoglycaemia and possibly improve lipid profile (Biovitum press release: www.leaddiscovery.co.uk/target-discovery/abstracts/PubMed-110305.html)

6.1.4 Animal studies

Studies in rodents have shown some comparable results regarding variability in metabolism of adipose tissue depots to those observed using human tissue. Altered activity of 11β-HSD1 appears to be tissue specific as seen in obese Zucker rats which exhibit increased activity of 11β-HSD1 in omental fat whilst activity is decreased in the liver. This can be compared to human tissue research where an observation of increased activity in subcutaneous adipose tissue has been made in conjunction with impairment in the liver enzyme (Rask *et al* 2001, Reynolds and Walker 2002). In addition it has been demonstrated that there are increased levels of 11β-HSD gene expression in both subcutaneous and visceral adipose tissue of obese subjects versus their lean counterparts (Stewart and Krozowski 1999). Over expressing 11β-HSD1 in adipose tissue of transgenic mice elevates the intra-abdominal and portal vein glucocorticoid concentrations without significantly affecting peripheral circulating levels (Seckl and Walker 2001). This is associated with a three fold increase in visceral adipose tissue (Masuzaki *et al* 2001), this increase may result due to hypertrophy and/or hyperplasia of adipose cells. Over expression of 11β-HSD1 via the aP2 promoter induced a disease state similar to the metabolic syndrome which is associated with obesity, hyperglycaemia, dyslipidaemia and hypertension (Masuzaki *et al* 2001, Masuzaki and Flier 2003). By contrast mice deficient of the enzyme show resistance to diet induced obesity and are protected against

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the associated hyperglycaemia, dyslipidaemia and hypertension (Morton *et al*, 2001). A similar protection against the development of obesity is observed in mice given selective inhibitors of 11β-HSD1. One hypothesis which is not generally supported proposes that cortisol reduction by suppression of 11β-HSD1 may in fact exacerbate visceral adiposity via promotion of pre-adipocyte proliferation: however evidence from Morton *et al* (2004) demonstrates a reduction in visceral fat accumulation; Morton *et al* (2004) suggest that in 11β-HSD1 null mice there is decreased visceral adiposity attained via a decrease in adipose tissue hypertrophy. The confusion here may reflect methodological inconsistencies in the measurement of cell number and cell size during measurements of adipose mass, (see chapter 4)

It has been shown that in obesity prone mice 11β-HSD1 levels are increased, with subcutaneous and peripheral fat depots exhibiting the highest levels even though receptor levels are higher in visceral depots (Morton 2004).

In cases of chronic high fat feeding there is a down regulation of 11β-HSD1 in adipose tissue without effects on muscle or liver isoforms. It is hypothesised that this is a homeostatic adaptation may protect against metabolic disease. However this adaptation may not occur in some humans or be overridden by other metabolic factors, resulting in a portion of the population having an increased susceptibility to metabolic side effects of increased levels of 11β-HSD1 when undertaking a westernised dietary regime (Morton 2004). This theory supports data showing a positive correlation between obesity and adipose tissue 11β-HSD1 levels.

Recent interest in 11β-HSD1 inhibitors has lead to the discovery of BVT.2733 (3-chloro-2-methyl-*N*-{4-[2-(4-methyl-1-piperazinyl)-2-oxoethyl]-1,3-thiazol-2-yl} benzenesulfonamide)

a selective inhibitor of 11β-HSD1 in mice. Studies show that this compound successfully reduces circulating plasma glucose levels in diabetic mice but does not affect wild-type; therefore suggesting it would not cause hypoglycaemia. Furthermore in the same study it was shown that there was a reduction in food intake suggesting an additional beneficial effect on adiposity (Alberts *et al* 2003). Biovitrum have recently announced a selective human 11β-HSD1 inhibitor BVT.3498 which is currently in phase III trials and is being marketed as an anti-diabetic agent (Biovitum press release: www.leaddiscovery.co.uk/target-discovery/abstracts/PubMed-110305.html).

Aims

The balance of evidence reviewed in the foregoing section of this chapter and is section 1.5.1.2 of chapter 1 indicate that 11β-HSD1 is a legitimate potential therapeutic target to reduce production of cortisol within adipose tissue. This in turn should reduce the metabolic effects of cortisol in adipose tissue which are associated with differentiation of pre-adipocytes to mature adipocytes and increase in lipid accumulation, leading to visceral obesity and other components of the metabolic syndrome.

To investigate this issue the present study examines the prevention of local cortisone activation to cortisol. Specifically by inhibition of 11β-HSD1 activity by glycyrrhetinic acid, BVT1 (BVT.2733), BVT2 (BVT.3498) or AZ121 on isolated mature adipocytes obtained from lean, obese or obese diabetic patients.

6.2 Methods

6.2.1 Sample preparation

Adipose tissue was obtained from human volunteers undergoing elective surgery (see section 3.2) and adipocytes were isolated (see section 3.3.2). Patients were aged between 25-75 years of which approximately 80% were female. Lean patients had a BMI of between 20-26 kg/m^2, overweight and obese patients had a BMI of between 28-33 kg/m^2. There were 5 diabetics in this cohort who were all obese. Isolated cells were counted and resuspended at approximately 1×10^6 cells per ml. Cell suspension (800µl) was added to 100µl test agent within the concentration range 1×10^{-4}-1×10^{-8} and 100µl cortisone (1µM). Samples were incubated at 37°C for 2h. Upon completion all samples were extracted via the addition of ethyl acetate (2.5ml), vortexed and allowed to stand for 1 min. The uppermost ethyl acetate layer was transferred to a fresh glass vial and evaporated at 40°C under nitrogen gas. The sample was resuspended in phosphate buffer (100µl, pH 7.4). Samples were analysed for cortisol content via an ELISA assay procedure as described in detail in chapter 3 section 3.10.2, 10µl of sample was added to 90µl of kit buffer for analysis.

6.2.2 Data analysis

After analysis of cortisol by the ELISA method (Chapter 3.10.2), the concentration of samples was determined via extrapolation of data from a standard curve. The data were expressed as amount of cortisol extracted pg/10^6 cells. Data were tabulated (see appendix to chapter 6), statistically analysed and graphically presented. Statistical analyses performed were standard error of the mean (SEM), analysis of variance (ANOVA) and Student's t test (see graph legends and appendix to chapter 6).

6.3 Results

6.3.1 Lean Tissue

Glycyrrhetinic acid significantly decreased cortisone conversion to cortisol in adipocytes isolated from visceral and subcutaneous tissue extracted from lean patients (figures 6.1 and 6.2). This decrease in conversion exhibited an apparent dose-dependent effect with a particularly steep decrease in conversion seen at 10^{-6}M glycyrrhetinc acid (see figure 6.3). There was no substantial difference between subcutaneous and visceral depot cells under pharmacologically accepted levels of this test agent. Surprisingly, a similar decrease in cortisol production was seen with addition of the Biovitum compound BVT1 (see figure 6.4). In the presence of BVT2 (see figure 6.5) there was a small but significant decrease of cortisol production, but the proportional decrease compared with control was less significant than with glycyrrhetinic acid and BVT1 .

6.3.1.1 Glycyrrhetinic acid

Effects of glycyrrhetinic acid on isolated human visceral adipocytes extracted from lean patients
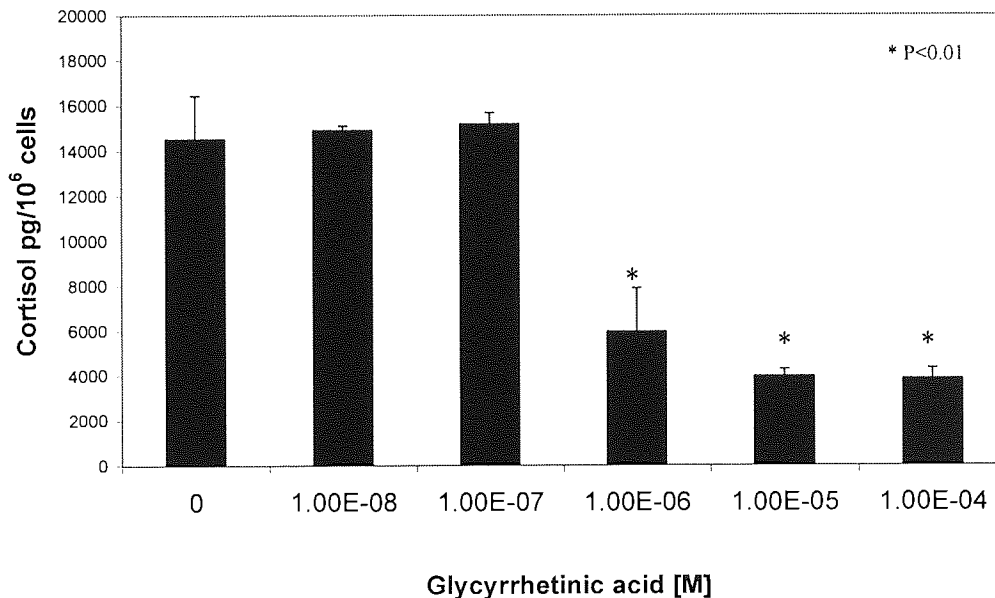
Figure 6. 1: Glycyrrhetinic acid on isolated visceral lean adipocytes

Detection of cortisol produced by isolated human visceral adipocytes from lean patients. Adipocytes were exposed to glycyrrhetinic acid (10^{-6}-10^{-8}) for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values $\pm$ SEM, n=4-15. (x axis: 1.00E-08=1x10^{-8}, 1.00E-07=1x10^{-7}, 1.00E-06=1x10^{-6}, 1.00E-05=1x10^{-5}, 1.00E-04=1x10^{-4}). Data were analysed by analysis of variance (ANOVA) and individual groups were compared using Student's t test. ANOVA showed strong significance (f=15.4, p> 0.00001, d.f= 30). Student's t tests showed that glycyrrhetinic acid concentrations of 10^{-8} and 10^{-7} M there was no statistical difference from control (0) and at concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M the results were highly significant (p>0.00001, see appendix) and differed vastly from control.

Effects of glycyrrhetinic acid on isolated subcutaneous human adipocytes extracted from lean patients

Figure 6. 2: Glycyrrhetinic acid on isolated subcutaneous lean adipocytes

Detection of cortisol produced by isolated human subcutaneous adipocytes from lean patients. Adipocytes were exposed to glycyrrhetinic acid $(10^{-6}\text{-}10^{-8})$ for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values ± SEM, n=2-8. (x axis: 1.00E-08=1x10^{-8}, 1.00E-07=1x10^{-7}, 1.00E-06=1x10^{-6}, 1.00E-05=1x10^{-5}, 1.00E-04=1x10^{-4}.) Data were analysed by analysis of variance (ANOVA) and individual groups were compared using Student's t test. ANOVA showed strong significance (F= 20.5, p>0.00001, d.f= 30). Student's t tests showed that glycyrrhetinic acid concentrations of 10^{-8} and 10^{-7} M there was no statistical difference from control (0) and at concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M the results were very significant (p>0.005, see appendix) and differed vastly from control.
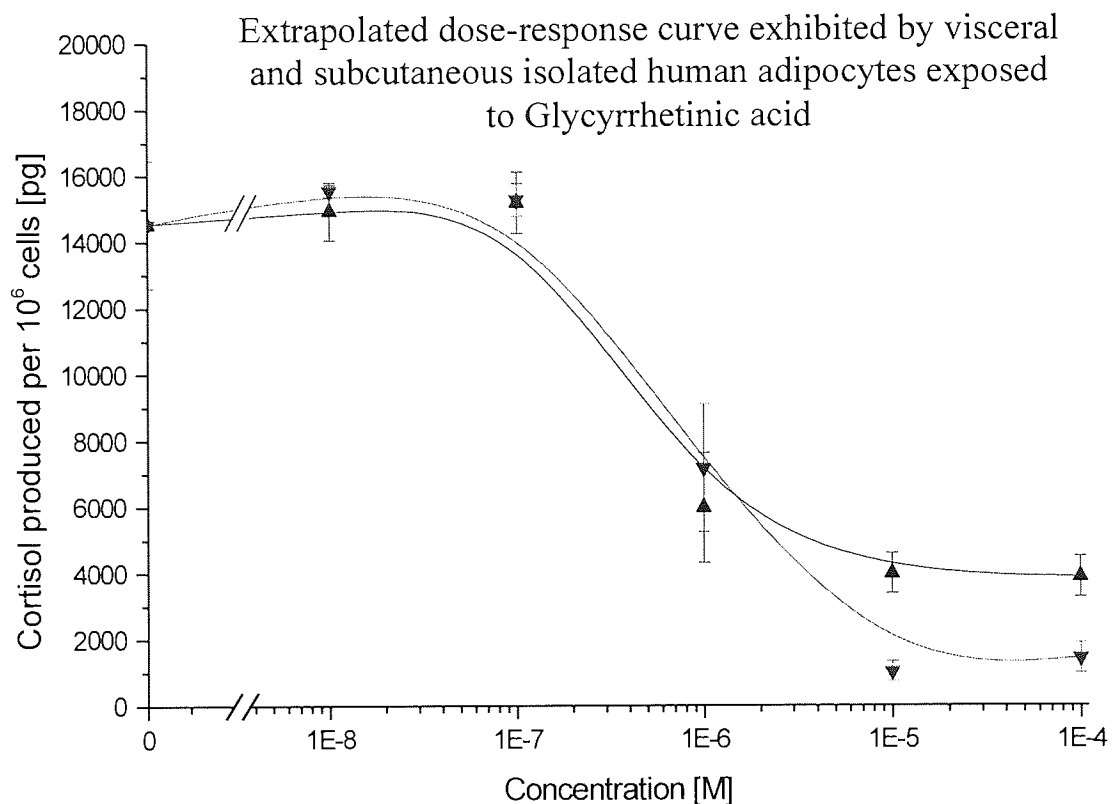
Figure 6. 3: Extrapolated dose-response effects of glycyrrhetinic acid on amount of cortisol produced by isolated human adipocytes from lean patients.

Adipocytes were exposed to glycyrrhetinic acid (10^{-4}-10^{-8}) for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values ± SEM, n=2-8. (x axis: 1.00E-08=1x10^{-8}, 1.00E-07=1x10^{-7}, 1.00E-06=1x10^{-6}, 1.00E-05=1x10^{-5}, 1.00E-04=1x10^{-4}.)

6.3.1.2 Biovitrum compounds

Effects of BVT1 on isolated human adipocytes from lean patients

Figure 6. 4: BVT1 on isolated lean adipocytes

Detection of cortisol produced by isolated human subcutaneous and visceral adipocytes from lean patients. Adipocytes were exposed to BVT1 (10^{-4}-10^{-6}) for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values $\pm$ SEM, n=3, d.f= variable see appendix. 1.00E-08=1x10^{-8}, 1.00E-07=1x10^{-7}, 1.00E-06=1x10^{-6}, 1.00E-05=1x10^{-5}, 1.00E-04=1x10^{-4}. Data were analysed by analysis of variance (ANOVA) and individual groups were compared using Student's t test. ANOVA showed no significance for both subcutaneous and visceral cells (F= 0.17, p=0.84; F= 0.96, p=0.43 respectively, d.f=8). Student's t tests showed that BVT1 concentrations of 10^{-6} and 10^{-5} M on visceral cells showed statistical difference from control (0, p>0.003, see appendix) but at concentrations of 10^{-4} this statistical significance was lost due to variation in data (p=0.164, see appendix). On subcutaneous cells all the data were highly significant (p>0.001, appendix).

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Effects of BVT2 compound on isolated human adipocytes extracted from lean patients
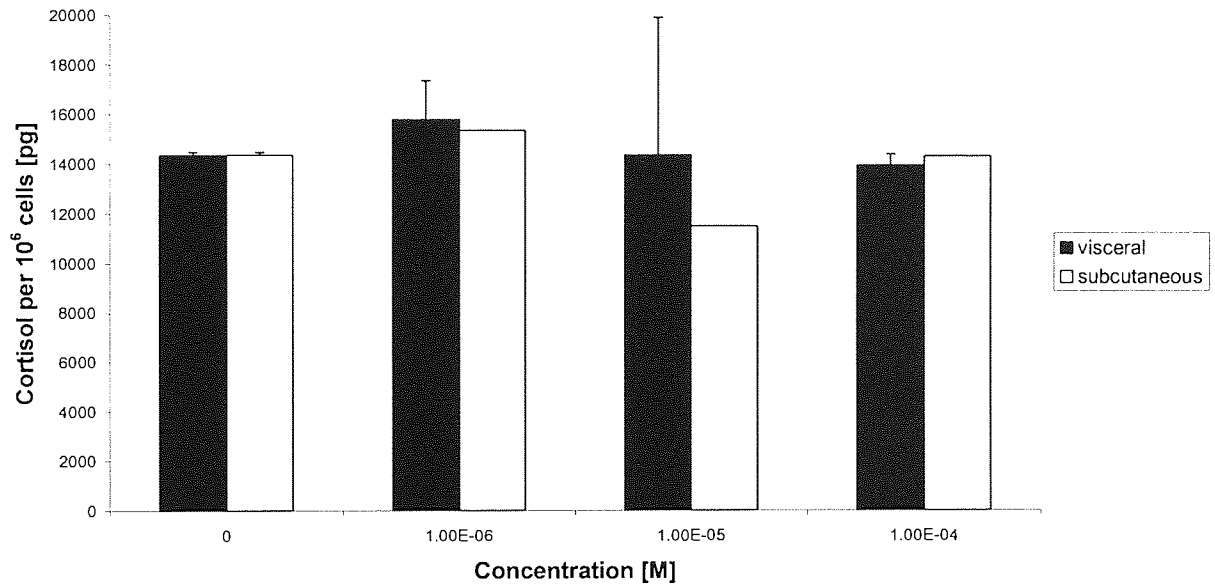
Figure 6. 5: BVT2 on isolated lean adipocytes

Detection of cortisol produced by isolated human subcutaneous and visceral adipocytes from lean patients. Adipocytes were exposed to BVT2 (10^{-4}-10^{-6}) for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values ± SEM, n=2. (x axis: 1.00E-08=$1x10^{-8}$, 1.00E-07=$1x10^{-7}$, 1.00E-06=$1x10^{-6}$, 1.00E-05=$1x10^{-5}$, 1.00E-04=$1x10^{-4}$.) Data were analysed by analysis of variance (ANOVA) and individual groups were compared using Student's t test. ANOVA showed no significance for subcutaneous but significant variance for visceral cells (F= 0.18, p=0.84; F= 41.4, p=0.0065 respectively, d.f=5). Student's t tests showed that BVT2 concentration of 10^{-6} M in visceral cells showed a negative t value indicating a significance in the opposite direction to that desired (p>0.01, see appendix). All other data expressed on the graph showed no statistical significance (p>0.1, see appendix). Due to low n numbers some error bars are negligible or cannot be shown.

6.3.2 Obese tissue

Glycyrrhetinic acid significantly decreased cortisone conversion to cortisol in adipocytes isolated from subcutaneous tissue extracted from obese patients (figure 6.6), however there were insufficient numbers of obese patients to acquire sufficient data to accurately determine a dose-response effect. Biovitum compound BVT2 (BVT.3498) (see figure 6.7) showed a significant reduction in cortisol production as did AstraZeneca compound AZ121. This reduction resulted in about 15% of the cortisol production observed with the control. The same effect of AZ121 was evident at each of the concentrations tested (10^{-4}-10^{-6}) indicating that lowering concentrations might also be effective. Unfortunately due to the quantities of tissue available for study and the increased risk associated with extraction of visceral tissue from obese patients, only subcutaneous samples were obtainable for investigation and as such no comparisons could be made with the visceral tissue response.

6.3.2.1 Glycyrrhetinic acid

Effects of Glycyrrhetinic acid on isolated adult human subcutaneous adipocytes extracted from obese patients
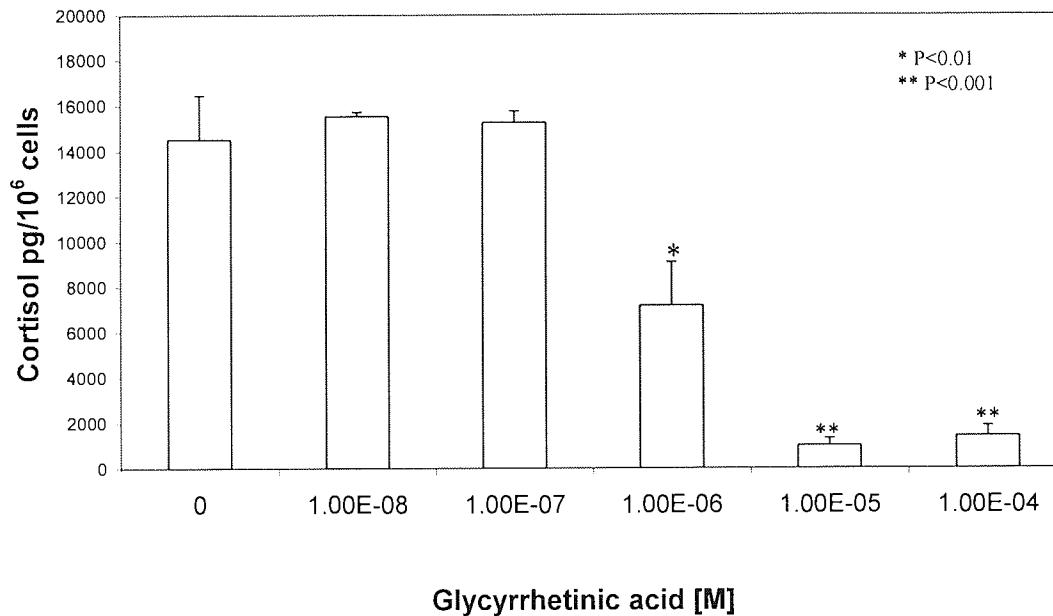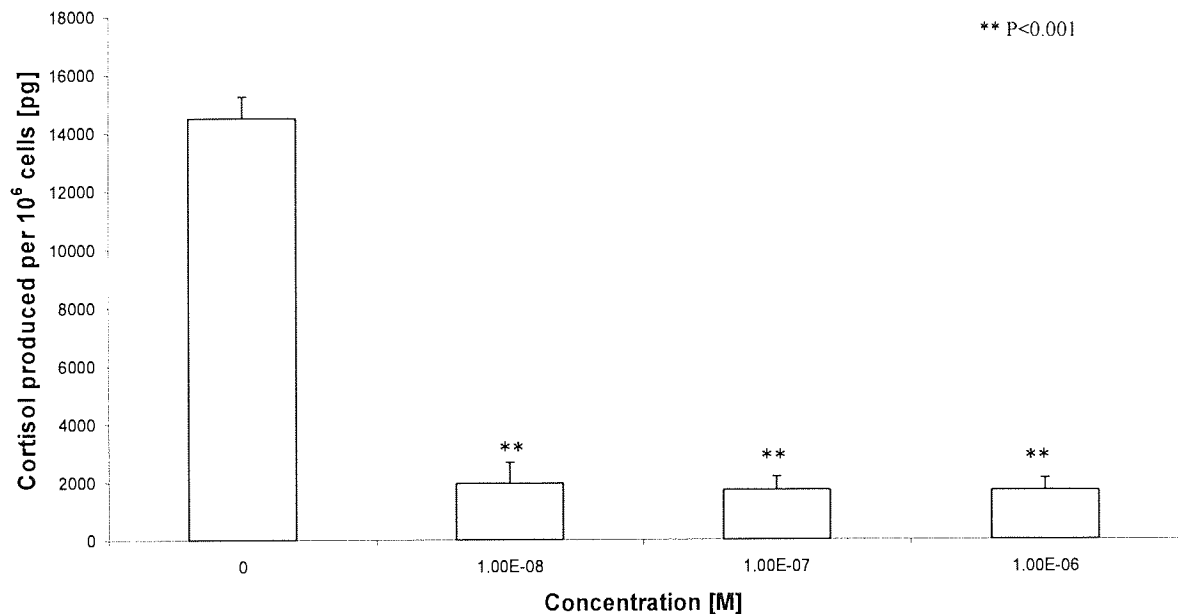

Figure 6. 6: Glycyrrhetinic acid on isolated subcutaneous obese adipoctes.

Detection of cortisol produced by isolated human subcutaneous adipocytes from obese patients. Adipocytes were exposed to glycyrrhetinic acid (10^{-6}-10^{-8}) for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values ± SEM, n=6-7. (x axis: 1.00E-08=1x10^{-8}, 1.00E-07=1x10^{-7}, 1.00E-06=1x10^{-6}). Data were analysed by analysis of variance (ANOVA) and individual groups were compared using Student's t test. ANOVA was highly significant (F= 96.3, p>0.00001 see appendix; d.f=24). Student's t tests showed that data from all glycyrrhetinic acid concentrations when compared with control were highly significant (p>0.000001 see appendix).

Effect of BVT2 on isolated adult mature subcutaneous adpocytes from obese patients
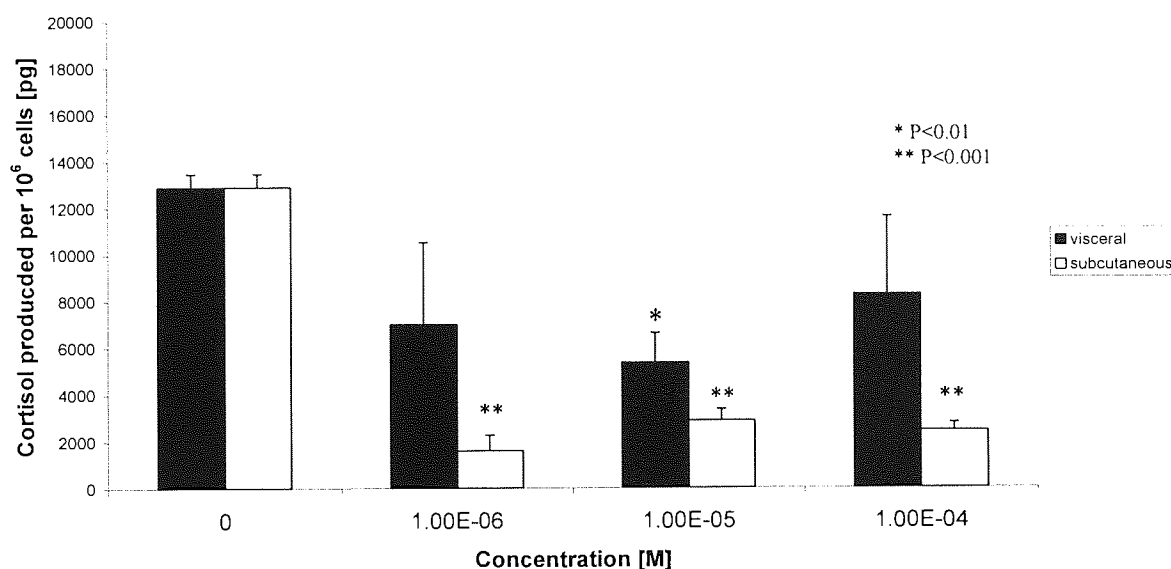
** P<0.001
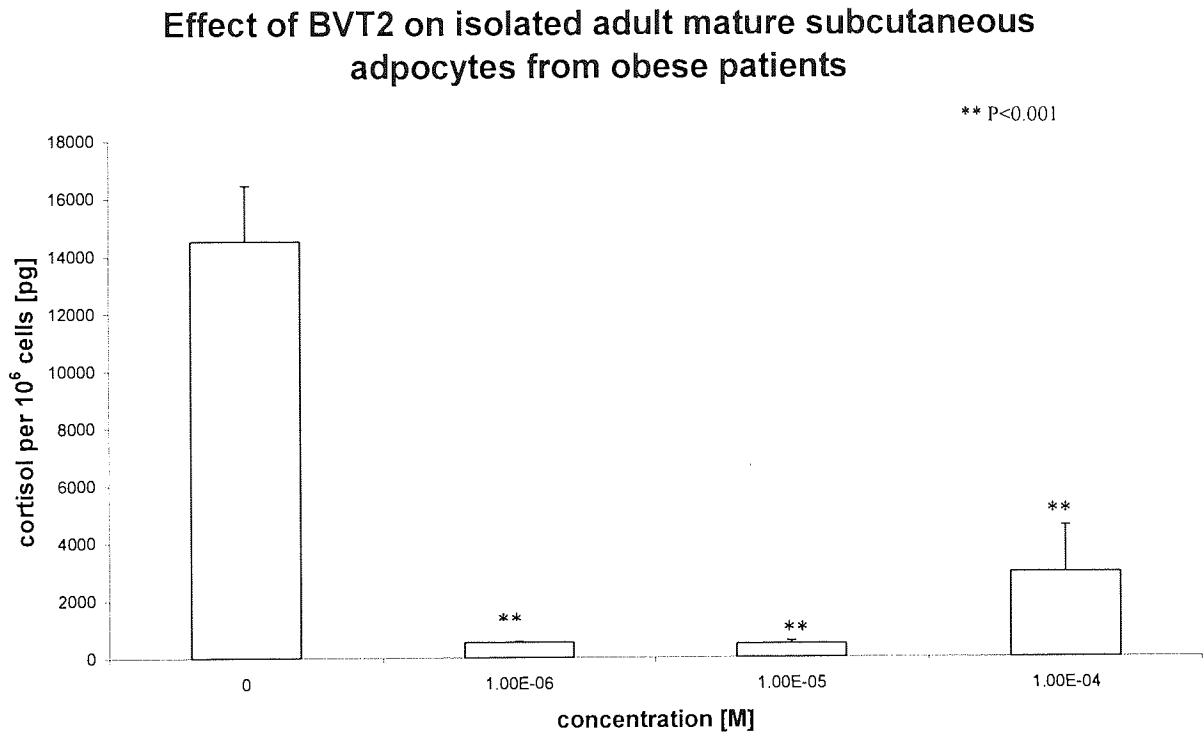
Figure 6. 7: BVT2 on isolated subcutaneous obese adipocytes

Detection of cortisol produced by isolated human subcutaneous adipocytes from obese patients. Adipocytes were exposed to BVT2 (10^{-6}-10^{-8}) for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values ± SEM, n=3. (x axis: 1.00E-06=1x10^{-6}, 1.00E-05=1x10^{-5}, 1.00E-04=1x10^{-4}). Data were analysed by analysis of variance (ANOVA) and individual groups were compared using Student's t test. ANOVA were highly significant (F= 54.8, p>0.00001 see appendix; d.f=15). Student's t tests showed that data from all BVT2 concentrations were highly significant (p>0.00001 see appendix).

6.3.2.3 AstraZeneca compound

Effects of AZ121 on isolated subcutaneous human adipocytes extracted from obese patients
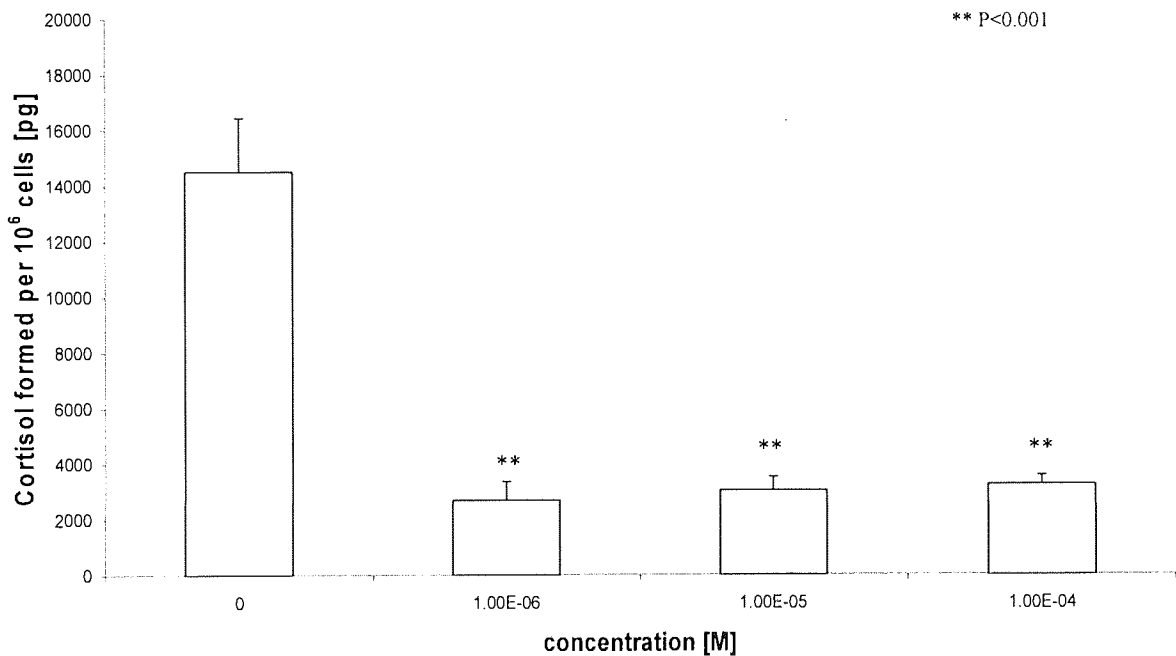
Figure 6. 8: AZ121 on isolated subcutaneous obese adipocytes

Detection of cortisol produced by isolated human subcutaneous adipocytes from obese patients. Adipocytes were exposed to Astrazeneca compound AZ121 (10^{-4}-10^{-6}M) for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values ± SEM (n= variable see appendix). (x axis: 1.00E-06=1x10^{-6}, 1.00E-05=1x10^{-5}, 1.00E-04=1x10^{-4}). Data were analysed by analysis of variance (ANOVA) and individual groups were compared using Student's t test. ANOVA were highly significant (F= 61.5, p>0.00001 see appendix; d.f=15). Student's t tests showed that data from all AZ121 concentrations were highly significant (p>0.00001 see appendix).

6.3.3 Obese diabetic tissue

Glycyrrhetinic acid decreased cortisone conversion to cortisol in adipocytes isolated from visceral and subcutaneous tissue extracted from obese diabetic patients (see figure 6.8). This decrease in conversion was dose-dependent effect in subcutaneous tissue. Data from visceral tissue exhibited a similar dose-dependent trend, although there was little reduction in cortisol production until the glycyrrhetinic acid concentration was elevated to 1×10^{-4}M. Although there were statistically significant differences between visceral these were marginal, and there was no continuity to this difference across the range of glycyrrhetinic acid concentrations tested. Similar reductions in cortisol production were seen with the addition of Biovitum compound BVT2 (see figure 6.4), however no dose-response was exhibited within the concentration range 10^{-4}-10^{-6}M. In contrast, adipocytes obtained from both subcutaneous and visceral depots of lean patients showed no significant reduction in cortisol production with the addition of BVT2 (see figures 6.12 and 6.13).

6.3.3.1 Glycyrrhetinic acid

Effects of Glycyrrhetinic acid on isolated adult adipocytes extracted from obese diabetic patients
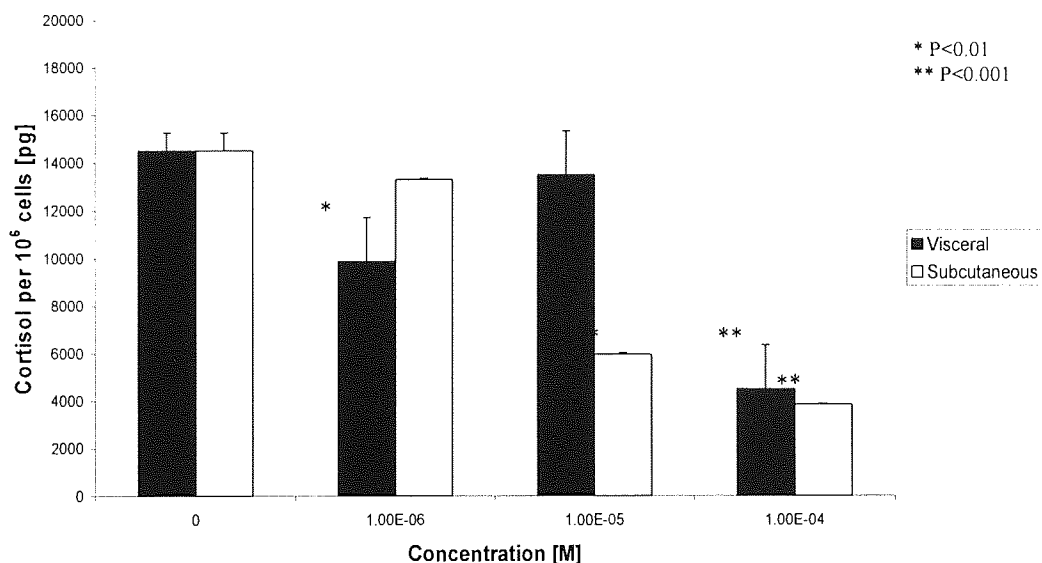
Figure 6. 9: Glycyrrhetinic acid on isolated obese adipocytes from diabetic patients. Detection of cortisol produced by isolated human subcutaneous adipocytes from obese patients. Adipocytes were exposed to glycyrrhetinic acid (10^{-4}-10^{-6}M) for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values $\pm$ SEM, n=3. (x axis: 1.00E-06=1x10^{-6}, 1.00E-05=1x10^{-5}, 1.00E-04=1x10^{-4}). Data were analysed by analysis of variance (ANOVA) and individual groups were compared using Student's t test. ANOVA were statistically significant for both visceral and subcutaneous cells (F= 7.8, p>0.01; F=9.05, p=0.012 respectively, d.f=15-18; see appendix). Student's t tests showed that 10^{-4} and 10^{-6} M glycyrrhetinic acid concentrations on visceral cells were statistically significant (p>0.01 see appendix). Concentration 10^{-5}M was not statistically different from the control (p=0.3). Results on subcutaneous cells reach significance at 10^{-5}M glycyrrhetinic acid (p>0.001, see appendix) and show a dose-dependent decline.

6.3.3.2 Biovitrum Compounds

Effects of BVT2 on isolated human adipoctes extracted from obese diabetic patients
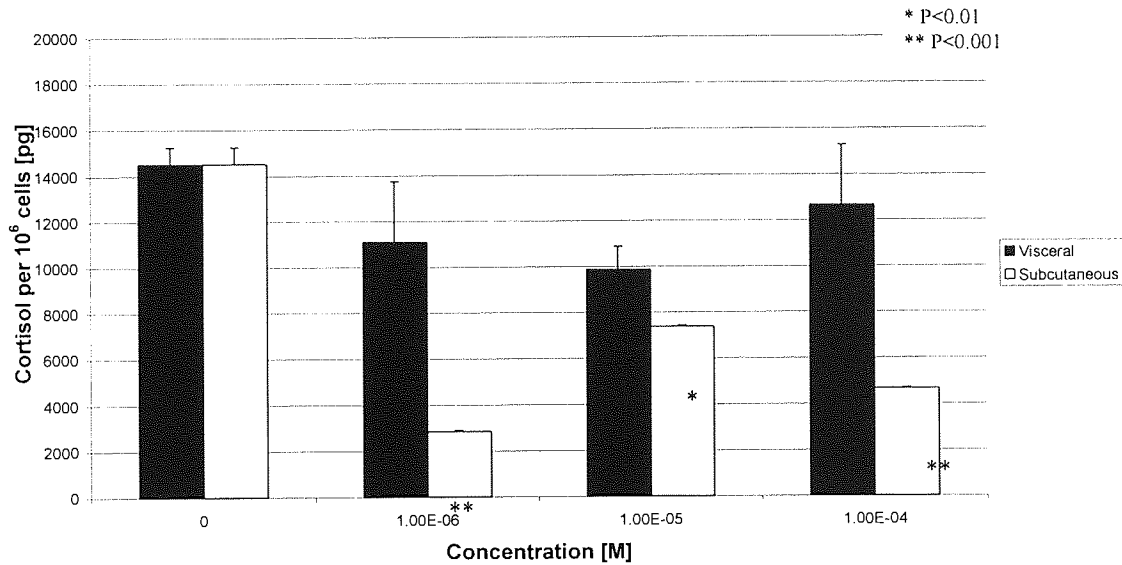
Figure 6. 10: BVT2 on isolated obese adipocytes from diabetic patients

Detection of cortisol produced by isolated human subcutaneous adipocytes from obese patients. Adipocytes were exposed to BVT2 (10^{-4}-10^{-4}) for 2h at 37^0C. Results were expressed as pg of cortisol extracted per 10^6cells. Mean values ± SEM, n=3. (x axis: 1.00E-06=1x10^{-6}, 1.00E-05=1x10^{-5}, 1.00E-04=1x10^{-4}). Data were analysed by analysis of variance (ANOVA) and individual groups were compared using Student's t test. ANOVA showed that visceral data showed no statistical significance, whereas subcutaneous data was highly statistically significant (F= 1.5, p=0.25; F=29.7, p> 0.00001 respectively; d.f=15-18, see appendix). Student's t tests showed that data from BVT2 concentrations 10^{-6} and 10^{-4} M on visceral tissue exhibited no statistical difference from control, however at 10^{-5}M significance was noted (p>0.01, see appendix). All data obtained from subcutaneous cells showed statistical significance (p>0.01, see appendix). Data from neither visceral nor subcutaneous cells exhibited a dose-dependent relationship.

6.3.4 Graphical summary

6.3.4.1 Visceral data

Summary of visceral data
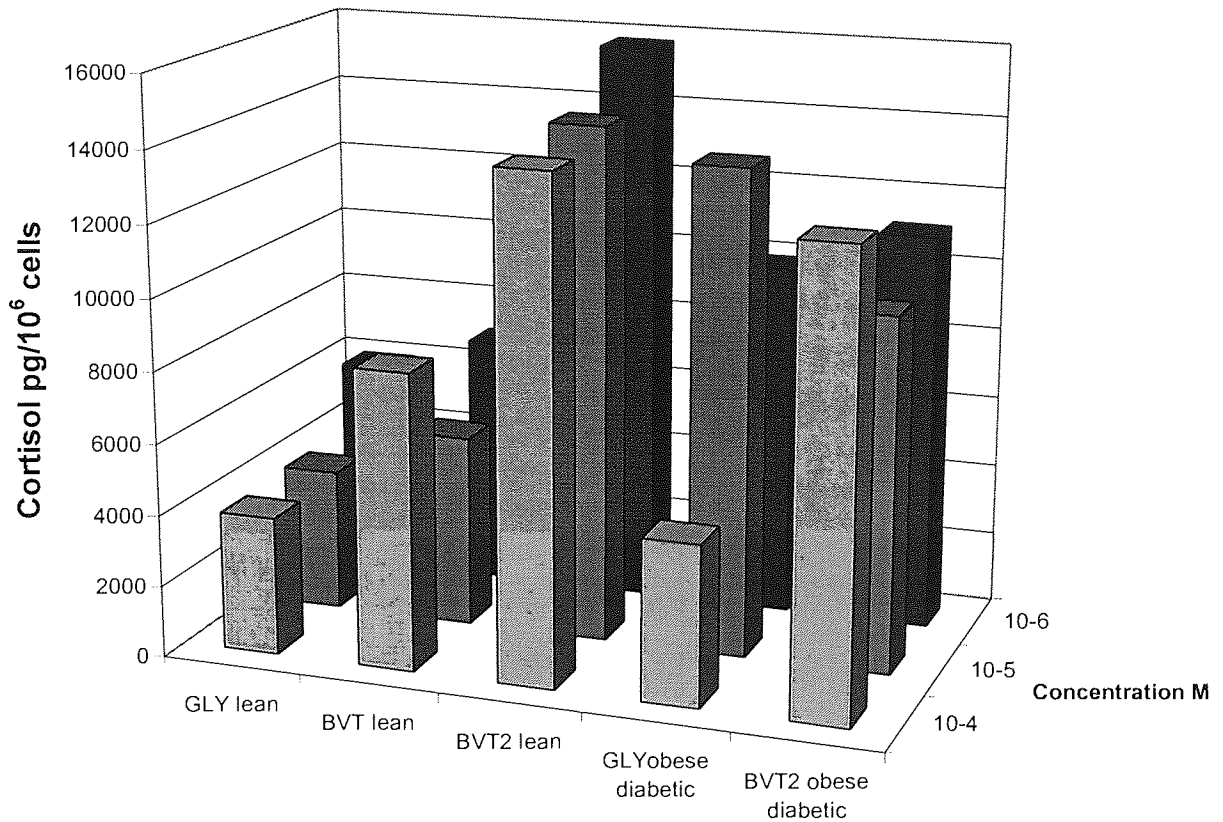
Figure 6. 11: Summary of data

Summary of data depicting cortisol production from isolated visceral adipocytes with the addition of 11β-HSD1 inhibitors glycyrrhetinic acid (GLY) and Biovitrum compounds BVT1 and BVT2, on tissues from lean obese or obese diabetic patients.

6.3.4.2 Subcutaneous data

Summary of subcutaneous data
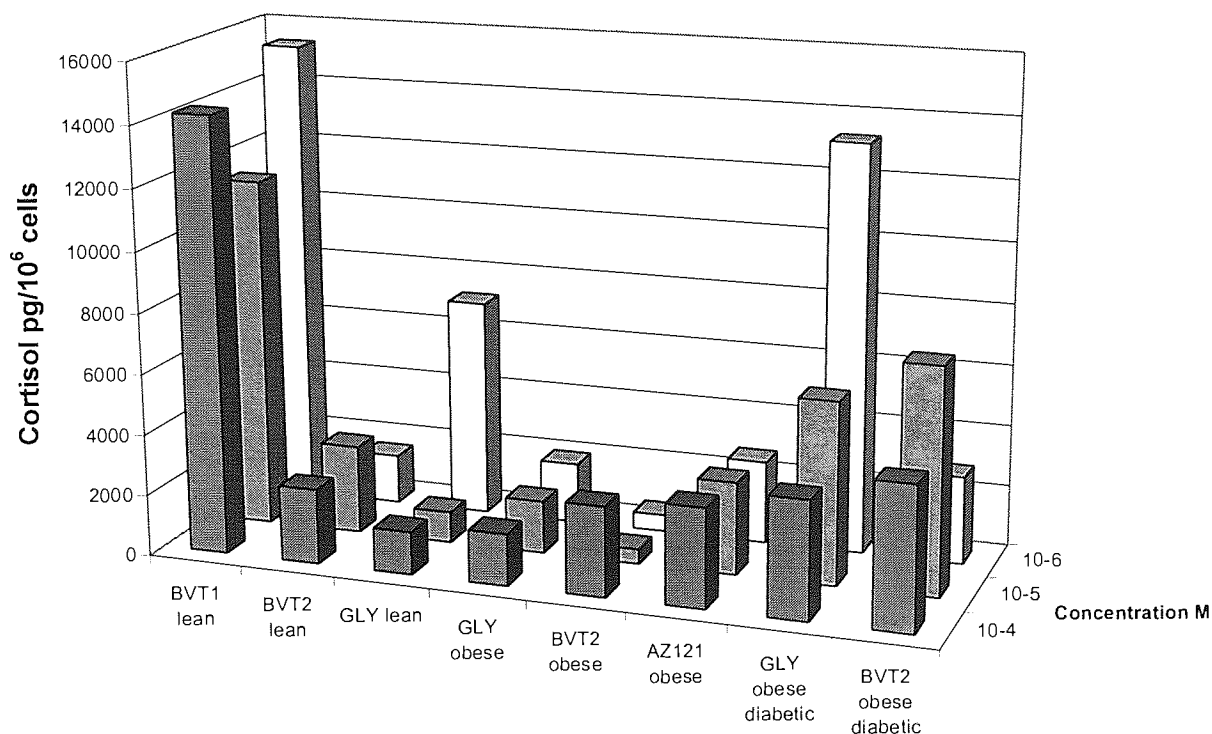
Figure 6. 12: Summary of data

Summary of data depicting cortisol production from isolated subcutaneous adipocytes with the addition of 11β-HSD1 inhibitors glycyrrhetinic acid (GLY), Biovitrum compounds BVT1 and BVT2, and Astrazeneca compound AZ121 on tissues from lean obese or obese diabetic patients.

6.4 Discussion

The inter-conversion of cortisone and cortisol in tissues such as adipocytes is controlled by the enzyme 11β-HSD. In adipose tissue the predominant direction of the reaction is responsible for local cortisone activation to cortisol (see Chapter 5 section 5.1.2). Glycyrrhetinic acid is a known inhibitor of 11β-HSD, and in adipose tissue glycyrrhetinic acid significantly decreased cortisol production in both lean and obese isolated human adipocytes. In addition investigations with lean tissue showed a significant decrease in cortisol production using both subcutaneous and visceral tissue depots in the presence of glycyrrhetinic acid. Investigations with obese tissue were not undertaken due to constraints on tissue supply (see Chapter 6 section 6.3.2), although other agents were tested in adipocytes from obese patients. Both depots showed a similar dose-response decline in cortisol production with little or no significant difference between them during exposure to glycyrrhetinic acid. These findings are not consistent with suggestions of a possible site-dependent variation in the reductase activity of 11β-HSD1 between lean and obese adipose tissue cited (Engeli 2003). Stewart and Krozowski (1999) found increased activity of 11β-HSD in subcutaneous and visceral depots of lean subjects compared with obese. The present study also conflicts with the viewpoint that there is a variation in cortisol production between visceral and subcutaneous depots as suggested in Morton 2004, as no such variation was observed. However, the present results are supported by findings from Stewart's laboratory (Tomlinson *et al* 2005), where it was observed that there were no differences between subcutaneous and omental depots, which also did not correlate with BMI. Further to this, it was reported that a decrease in mRNA levels and activity of 11β-HSD1 occurred in obese patients and inhibition of the enzyme resulted in increased preadipocyte proliferation. The effects of glycyrrhetinic acid on isolated visceral

and subcutaneous adipocytes from obese diabetic patients are much less a dose-dependent decrease of cortisol production was still evident. These data show greater significance for subcutaneous depots over visceral depots; a possible explanation for this variation may lie with the action of insulin.

Patients with T2DM have impaired insulin action: this is believed to be related to insensitivity of tissues to insulin action as outlined in chapter 1 (section 1.3). Insulin is a metabolic inhibitor of 11β-HSD (Reynolds and Walker 2003). The suppression of 11β-HSD in insulin resistance T2DM may therefore obscure or override the suppression by other test compounds in the present study. This could contribute to differences of effects between obese diabetic and non-diabetic tissues examined herein.

Biovitrum compounds BVT1 (BVT.2733) and BVT2 (BVT.3498) had contrasting results which varied according to the tissue depot and patient type. BVT1 is a murine specific 11β-HSD1 inhibitor and BVT2 is a human 11β-HSD1 inhibitor which also shows effect in murine tissue.

In tissue from lean patients there was a decrease in cortisol production with BVT1 as seen in glycyrrhetinic acid, but to a lesser same extent. This effect was unexpected as BVT1 was understood to be selective only for murine tissues. In lean patients BVT2 showed no significant decrease in cortisol production. An explanation for this may reside with either the cellular degradation or mode of action of the compounds. For example different metabolites of the compounds could have different effects on oxo-reductase versus dehydrogenase activity. Recent press releases from biovitrum (Nov 2003) suggest that studies have supported this finding and as a result this has protected against hypoglycaemia even with BVT.3498 with

treatment   (www.leaddiscovery.co.uk/target-discovery/abstracts/PubMed-110305.html).

However there are no published data to inform about the cellular metabolism and metabolites of these agents, or their molecular interaction with 11β-HSD, therefore this potential route to explain differences in the actions of these compounds cannot be explored further at this time. The effect of BVT2 on obese diabetic tissue shows a comparable decrease in cortisol production to that seen with glycyrrhetinic acid, except there is no obvious dose dependent effect within the concentration range of the drug tested. This suggests that BVT2 is likely to be an effective suppressant of 11β-HSD1 activity in subcutaneous obese adipocyte, although there was no apparent activity in subcutaneous or visceral lean adipocytes. Having already noted that other agents are producing similar effects in lean and obese adipocytes, it is particularly interesting that this agent should show different effects between tissues. This might be due to differences in the control of 11β-HSD, but the conformity of the tissues in response to other suppressants of 11β-HSD would suggest that the 11β-HSD is similarly controlled in the two adipose depots. Therefore it is more likely that the BVT2 is metabolised either to different metabolites or degraded at different effects on 11β-HSD.

Addition of AstraZeneca compound AZ121 to isolated human adipocytes from obese patients showed a knock down of 11β-HSD action comparable to that exhibited by glycyrrhetinic acid. The effect was similar at all concentrations tested (10^{-4}-10^{-6}M) suggesting a potent effect that is likely to occur at concentrations below 10^{-6}M, although there was insufficient tissue to conduct these experiments.

Some of the studies within this field have been directly contradictory in their results. Stewart *et al* (2001) propose that there is no expression of 11β-HSD2 in adipose tissue; however this is

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contradicted by Engeli *et al* (2004) who recorded detectable levels of 11β-HSD2 mRNA in both adipose tissue biopsies and isolated human subcutaneous and abdominal adipocytes.

The over-expression of 11β-HSD2 is known to bring about an increase in proliferation of pre-adipocytes. In contrast 11β-HSD1 inhibits pre-adipocyte proliferation whilst increasing differentiation and lipid accumulation. Thus it follows that if 11β-HSD1 is persistently inhibited there would be a substantial increase in pre-adipocyte number, and following termination of treatment the patient would be pre-disposed to diet induced obesity. By analogy with thiazolidinedione (TZD) drugs which activate PPARγ, there is some support for this possible interpretation. The TZD PPARγ agonists cause adipogenesis leading to differentiation of pre-adipocytes to adipocytes. Increase in the numbers of small adipocytes in subcutaneous depots due to the PPARγ-stimulated adipogenesis results in an increase in overall fat mass depot and a gain in weight (Boden *et al* 2003). Contrary to this hypothesis rodent models, primarily 11β-HSD knock out mice, show resistance to diet induced obesity (Morton *et al* 2001, Kershaw *et al* 2005). Theoretically if these mice were to subsequently acquire a functional 11β-HSD enzyme their weight gain would be greater than that of wild type mice due to an increased number of pre-adipocytes (Tomlinson 2005). The human comparison of this model is patients with apparent cortisone reductase deficiency (Draper *et al* 2003, Jamieson *et al* 1999). In such cases there is a deficiency in the HSD11B1 gene rendering it non-functional. In these patients there is a tendency towards adiposity (Tomlinson 2005, Draper *et al* 2003), in particular in the central region (Jamieson *et al* 1999).

In light of increased pre-adipocyte proliferation seen after the administration of 11β-HSD1 inhibitors, the pharmaceutical agents would probably be unsuitable as obesity reducing agents unless administered for life. However, they could be used therapeutically for morbidly obese

or obese individuals who require acute weight loss prior to surgical intervention in combination with a calorie controlled diet. The increased number of subcutaneous pre-adipocytes would be removed during surgery, leaving only an intra-abdominal increase in pre-adipocytes. These could potentially be reduced via other treatments, including continued calorie constriction.

In conclusion, the present data outline four potential inhibitors of 11β-HSD1. Glycyrrhetinic acid is a potent inhibitor of 11β-HSD1 but is not a potential therapeutic agent due to its lack of selectivity for adipocytes. In the present study data obtained from the addition of glycyrrhetinic acid can be used as a bench mark of potency for the remaining selective agents. Overall the test agents were most potent in subcutaneous depots compared with visceral depots, although further investigation with AZ121 is required since visceral tissue was unavailable for comparison. Potency of BVT1 and AZ121 was comparable to that seen with glycyrrhetinic acid in subcutaneous and visceral tissue as indicated by the preliminary and limited data available from the present study. The most notable discovery was seen with BVT2 followed by AZ121; significant inhibition was only observed in obese and to a lesser extent obese diabetic tissue (BVT2) and not in lean tissue. This trend was seen primarily in subcutaneous tissue depots. It is possible that the effects of BVT2 on obese diabetic patient tissue could be enhanced via the addition of an insulin sensitizing agent such as a TZD. Understanding the difference in pharmacokinetics, down steam metabolic effects and metabolites of BVT2 upon these two tissue depots could reveal further potential for combinational therapeutic targets and agents for obesity.

Chapter 7: Discussion

7.1 Overview

This research programme set out to investigate some of the molecular signalling mechanisms that control total energy balance and regulate adiposity. The studies undertaken utilised human visceral and subcutaneous adipose tissue removed with appropriate permission during elective surgical procedures. Tissue was processed (see chapter 3) for use in characterisation studies and for more detailed investigation of 11β-HSD1. These studies have provided novel information regarding the nature of mature adipocyte development, suggesting that these cells have the capability to undergo division. It appears that the rate of division is dependent upon a density threshold of cells. Genetic information suggested that subcutaneous tissue may especially contribute to the preponderance of adipokine signalling molecules and could be the type of adipose tissue responsible that is more strongly for sensing overall adipose energy storage and initaiting adipokine regulation Visceral tissue, by comparison, ensures the turnover of fatty acids. Studies investigating 11β-HSD1 demonstrated a reduction in activity of this enzyme in the presence of inhibitors (BVT1, GLY and AZ121), with BVT1 and AZ121 being the most potent. Suppression was greatest in subcutaneous adipocytes isolated from both lean and obese individuals.

7.2 Rationale

The aims of this study were to determine the subtle differences in visceral and subcutaneous adipose tissue function and to propose how these differences could be associated with sensing-signalling activities, metabolic disease, cardiovascular risk and insulin resistance. In addition, knowledge attained from the study could provide important insights into depot specific targets for potential new therapeutic interventions against obesity.

7.3 Evaluation of novel methods

The ability of adipocytes to retain key functions (lipolysis, division and adipokine production) that are associated with the cell type is vital to this study. Therefore given the difficulty and viable success rate depicted in the literature a gentle isolation technique (chapter 3) and surgical method of tissue removal was utilised to reduce tissue damage and provide maximal viable isolated cells. The present method of whole tissue excision was selected in favour of liposuction techniques to preserve function. Many earlier studies designed to investigate key functions of adipocytes *ex vivo* have used tissue obtained by liposuction. This procedure may be damaging to the tissue due to the physical forces subjected to the cells. The procedure also uses a variety of chemicals including sympathomimetic agents to constrain local bleeding. Studies on functions of such tissues (measuring lipolytic, division and adipocyte function) may not therefore give a representative reflection of prior physiological performance of the tissue *in vivo*. For example tissue acquired via liposuction that is artificially stimulated with noradrenaline during removal (Brown *et al* 2004) may not show a normal response to restimulation as typified by the reduction in sensitivity to repeated stimulation by catchecholamines seen during adrenergic synaptic stimulation (Wingard *et al* 1991). Also

much of the tissue has been lysed or disrupted by sonication leaving a greater distribution of smaller cells (Pu *et al* 2005, Boschert *et al* 2002). Therefore this method of tissue acquisition may have given rise to artifactual data and a non-physiological responsiveness.

The primary aims of this study were dependent upon superior adipocyte isolation techniques which were adapted for human tissue from the rodent adipose isolation procedure of Rodbell (1963). Some previous studies have relied upon pre-adipocyte cell lines (see chapter 4) which can be induced to differentiate, however morphologically they do not resemble mature fat cells and functionally produce only a fraction (1-2%) of leptin seen in mature adipocytes (Flier 1997), indicating a substantial difference in signalling activity.

Isolation and culture of mature human adipocytes has proven to have several difficulties including the ability to float and the multitude of cells comprising adipose tissue. To attain greatest quality of data it was imperative to achieve clean separation of cells into distinct fractions in order to isolate pre-adipocytes from mature adipocytes and to remove the majority of the lipid released from lysed cells. Alternative techniques were explored including a ceiling culture methodology proposed by Sugihara *et al* (1986) with varying degrees of success. The final novel option of free floating cultures for mature cells was selected for several reasons. Initially, as expected from the literature (Zhang et al 2000), adipocytes suffered major cell lysis during the first 72hrs. Modification of the protocol by changing the depth of the media and culture vessels greatly reduced this cell lysis. This provided the basis of a novel and reproducible protocol for mature adipocyte cell culture. Use of this technique ensured there were good consistency of results and a reproducible of environment allowing cells to divide freely (see chapter 4). As the procedure was not dependent on cell adherence, known to be comparatively low in both cell subtypes (visceral and subcutaneous) of mature adipocytes, the

procedure allowed comparison of the subtypes in an environment that was apparently more conducive to their survival. Maintaining cells in their natural 3D-structural form retained their ability to grow and divide for at least 14 days which is indicative of a healthy growth culture environment.

As a consequence of this novel approach to adipocyte culture, a computerised method of cell counting had to be devised (see chapter 3.5). This enabled cells to be counted in situ, within the cultures, and the method was validated by comparison with haemocytometric methods. In addition an approximation of cell number had to be determined via a modified haemocytometric method (Chapter 3.5) for plate seeding. The new counting methods developed were comparable to conventional procedures. Accuracy and reproducibility of methodology ensured data were reliable and consistent in quality.

7.3 Discussion of Key findings

7.3.1 Isolation and culture of adipocytes (Chapter 3 and 4)

As the focus of this study was concerned specifically with human genetic and molecular responses to adiposity, 'human' was the primary source of tissue and all data described were derived from human tissue samples (chapter 3.2) in the procedure outline in chapter 3. Isolated mature and pre-adipocytes were used throughout the studies, and have been characterised in chapter 4. Primary culture of pre-adipocytes was accomplished in only visceral samples due to a reduced adherence ability of subcutaneous cells and constraints on availability of tissue after the technique had eventually been developed. Observation of subcutaneous cells isolated at the same time showed reduced primary adherence and subsequent lack of proliferation. In addition, cells remained in spherical form suggesting that they may have increased susceptibility to early differentiation. Bakker *et al* (2004) recently observed similar disparity

in the adherence of pre-adipocytes between individuals and between tissue depots. They suggested that the differences may be attributed to age or adiposity. In addition they suggested that pre-adipocyte numbers are regulated at a systemic and/or genetic level. Increased ability of the visceral depot to create a larger pool of pre-adipocytes may enable this subtype of adipocytes to retain smaller insulin sensitive cells during periods of fatty acid increase. Enhanced susceptibility of pre-adipocyte differentiation may promote the storage of excess fat in the subcutaneous fraction.

Mature adipocyte isolation and characterisation was described in chapter 4. Main findings showed that mature adipocytes have the capacity to divide and additionally this mitosis has been shown to have a threshold onset. Although previously presumed to be unable to undertake further division post the "terminal stage" stage of development (Prins and O'Rahilly 1997) the process has been described tentatively recently by Zhang *et al* (2000). This thesis develops these initial findings suggesting that the onset of mature cell number increase is sensitive to an increase in fatty acid uptake and subsequent reduction in available space between the existing cells or the pressure exerted against adjacent cells. In recent time adipose tissue has been recognised for its role in providing regulatory endocrine, paracrine and autocrine signals (Kim and Moustaid-Moussa 2000, Rasmussen *et al* 2006, Fukumura *et al* 2003). Therefore it is reasonable to assume that increased mature cell division may be induced by a change in cell density and/or paracrine communication through local adipokine concentration. Recent discoveries suggest that AdipoR1, an adiponectin receptor, is highly expressed in human adipose tissue, and this has been proposed to be involved in autocrine/paracrine signalling (Rasmussen *et al* 2006). In addition both AdipoR1 and adiponectin (chapter 5) are reduced in obesity. Future investigation could consider whether

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this decrease in local adiponectin signalling results in an increase in mature adipocyte cell number, and could in part explain the conundrum surrounding adiponectin (Haluzik *et al* 2003). This theory suggests that the decrease in adiponectin seen in obesity is a local protective mechanism in order to increase cell number and thus increase capacity to store fat (Koerner *et al* 2005).

7.3.2 Gene expression of selected adipokines

The growing prevalence of obesity has expedited the requirement for therapeutic intervention. The need to discover potential therapeutic targets has in part focused upon the release and function of adipokines. In chapter 5 the gene expression distribution pattern of selected adipokines was investigated. Genes encoding proteins involved in adipocyte control, fat storage and sensing of energy deposition were explored. The main findings of this research suggest that subcutaneous fat is the primary depot of adipokine release and by extrapolation, long-term energy management. For some of the selected genes this control varies between gender providing insight into possible pathways involved in the dimorphic differences observed between men and women. Notably the expression of genes involved in fat storage (SCD, GPAT, and DGAT) is greater in the subcutaneous depot in females versus males in lean subjects. These expression effects were less apparent with obesity in all participants. This could be because the body has an increase in adipose cell number, and therefore the rate of fatty acid storage for a given cell number may appear lower even though over all uptake is increased. Further study would be required using labelled fatty acid incorporated into triglyceride to monitor fatty acid uptake to determine if this hypothesis can be supported. There is little information to support these expression data as much of the related work has

only been carried out in rodent models or on rodent tissue, and this information has not been separated by tissue depot or gender.

DGAT knock out mice support a decrease in triglyceride, suggesting that inhibitors of DGAT1 and DGAT2 could provide novel agents against obesity (Chen and Farese 2005). There was no significant variation in expression patterns observed within data from the visceral depot, suggesting lipolytic increase described in the literature (Arner 2005) is attributable to an increase in cell number. This would therefore increase with increased adiposity. To complete the picture further, expression information regarding lipogenic enzymes would have to be acquired. One could postulate that expression should be highest in the visceral depot and this would support both lipolytic activity (Frayn 2002) and increased fatty acid turnover in visceral tissue. The synergistic relationship between the visceral and subcutaneous function has yet to be completely explored, however the present preliminary findings suggest that fat intake is turned over rapidly by the visceral depot.

Surprisingly there was no statistical evidence suggesting the expected increase in 11β-HSD1, which would increase local cortisol levels and thus increase differentiation of pre-adipocytes to mature adipocytes (Chapter 5). However consistent with earlier observations expression levels are increased in the subcutaneous depots of female participants. This is consistent with local control of dimorphic gender differences. This pattern of expression is also seen in male participants, but to a lesser extent, which supports the greater importance of subcutaneous tissue as an overall lipid store. However, it should be emphasized that although mature cell division is increased in the visceral depot (chapter 4), further investigation will be necessary to determine any possible gender differences. Expectation would be that males would have a greater capacity for division than females in the visceral depot, thus conforming to gender

dimorphic patterns (apple and pear) well recognised and described in the literature (Krotkiewski *et al* 1983, Albright and Stern 1998, Jensen *et al* 2003).

Expression of leptin greatly supported initial theories that the subcutaneous depot is primarily responsible for signalling energy levels to the brain. Leptin expression was greater from subcutaneous depots although this finding is highly controversial as several studies have indicated that visceral fat makes a higher contribution to for the fluctuations in leptin concentrations (Casabiell *et al* 1998, Park *et al* 2004). However studies by Van Harmelen *et al* (1998) and Hube *et al* (1996) support data from this study finding that subcutaneous fat provided increase leptin signalling compared with visceral. Furthermore it would be logical from an evolutionary perspective for the more stable form of adipose tissue (subcutaneous) to be the source of adipokine signalling to notify the brain of lipid energy stores. In general expression levels do not change with obesity, however the increase in subcutaneous adipose tissue should result in an overall plasma leptin increase. Furthermore it has been documented that total subcutaneous fat correlates better with circulating leptin levels than visceral (Woods *et* al 2003). Additional findings to support local control of sexual dimorphic fat distribution have been derived from leptin expression data that show expression levels were significantly increased in females versus males. Information from Woods *et al* (2003) supports this claim, although these authors have used the data to propose that adiposity control in females is more reliant on leptin, while males are more reliant on insulin. The acute fluctuations of insulin, and the interactions of leptin and insulin on nutrient metabolism (Bates and Myers 2003) make it difficult to test this concept. Indeed previous studies attempting to identify a role of insulin in signalling energy store size (e.g. measuring insulin in the CSF) have given equivocal results (Baile *et al* 1983).

Adiponectin expression was lower in subcutaneous cells, this is particularly apparent in tissue from male participants. This is supported by the literature which suggests women naturally have increased levels of adiponectin (Kern *et al* 2003). Gender differences in adiponectin may be closely related to control mechanisms for the distribution of adipose stores. Earlier proposals have suggested that leptin and insulin control have a gender bias (Woods *et al 2003*). A decrease in adiponectin and increase in insulin resistance are often associated and have a gender bias towards males. Both a decrease in adiponectin and increase in insulin resistance are associated with increase cardiovascular risk (Haluzik *et al* 2003, Goldstein and Scalia 2004, Schondorf *et al* 2005). In addition male gender is associated with an increase in visceral adiposity; a previous hypothesis has suggested that decreased adiponectin levels may help increase adipocyte cell number in the subcutaneous depot. The reduction in levels of adiponectin in males may be indicative of a protection mechanism to re-direct fat distribution away from the visceral compartment.
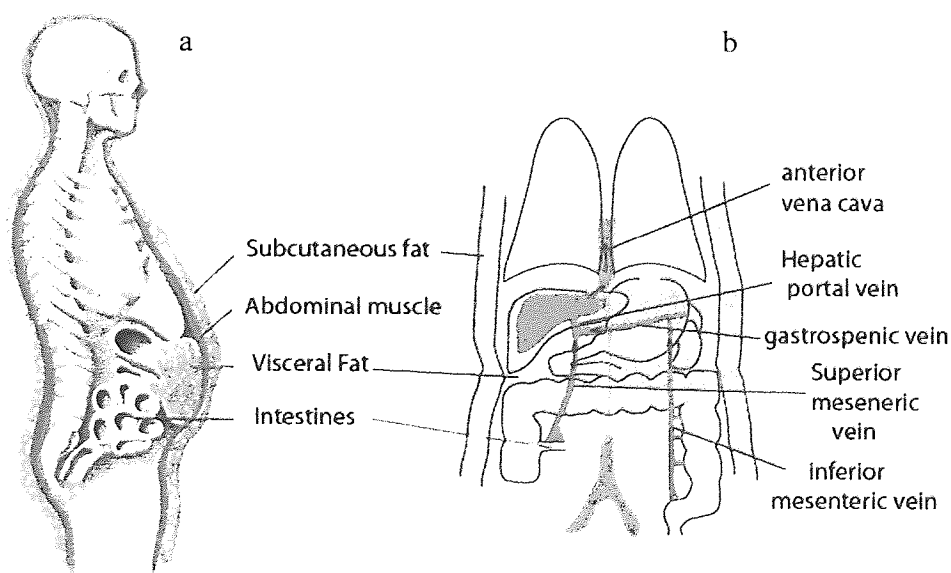
Figure 7. 1 'Central' Distribution of adipose tissue depots (subcutaneous and visceral)

Figure 7. 2 Association of vasculature and organs with adipose tissue depots

Physiologically, these hypotheses conform to the morphology of the human body. Figure 7.1 and 7.2 illustrate the association of the circulatory system and major organs with the two main central adipose tissue depots and 7.2 demonstrates where free fatty acids most commonly enter the circulation. This illustration demonstrates that the liver is ideally located to be the primary organ to process fatty acids from the diet. Furthermore the proximity and vascularisation of visceral adipose tissue enables it to act as the primary buffer for dietary fatty acids during periods of energy intake. This could alleviate initial stress on the liver during periods of excess fat consumed in the diet (Frayn 2002). Visceral adipose tissue has been strongly associated with fatty acid flux to the liver and evidence in support describes the hepatic portal vein as the route of transport (Bjorntorp 1991, Kabir *et al* 2005). Visceral tissue is suitably positioned to take up fatty acids directly from the diet via the mesenteric veins that drain into the hepatic portal vein. This enables controlled fatty acid transport to the liver. Depending on the body's requirements fatty acids could then be released safely bound to albumin or a fatty-acid transporter protein (FATP). However, if fat intake is constantly increased these two organs cannot retain control, resulting in increased free fatty acid circulation in the blood. These free fatty acids contribute to the detrimental effects associated with metabolic syndrome including insulin resistance, atherogenesis and cardiovascular complications (Eckel *et* al 2005, Miles *et* al 2005, Arner 2001). Excess accumulation of fatty acids is also likely to occur in the liver, giving rise to the prevalence of NAFLD (non-alcoholic fatty liver disease, also known as non-alcoholic steatohepatitis) that is associated with visceral adiposity and cardiovascular disease (Watanabe *et al* 2005, Gasbarrini *et al* 2005). Also, excess circulating fatty acids will increase intramyocellular fat, which is increasingly appreciated as a cause of insulin resistance in muscle (Kovacs and Stumvoll 2005, Shaffler *et al* 2005)
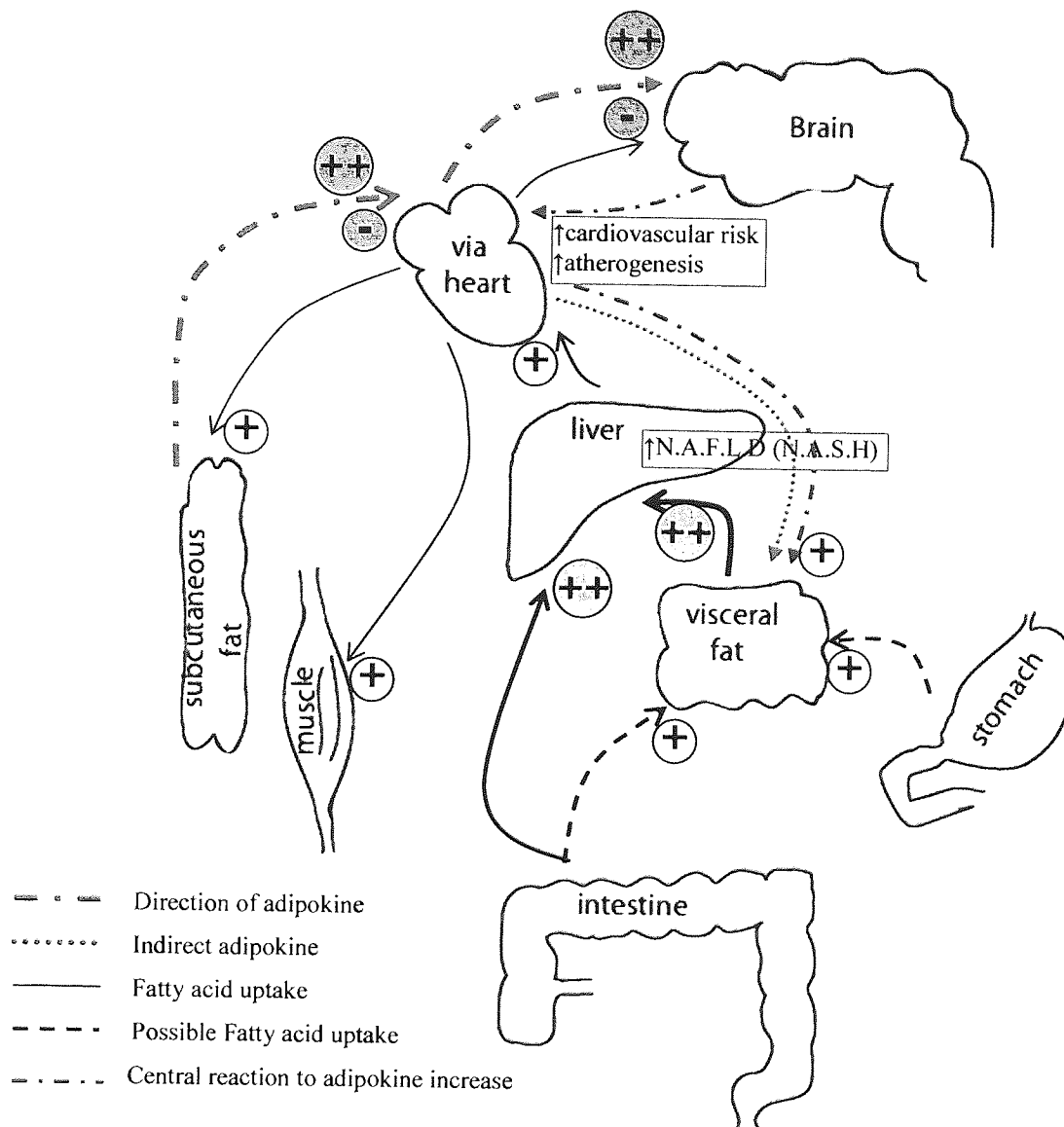
Figure 7. 3: Effects of obesity (excess adiposity) on fatty acid uptake and adipokine distribution

Adipokines have been shown to increase with obesity, including leptin. Adipokines known to decrease include adiponectin. Local adipokines that have been shown to decrease with obesity include Stearyl-CoA dehydrogenase (SCD1), Glucose-3-phosphate acyl-transferase (GPAT) and Di-glycerol acyl-transferase (DGAT). Local adipokines that increase include 11β-hydroysteriod dehydrogenase (11β-HSD1). Increases in fatty acid transported around the body can increase insulin resistance, non-alcoholic fatty liver disease (N.A.F.L.D) or non-alcoholic steatohepatitis (N.A.S.H), atherogenesis and cardiovascular risk

Figure 7.3 illustrates the direction of fatty acid transport and highlights the primary route of adipokine communication proposed by this study. The layout of the circulatory system and data from this thesis suggest that in lean humans the majority of dietary fat is adequately managed by the liver and visceral adipose tissue before being released for use by the rest of the body. In the event that the lipid capacity and requirement is modestly exceeded, fat would be stored in subcutaneous tissue. One could hypothesise that upon receipt of an increase in fat for storage the subcutaneous depot alters its adipokine profile to implement a decrease of energy intake into the body and as the body utilised energy there may be a small but significant decrease in fat the subcutaneous depot which would alter gene expression to promote energy uptake. When in energy balance, one could postulate that this system would mirror and complement the glucose/glycogen energy system (see figure 7.4) to maintain uniform fuel availability for very acute (glycogen) and slower but more protracted (triglyceride) energy supply (Frayn 2003 chapter 6). However, when there is a consistent excess of fat intake the body must increase its storage capacity which in turn disrupts the balance of control. Consideration of primary storage location must also be acknowledged, fat deposition depends upon the body's dimorphic properties; much of the literature suggests males are more susceptible to gaining storage capacity in the visceral abdominal region and women in the subcutaneous truncal region (Krotkiewski *et al* 1983, Albright and Stern 1998, Jensen *et al* 2003). In addition to these roles as a source of energy during periods of food deprivation, fat provides insulation and protective cushioning to internal and external forces and ensures fatty acids are not free to accumulate in non-lipid storing tissues (e.g. muscle, vasculature and liver) thereby protecting from cardiovascular events and insulin resistance (Albright and Stern 1998, Arner 2005).
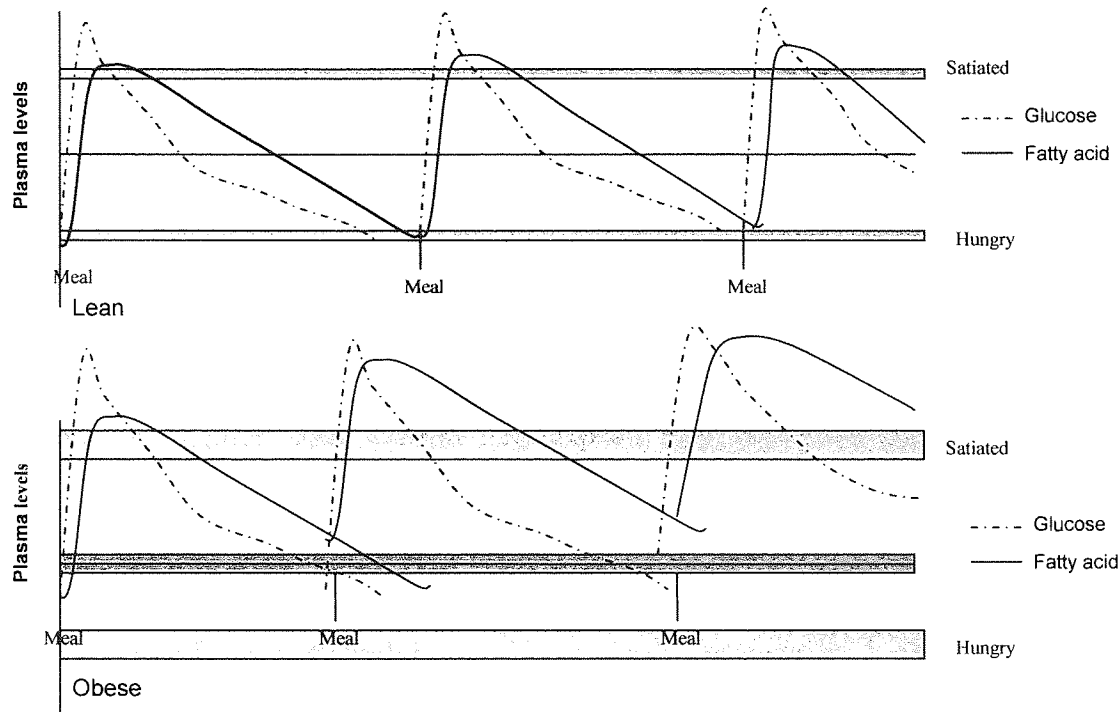
Figure 7. 4: Hypothesised effects of increase energy intake in lean and obese adult humans.

The issue of protracted nutrient deprivation is not applicable for the majority of people in current economical climates of western society. The insulation effects occur increasingly with greater adiposity but may become detrimental to temperature control and would exert pressure on internal organs with increases above the ideal. Although increased temperature would increase metabolic rate, it is the issue of fatty acids in the circulation which creates metabolic difficulty for patients and is thus the focus of much research.

Genetic data from this thesis showed a reduction in expression of triglyceride storage genes (SCD1, GPAT, DGAT1 and DGAT2) with increased adiposity in the subcutaneous tissue depot from male and female participants. However, if estimated for the increased tissue mass, the overall expression level in obese compared to lean participants should be increased. Furthermore, the expression levels of leptin were increased in women. Such findings which

have been implicated by others in the literature (Woods *et al* 2003) and may be attributable to the function of fat as a source of nutrients in female reproduction, and suckling (Mitchell *et al* 2005). Although expression levels of leptin were similar across the tissue depots from lean and obese participants, with consideration to the dimorphic increase in adiposity, plasma levels of leptin are elevated in obese patients especially women and therefore women may be more susceptible to leptin resistance than men (Saad *et al* 1998). Ambiguous or disregarded dimorphic adipose tissue patterns may explain why there have been contradictory reports regarding plasma leptin levels in the literature (Woods *et al* 2003, Park *et al* 2004).

7.3.3 11β-HSD1 activity findings

The main findings from the present studies of 11β-HSD1 activity in isolated adipocytes show that the most potent inhibition of 11β-HSD1 occurred in subcutaneous cells with AZ121. Overall when compared with visceral tissue; subcutaneous tissue showed greatest inhibition with most of the test agents. Although potency on subcutaneous tissue is not considered therapeutically ideal, in light of earlier hypotheses regarding subcutaneous signalling a reduction of subcutaneous tissue may indirectly cause a reduction of energy intake.

The capacity of 11β-HSD to operate both an oxo-reducatase reaction (11β-HSD1: cortisone to cortisol) and a dehydrogenase reaction (11β-HSD2 and 11β-HSD1: cortisol to cortisone) is based on the presence of two separate genes with 21% homology (Draper and Stewart 2005). Expression of these two enzymes varies between tissues such that adipocytes express mainly 11β-HSD1, and there remains some controversy over potential differences in the levels of expression and activity of this enzyme in different adipose depots (Tomlinson 2005). Previous evidence shows that glycyrrhetinic acid can inhibit both 11β-HDS1 and 2 whereas other agents such as BVT.3498 can selectively inhibit 11β-HSD1. This has prompted interest in the

therapeutic potential of selective inhibition of 11β-HSD1 to reduce tissue accumulation of active cortisol and consequently reduce adipocyte differentiation, lipid accumulation and various associated features of metabolic syndrome (Biovitrum annual report 2004, Tomlinson 2005). Particular therapeutic interest is focused on reducing visceral adiposity because excess intra-adbdominal fat has been strongly associated with metabolic syndrome, particularly insulin resistance, T2DM and cardiovascular diseases (Matsuzawa 2005). While the present studies have provided support for the selective action of BVT1 and AZ121 to reduce formation of cortisol in adipose tissue, there was generally a greater suppression of 11β-HSD in subcutaneous than visceral fat. This is not the preferred balance for optimal therapeutic efficacy. Moreover cortisol normally promotes pre-adipocyte proliferation, and in the present studies it has been noted that cessation of therapeutic suppression of 11β-HSD1 could result an increased pool of pre-adipocytes which could predispose to diet-induced obesity. Also it is not clear whether suppression of 11β-HSD1 oxo-reductase activity would also suppress its dehydrogenase activity. The structural analysis of 11β-HSD's active sites has yet to be confirmed (Draper and Stewart 2005), and the balance of these enzyme activities, known to normally favour the oxo-reductase *in vivo,* may be detrimentally disrupted. Therefore, while use of a selective suppressant of 11β-HSD1 provides an interesting therapeutic opportunity to reduce production of local cortisol, and associated metabolic disease predisposition, these are reasons for caution described above which leave open the possibility of homeostatic readjustment of the balance between oxo-reductase and dehydrogenase activity and the opportunity for over-compensation of adipose mass if therapy is relaxed or withdrawn.

7.4 Conclusions

This thesis has provided evidence base to support a hypothesis for the appreciation of subcutaneous fat as the major adipokine signaling depot whilst retaining its role as a long term energy storage unit. Furthermore it has reinforced the role of the visceral depot as an energy control centre in the form of a fatty acid turnover 'machine'. This thesis has outlined potential molecular mechanisms by which each depot can perform its function and postulated how that role is interpreted and utilized by the body. The experimental programme herein has produced a novel reproducible culture technique for mature adipocytes. This technique has enabled investigation to support mature adipocyte cell division, and pointed to further experimental work to investigate the control of this cell division and its possible dependence on cell proximity. The conclusions of this thesis have been summarized within the foregoing discussion. Major findings indicate mature adipocyte cells can divide post 'terminal' differentiation, and there is an important role of the subcutaneous adipose tissue depot in the metabolic control of adipose tissue sensing and signalling function to control overall adipose mass. Potential inhibitors of 11β-HSD1 offer a possible approach to the containment of visceral adiposity. Further research and targets for therapeutic intervention are re-emphasized as opportunities to control the depot-specific effects of visceral and subcutaneous adipocytes in sensing, signalling and energy homeostasis.

8.0 Reference section

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9.0 Appendices

Appendix to chapter 3: Ethics; Patient information sheet and consent form

Study Title: Causes and Treatment of Abdominal Obesity

Information and Consent form

Before you decide whether you agree to assist us in this study, please take your time to read the following information. Please feel free to discuss this and raise any questions with the surgeons before the operation.

Background and Purpose
Sandwell and West Birmingham Hospitals, in collaboration with Aston University, is conducting a research programme into the causes and treatment of excess fat tissue in the abdomen (tummy), as this may be important for developing new treatments for obesity. As part of the research, we wish to determine how quickly the fat tissue will release and replace its fat content in response to different stimuli such as hormones and other chemicals. Over time we hope to build a database from patients of all weights to see if any changes in fat metabolism occur when people put on weight. We will also investigate new ways of increasing the release of fat from the fat tissue.

What will happen to you?
During your operation we would like to take a small amount of fat tissue which will be removed from the site of incision (cut) and from within your abdomen (tummy). In most cases this fat would normally be destroyed, but in a small number of cases it would be an extra amount removed, but this will do you no harm. The use of your fat tissue will not affect your operation in any way, and carries no risks.

What happens to the fat samples?
The fat samples that you donate will be used for the research of the Diabetes Research Group at Aston University. Some of the experiments may be undertaken in collaboration with sponsors such as the Biotechnology and Biological Science Research Council (BBSRC) and sponsors from pharmaceutical organisations.

Confidentiality
The samples will not be labelled with your name. Each sample will have a code that will link it to information from your clinical notes and this will be retained on file. Your identity will be accessible only to Dr Bailey and Mr Nicholl and their research colleagues.

No researcher will be able to identify you from the tissue samples provided.

Voluntary participation and right to withdraw

Participation in this study is voluntary and entirely up to you. If you decide that you do not want to provide tissue for the study, you don't have to give a reason for this. It won't be held against you in any way.

You can withdraw your consent for tissue collection, without giving any reasons, up to the time of use of the sample by informing the surgical staff.
The information from the study may be shared with other scientists, and published in medical and scientific journals. No personal details that could identify you will be used in this.

Research outcomes
Sandwell and West Birmingham Hospitals, Aston University and sponsors of the research may use some of this information, with the test results from other donors, to help research and development for new treatments or diagnostic tests for obesity.
By donating your tissue for this research you do not have any claim on any new treatments and patents that may be directly or indirectly linked to this research.

If you have any further questions about this study please do discuss them with:

Aston University

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Patient Consent Form

Causes and treatment of abdominal obesity
Sandwell and West Birmingham Hospitals NHS Trust, in collaboration with Aston University, is conducting a research programme into the causes and treatment of excess fat in the abdomen.

Details of the research programme are given on the accompanying Patient Information Sheet.

You are invited to participate in this study. Fat tissue which would normally be removed and discarded during your operation will be used in the research programme to determine how quickly it will release its fat content.

If you agree to participate in this study please could you sign and date this consent form.

I have read and understood the Patient Information Sheet entitled " Causes and treatment of abdominal obesity". I have had an opportunity to ask questions about the study. I agree to participate in the study.

Name of Patient   Signature of Patient   Date
....
Name of Doctor   Signature of Doctor   Date
....
Patient Copy
(duplicate copy for patient file)

Appendix to chapter 4: Graticule photograph and generated computer grid
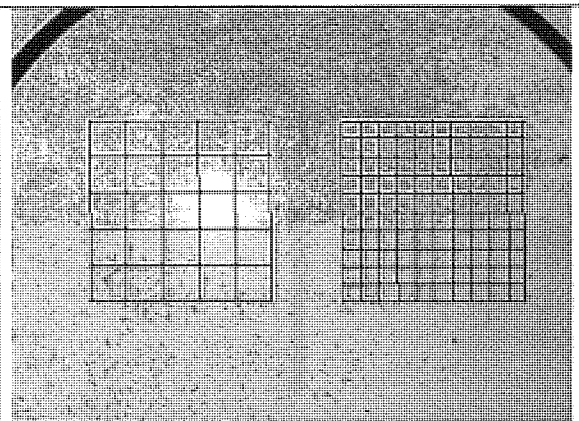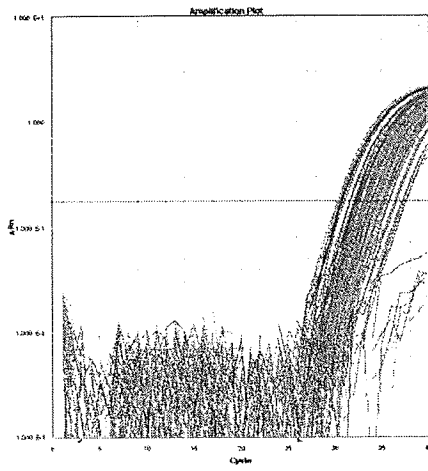
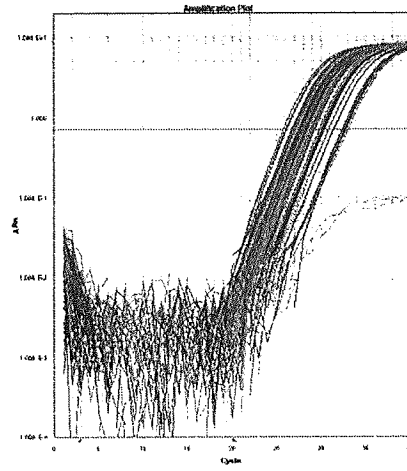Graticule
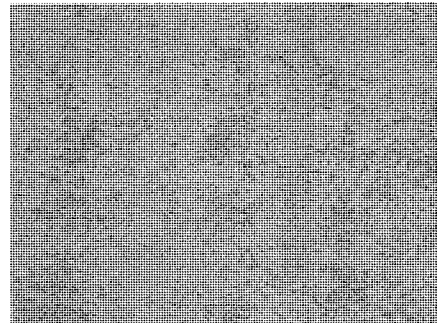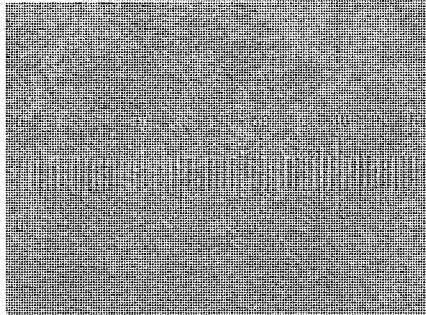
Grid
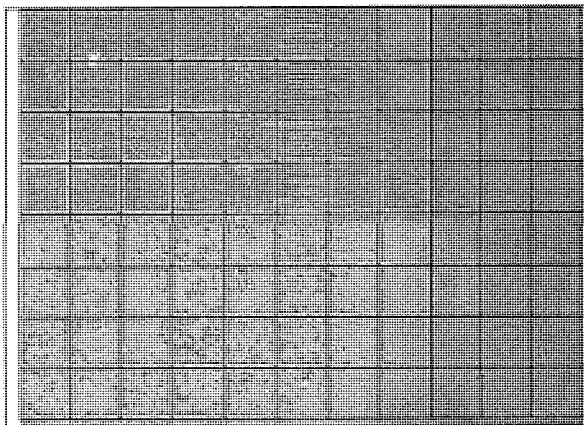
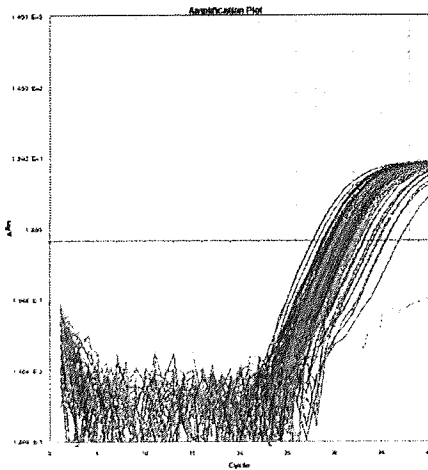Grid (x10) with graticule photograph | Grid (x8) with graticle photograph

Appendix to chapter 5: Amplification Plots

SCD1

GPAT

DGAT1

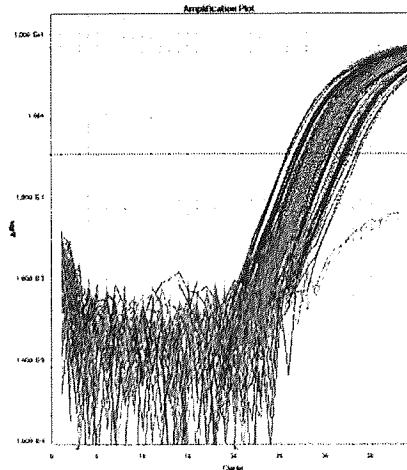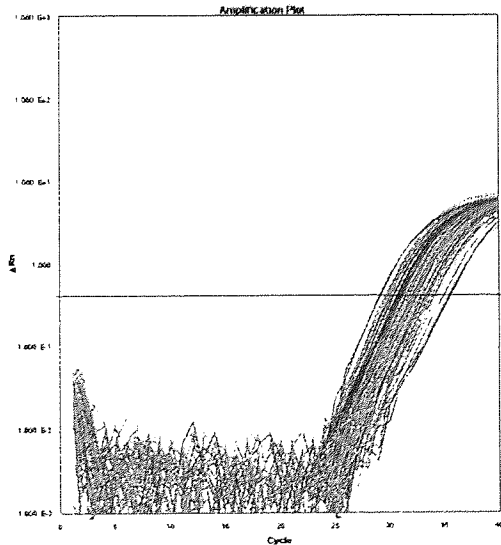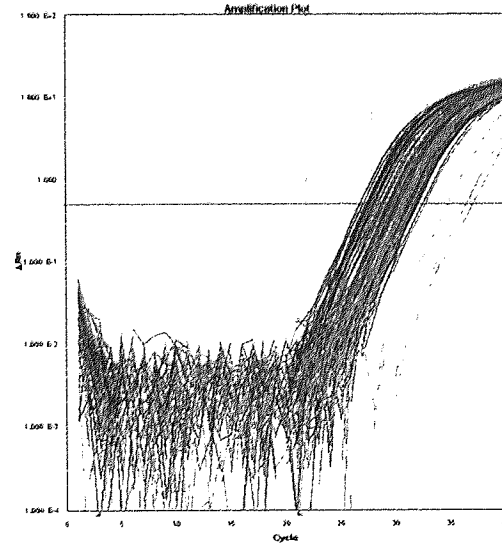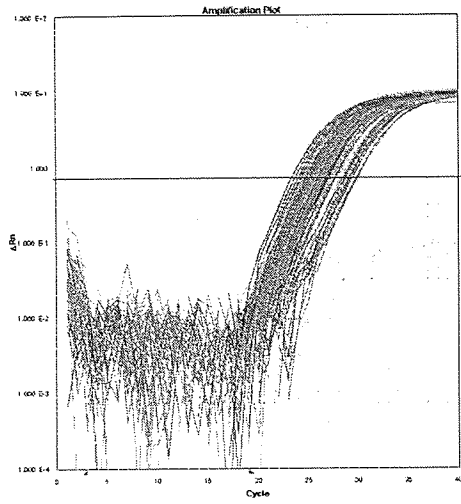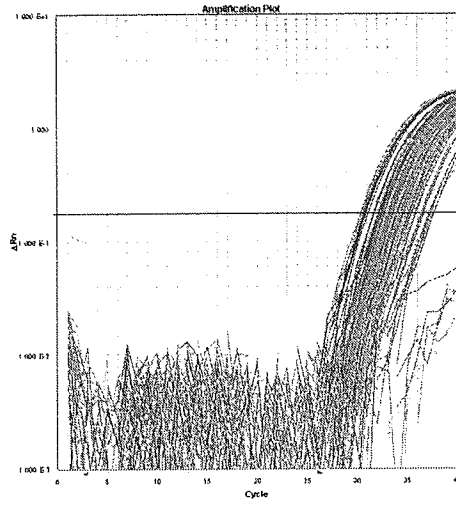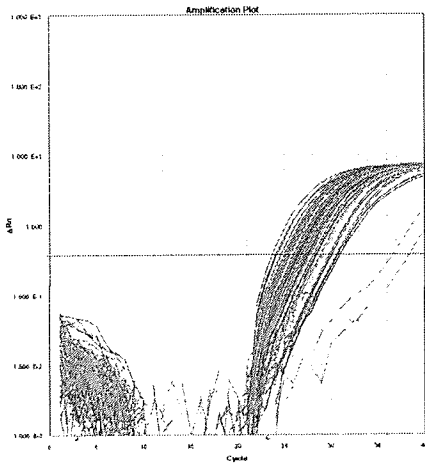DGAT2

11β-HSD1

eptin

Adiponectin

GPR41

Ribosomal Protein
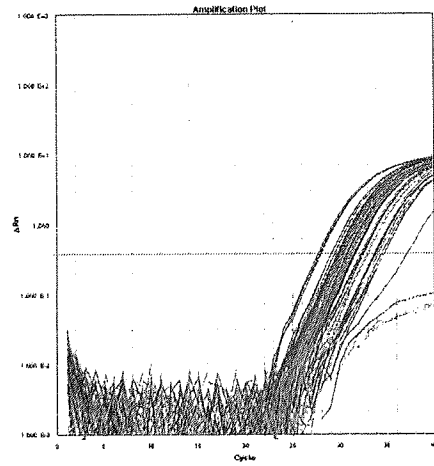
B-Glucosidase

Appendix to chapter 6: Raw Data and Statistics

Table 6.1 Data corresponding to figures 6.1, 6.2 and 6.3

| | Concentration of Glycyrrhetinic acid (M) | | | | | |
	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
visceral Tissue	14524	14913	15175	5960	3972	3864
$\delta/\sqrt{n}$	1929	875	937	1657	603	613
Subcutaneous Tissue	14524	15538	15263	7163	1015	1414
$\delta/\sqrt{n}$	1929	186	504	1930	304	461

Table 6.2 Data corresponding to figure 6.4

| | Concentration of Biovitrum compound acid (M) | | | |
	0	10^{-6}	10^{-5}	10^{-4}
visceral Tissue	12897	7018	5362	8289
$\delta/\sqrt{n}$	584	3487	1275	3327
Subcutaneous Tissue	12897	1605	2898	2468
$\delta/\sqrt{n}$	584	670	478	326

Table 6.3 Data corresponding to figure 6.5

| | Concentration of Biovitrum compound acid (M) | | | |
	0	10^{-6}	10^{-5}	10^{-4}
visceral Tissue	14375	15800	14363	13938
$\delta/\sqrt{n}$	124	1556	5524	433
Subcutaneous Tissue	14375	15350	11488	14288
$\delta/\sqrt{n}$	124	0	3	11

Table 6.4 Data corresponding to figure 6.6

| | Concentration of Glycyrrhetinic acid (M) | | | |
	0	10^{-8}	10^{-7}	10^{-6}
Subcutaneous Tissue	14524	1974	1728	1712
$\delta/\sqrt{n}$	5489.502	805.9298	705.4939	699.064

Table 6.5 Data corresponding to figure 6.7

| | Concentration of Biovitrum compound (M) | | | |
	0	10^{-6}	10^{-5}	10^{-4}
Subcutaneous Tissue	14524	540	481	2947
$\delta/\sqrt{n}$	1929	25	109	1625

Table 6.6 Data corresponding to figure 6.8

Concentration of Astrazeneca compound 121

	0	10^{-6}	10^{-5}	10^{-4}
Subcutaneous Tissue	14524	2694	3028	3256
$\delta/\sqrt{n}$	1929	659	489	322

Table 6.7 Data corresponding to 6.9

Concentration of Glycyrrhetinic acid (M)

	0	10^{-6}	10^{-5}	10^{-4}
visceral Tissue	14523.86	9890.9	13518.17	4493.9
$\delta/\sqrt{n}$	743.6363	1847.072	1812.766	1848.797
Subcutaneous Tissue	14523.86	13312.03	5954.033	3854.133
$\delta/\sqrt{n}$	743.6363	57.41922	67.12716	38.70357

Table 6.8 Data corresponding to figure 6.10

Concentration of Biovitrum compound acid (M)

	0	10^{-6}	10^{-5}	10^{-4}
visceral Tissue	14524	11106	9885	12673
$\delta/\sqrt{n}$	744	2641	1010	2619
Subcutaneous Tissue	14524	2857	7381	4683
$\delta/\sqrt{n}$	744	57	67	39

Statistical tests

Table 6.9 Annova : Statistical test results shown in order of graphs

Section Number	Visceral			Subcutaneous		
	F value	d.f	p value	F value	d.f	p value
5.3.1a	15.4	30	6×10^{-7}	20.5	30	4×10^{-8}
5.3.1b	0.17	8	0.84	0.96	8	0.43
5.3.1b	41.4	5	0.0065	0.18	5	0.84
5.3.2a				96.3	24	1.95×10^{-12}
5.3.2b				54.8	15	2.8×10^{-7}
5.3.2c				61.5	15	1.5×10^{-7}
5.3.3a	7.8	15	0.0037	9.05	18	0.0012
5.3.3b	1.5	15	0.25	29.7	18	1.49×10^{-6}

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Table 6.10: Student's t test visceral data

Section Number	Visceral														
	10^{-8}			10^{-7}			10^{-6}			10^{-5}			10^{-4}		
	t	d.f	p	t	d.f	p	t	d.f	p	t	d.f	p	t	d.f	p
5.3.1a	-0.23	7	0.41	-0.39	7	0.35	4.2	13	5×10^{-4}	9.9	11	4×10^{-7}	10.0	11	3.8×10^{-7}
5.3.1b							6.8	3	0.003	4.4	4	0.005	1.11	4	0.164
5.3.1b							-8.14	2	0.007	0.07	2	0.47	1.4	2	0.15
5.3.2a															
5.3.2b															
5.3.2c															
5.3.3a	2.4	8	0.01	0.5	8	0.3	5.4	8	0.0003						
5.3.3b	1.5	8	0.08	3.16	8	0.007	0.9	8	0.2						

Table 6.11: Students T test subcutaneous data

Section Number	Subcutaneous														
	10^{-8}			10^{-7}			10^{-6}			10^{-5}			10^{-4}		
	t	d.f	p	t	d.f	p	t	d.f	p	t	d.f	p	t	d.f	p
5.3.1a	-0.64	7	0.27	-0.5	7	0.33	3.6	13	0.0017	4.4	11	6.4×10^{-9}	1.1	11	1.6×10^{-8}
5.3.1b							10.5	3	0.0009	10.3	4	0.002	12.7	4	0.0001
5.3.1b							-0.44	2	0.35	0.37	2	0.37	0.14	2	0.45
5.3.2a	11.1	11	1.3×10^{-7}	12.9	11	2.6×10^{-8}	13.2	11	2.2×10^{-8}						
5.3.2b							11	8	2×10^{-6}	11	8	2×10^{-6}	6.7	8	8×10^{-5}
5.3.2c							8.7	8	1.2×10^{-5}	8.7	8	1.2×10^{-5}	8.7	8	1.2×10^{-5}
5.3.3a							0.44	8	034	3.37	8	0.004	6.8	8	6.8×10^{-5}
5.3.3b							7.14	8	4.9×10^{-5}	4.2	8	0.0014	6.7	8	7.5×10^{-5}