Role of adipose tissue as an endocrine organ in systemic inflammation

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AUTHOR'S DECLARATION

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

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Abstract

Introduction: The escalating public health problem represented by obesity has spurred multidisciplinary research into adipose tissue and importantly, the molecular biology of the adipocyte. The concept of adipose tissue as an endocrine organ, in addition to an energy storage compartment, is now pivotal in linking excess adiposity to disease states. Recent studies suggest that obesity related metabolic disorders are characterised by mild chronic inflammation as a result of adipocytokine production from fat tissue leading to dysregulation in the pro/anti-inflammatory systemic balance. Adipokines and pro-inflammatory markers are implicated in insulin insensitivity, blood glucose dysregulation, inflammation and atherosclerosis. There is a considerable amount of research into the characterization of adipokines and pro-inflammatory cytokines, the anti-inflammatory adipocytokines warrant further exploration.

Research studies and design: This PhD research was set out to investigate potential anti-inflammatory molecules that could be used as markers of, and therapeutics for, metabolic syndrome associated maladies. This PhD consists of three studies including **Study 1:** a characterisation study of pro/anti-inflammatory mediators carried out on 116 men of various BMI and body composition; **Study 2:** an in vitro study design carried out in human Simpson Golabi Behmel Syndrome (SGBS) adipocyte cell line investigating a glucocorticoid regulated anti-inflammatory protein, annexin A1 (AnxA1) and its role in fat tissue function; and **Study 3**: a double-blind cross-over randomised trial in 15 borderline metabolic syndrome males investigating the effect of a supplemental anti-inflammatory agent, resveratrol (250 mg/day for two weeks, from 500 mg of *Polygonum cuspidatum* (from root)), on metabolic parameters.

Key findings: Study 1: We demonstrated for the first time that AnxA1 is significantly inversely correlated with increasing BMI (R = -0.424**, P < 0.001), increasing body fat % (R = -0.192, P = 0.037) and a larger waist size (R = -0.390**, P < 0.001) in 118 men aged 19 to 61 years, with BMI between 16.8 – 56.4 kg/m^2, BF % between 4.3 to 51.8 %. The negative correlation of decreasing plasma AnxA1 was strongest statistically when compared with WHR, rather than total body fat, suggesting that centrally located fat may be more influential at reducing plasma AnxA1 concentrations. **Study 2:** We have shown that ANXA1 gene is expressed in human SGBS adipocytes and hypoxia reduces the expression of ANXA1 gene showing that AnxA1 may act as a counter regulator of adipose tissue inflammation. We found that CRP expression was significantly down-regulated following 4 (P=0.015), 8 (P=0.035) and 24 (P=0.037) of hypoxia treatment in the cells also treated with Ac2-26 peptide compared to vehicle alone. IL-6 was also found to be significantly down-regulated after 24 hour hypoxia treatment in the Ac2-26 treated cells compared to vehicle (P=0.022).

Study 3: The effect of resveratrol on metabolic function had no significant effect on the metabolic markers measured including blood pressure, blood glucose, blood cholesterol and glycated LDL.

Conclusion: These data demonstrate that AnxA1 could potentially represent a (fat) depot specific biomarker whose decline with increasing central adiposity may relate to the phenomena of increasing systemic inflammation and associated disease risk. We also demonstrate for the first time that an AnxA1 is expressed in human SGBS preadipocytes and mature adipocytes and AnxA1 mimetic, Ac2-26 peptide, regulates pro-inflammatory markers in human SGBS adipocytes. We showed that it may be difficult to improve the metabolic profile of individuals through supplementation of exogenous anti-inflammatory agent, resveratrol. Whilst anti-inflammatory agents such as AnxA1 may propose novel therapeutics for metabolic syndrome associated diseases, to date regular exercise and weight loss remain the main interventions that significantly cut the risk of developing chronic long-term conditions and obesity-associated maladies.

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Publications and presentations

A Kosicka, D Renshaw, M G Zariwala, (2013) **DPPIV and macronutrients regulate the expression of PYY3–36 in human gut epithelial cells,** Endocrine Abstracts, 31, P197 **(Abstract).**

A Kosicka, A Cunliffe, R Mackenzie, M G, Zariwala, M Perretti, R Flower, D Renshaw, (2013) **Attenuation of plasma annexin A1 in human obesity.** FASEB J, 27, 368-78.

A Kosicka-Knox, A Cunliffe, R Mackenzie, M G Zariwala, D Renshaw, (2012) **Ac 2-26, an annexin A1-derived peptide, reduces inflammation in human SGBS adipocytes after hypoxia treatment.** Endocrine Abstracts, 29, P1172 **(Abstract).**

A Kosicka-Knox, A Cunliffe, R Mackenzie, M G Zariwala, D Renshaw, (2012) **Annexin A1, an anti-inflammatory regulator, is expressed in human adipocytes.** Endocrine Abstracts (2012) 28, P193 **(Abstract).**

A Kosicka-Knox, A Cunliffe, R Mackenzie, R Flower & D Renshaw, (2011) **Plasma Annexin A1 (AnxA1): A possible biomarker of central fat mass in humans.** The Endocrine Society 93rd Annual Meeting**,** Boston**,** USA June 2011 **(Abstract).**

A Kosicka-Knox, A Cunliffe, R Mackenzie, R Flower & D Renshaw, (2011) **Plasma Annexin A1 (AnxA1) is inversely correlated with waist to hip ratio in healthy males** Endocrine Abstracts 25, P133 **(Abstract).**

Abbreviations

AcCoA	Acetyl coenzyme A
AcRP30	Adipocyte complement-related protein of 30 kDa
AC	Adenylate cyclase
ACTH	Adrenocorticotropic hormone
AdipoQ	Adiponectin
ADSF	Adipocyte secreted factor
AgRP	Agouti-related protein
AnxA1	Annexin A1
ANXA1	Annexin A1 gene
AP	Area postrema
ApM1	Adipose most abundant gene transcript 1
AR	Adrenergic receptors
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
A^y	Yellow agouti
BAT	Brown adipose tissue
BCDF	B-cell differentiation factor
B-SF-2	B-cell stimulatory factor - 2
B-LPH	B-lipotropin
BMI	Body mass index
BMR	Basal metabolic rate
BST	Bed nucleus of the hypothalamus
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CEA	Central nucleus of the amygdala
CCK	Cholecystokinin
cDNA	Complimentary deoxyribonucleic acid
CLIP	Corticotropin-like intermediate lobe peptide
CNTF	Ciliary neurotropic factor
CRP	C-reactive protein
CVD	Cardiovascular disease
D2	2,5’ monodeiodinase
DCN	Cleavage product of decorin
DMV	Dorsal motor nucleus of the vagus
18F-FDG	18F-fluorodeoxyglucose
ER	Endoplasmic reticulum
Fen/phen	Phenteramine
FFA	Free fatty acids
GABA	Gamma-aminobutyric acid
GHS-R	Growth hormone secretagouge receptor
G-6-P	Glucose-6-phosphate
GLP-1	Glucagon-like peptide
Gs	G-proteins
HIF-1α	Hypoxia inducible factor-1 α
HPA	Hypothalamic-pituitary-adrenal
HSL	Hormone sensitive lipase
IL	Interleukin
JAK	Janus kinase
Lep-R	Leptin receptor

LIF	Leukaemia inhibitory factor
LH	Lateral hypothalamic area
LPB	Lateral parabrachial nucleus
gp	Glycoprotein
MC_4	Melanocortin receptor 4
MSC	Mesenchymal stem cell
MSHα	Melanocortin stimulating hormone α
NE	Noradrenaline
NEFA	Non-esterified fatty acid
NPY	Neuropeptide Y
NSAID	Non-steroidal anti-inflammatory drugs
NST	Non-shivering thermogenesis
OTC	Over the counter
P	Phosphate
PBEF	Pre-B cell enhancing factor
PET-CT	Positron emission tomography (PET) and computerised tomography (CT)
PKA	Protein kinase A
PGC-1	PPAR γ co-activator-1
POMC	Proopiomelanocortin
PPAR	Peroxisome proliferator-activated receptors
PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide YY
R	Receptor
RELMs	Resistin-like molecules
ROR	Reactive oxygen species
SCF	Sertoli cell factor
SGBS	Simpson Golabi Behmel Syndrome
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STAT	Signal transduction and transcription
TAGs	Triglycerides
T_3	Triiodothyronine
TNFα	Tumour necrosis factor α
UCP-1	Uncoupling protein-1
VLDL	Very low density lipoprotein
VMH	Ventromedial hypothalamus
WAT	White adipose tissue
WHO	World Health Organisation
WHR	Waist to hip ratio

Chapter 1. The role of adipose tissue as an endocrine organ in systemic inflammation

1.1. Introduction

1.1.1 Obesity and energy expenditure regulation

The prevalence of obesity has reached an alarming number over the past three decades in the United States and industrialized countries. Obesity, however, is not a recent phenomenon and can be traced as far back as ancient civilizations (Boriani et al., 2005). Obesity in humans was portrayed in Stone Age drawings (Bray, 1990) and illustrations of obese females date back thousands of years e.g. Venus of Willendorf circa 23000 B.C. (Haslam, 2007). The health complications associated with over fatness were recognized as early as 4th century B.C. by Hippocrates who noticed that '' sudden death is more common in those who are naturally fat than in the lean'' (Bray, 2003) and referred to obesity as ''not only a disease itself but the harbinger of others''(Bain, 2006). Whilst excess body weight was documented since the rise of ancient civilizations, only in the last three decades, it reached pandemic proportions. According to the World Health Organization (WHO) 2012 report obesity rates have more than doubled since 1980 across developed and developing countries. Twenty one per cent of world 20 and older population were overweight in 2008. A large proportion of these (14 % men and 21 % women) were classed as obese. Overweight and obesity has reached pandemic proportions and a majority of (65 %) the world's population reside in countries where metabolic syndrome associated diseases kill more people than undernutrition and underweight. What is more, excess body weight is now more frequently seen in children with the prevalence of overweight children under the age of five reaching more than 40 million in 2010. Overweight and obesity are described as abnormal or excessive body fat accumulation that may have debilitating effects on health. Body mass index (BMI) is a straightforward proxy of weight-for-height that is widely used by researchers and health professionals to classify overweight and obesity in adults (Table 1). BMI can be calculated by dividing a person's weight in kilograms by the square of his/her height in meters (kg/m^2). The WHO describes the range of BMI as follows:

- A BMI between 18.5 and 25 is classed as normal

- a BMI above or equal to 25 is classed as overweight
- a BMI above or equal to 30 refers to obesity class I
- A BMI above or equal to 35 refers to obesity class II, (WHO, 1995, WHO, 2000, Gostynski et al., 2004).

Table 1: The international classification of adult underweight, overweight and obesity according to BMI.

Classification	BMI (kg/m^2)	
	Principal cut-off points	**Additional cut-off points**
Underweight	**< 18.5**	**< 18.5**
Severe thinness	< 16.00	< 16.00
Moderate thinness	16.00 - 16.99	16.00 - 16.99
Mild thinness	17.00 - 18.49	17.00 - 18.49
Normal range	**18.50 - 24.99**	**18.50 - 22.99**
		23.00 - 24.99
Overweight	**≥25.00**	**≥25.00**
Pre-obese	25.00 - 29.99	25.00 - 27.49
		27.50 - 29.99
Obese	**≥ 30.00**	**≥ 30.00**
Obese class I	30.00 - 34.99	30.00 - 32.49
		32.50 - 34.99
Obese class II	35.00 - 39.99	35.00 - 37.49
		37.50 - 39.99
Obese class III	≥ 40.00	≥ 40.00

Source: (Adapted from WHO, 1995, 2000 and WHO, 2004). The table shows BMI classification for the underweight, normal, overweight and obese. The principal cut-off points are generally used to identify a normal weight for height in Caucasians. The additional cut-off points are provided for individuals of Asian ethnicity as research shows the risks associated with excess body weight vary between different ethnic groups.

Overweight and obesity have been associated with many diseases, including some forms of cancer (Michaud et al., 2001, Ryan et al., 2011, Harvey et al., 2011), type 2 diabetes (Maegawa and Kashiwagi, 2009, Eid, 2011, Sobti et al., 2012, Lukacs et al., 2012), stroke (Rexrode et al., 1997), coronary heart disease, hypertension, dyslipidemia, gallbladder disease, sleep apnoea (Zalesin et al., 2011, Gabbay et al., 2012) and osteoarthritis (March and Bagga, 2004). The most common types of cancers associated with obesity are endometrial, esophageal adenocarcinoma, colorectal, postmenopausal breast, prostate, and renal (De Pergola and Silvestris, 2013). A number of impoverished and developing countries are now presented with

a "double burden" of disease (Dhillon et al., 2012, Usfar et al., 2010, van der Sande et al., 2001). While they still try to tackle the difficulties of infectious disease and under-nutrition, at the same time they are facing a rapid increase in non-communicable disease risk factors such as obesity and people who are overweight, especially within the rural environment. It is now a commonplace to encounter under-nutrition and obesity prevailing simultaneously within the same nation, the same social group and even the same household (Khan and Khoi, 2008).

The underlying determinants of this overweight and obesity pandemic are multifactorial and profound. Genetic predisposition and certain gene mutations e.g. to the melanocortin-4 receptor (MC_4) both have a role in the development of excessive adiposity (Bouchard and Tremblay, 1997, Nieters et al., 2002). Dietary factors, such as the accessibility of energy-rich food, and the marked reduction in physical activity evidently also provide important contributions (Wadden et al., 2012, Unick et al., 2011). Most recent studies point to the evidence that consumption of diets rich in added sugar such as table sugar (sucrose) and high fructose corn syrup are closely linked with the rising obesity, cardiovascular disease (Tran et al., 2009, Sievenpiper et al., 2014) and type 2 diabetes pandemic (Johnson et al., 2013). Fructose is a major component of widely available food products and it has been linked with uric acid generation providing a molecular link for the pathogenesis of obesity, cardiovascular disease and type 2 diabetes (Johnson et al., 2009). Moreover, fructose-stimulated uric acid production was shown to induce mitochondrial oxidative stress that in turn increases fat deposition irrespective of excessive caloric intake. These novel and important studies indicate that perhaps "a calorie is not just a calorie" and show that the qualitative properties of food are as important as their energy content. Whilst the obesity pandemic is on the rise, research scientists investigate ways of regulating energy expenditure to curb excess adiposity and alleviate the panoply of diseases associated with excess body fat.

1.1.1.1 Energy homeostasis

To date scientists have not found a system where energy can be created or destroyed. Therefore, scientists have been on quest to explore possibilities which

could modulate energy intake (food) and consequently not lead to commensurate increases in body weight (Jequier and Tappy, 1999). The apparent simplicity of the energy balance equation e.g. $Balance = Intake - Expenditure$, belies the complexity of the expenditure component (McMinn, 1984). Intake being easily measured as calories in food and drinks consumed, whereas the calorie cost of life is portioned between basal requirements, physical activity, post-prandial thermogenesis and thermoregulation (Joosen and Westerterp, 2006). In addition it is apparent that the engine of cellular respiration while efficient does not convert 100 % of dietary energy to either adenosine triphosphate (ATP) or body tissue (Heymsfield et al., 1995). Observations of energy balance in man therefore, require an understanding of these components and an appreciation of the metabolic fate of dietary macronutrients. Observations of (relative) long-term weight stability in free living humans suggest strongly a set point of some sort, albeit one which frequently appears to be overridden (Levine, 2007). This defence of body weight can largely be explained through systems regulating the hunger-satiety cycle and feedback from energy (adipose) stores through circulating products of fat cells (e.g. leptin) providing proportional feedback to central centres involved in modulating feeding behaviour and energy expenditure (Flier and Maratos-Flier, 1998, El-Haschimi and Lehnert, 2003). It is clear, however, that inter-individual differences also occur and that qualitative aspects of dietary choice, variations in metabolic response to food ingestion and different inherited tendencies towards body composition can all influence energy balance in the whole person (Zheng et al., 2009). Dietary protein for example stimulates greater post prandial thermogenesis than fat or carbohydrate (Johnston et al., 2002), and metabolic rates vary between individuals and also within individuals according to body mass and composition.

One of the first theories of a mechanism to defend a set body weight concerned the phenomenon of post-prandial thermogenesis. Scientists thought that excess calories would be ‘given off’ as heat in a process then termed Luxuskonsumption (Ravussin et al., 1985, Garrow, 1983). While it is true that larger meals produce larger thermogenic responses (Tai et al., 1991), it is evident upon measurement, that this does not equal the excess of energy in an habitually hypercaloric diet. In addition,

while basal metabolic rate (BMR) does increase with increased body mass, this in itself is a reflection of the increase in body mass and not a mechanism to regulate it. It is of significance though that newly acquired tissue has a differential influence on BMR, with muscle tissue creating a larger increase gram for gram than adipose tissue (Ravussin and Bogardus, 1992). Energy as heat is also produced via adipose tissue itself when uncoupling occurs in the final stages of energy metabolism (oxidative phosphorylation) at the level of the mitochondria (Kozak and Harper, 2000). It is thought that this effect is mostly confined to brown adipose tissue (BAT) and positron emission tomography (PET) studies suggest that BAT is more extensively represented in leaner individuals (van Marken Lichtenbelt et al., 2009) suggesting a possible component of their leanness in what is increasingly seen as an obesogenic environment.

There are then clearly a number of potential fates for excess energy beyond that of being stored as fat. Manipulation of macronutrients to exaggerate the protein component (e.g. Atkins diet) has largely proved unsuccessful in the long-term quest for weight reduction and control; as such diets are difficult to comply with (Gardner et al., 2007). In addition many studies have suggested that a decrease in total calories eaten is responsible for weight loss when it is observed, rather than the relatively higher energy cost of metabolizing protein compared to fat or carbohydrate (Poehlman et al., 1991a, Stiegler et al., 2008, Redman et al., 2009).
In a population where a rising number of individuals are becoming overweight and obese, it is of considerable interest to better understand the differences observed in responses to exposure in challenges to defence of a stable and healthy body weight. Anecdotal reports of ‘pair feeding’ between individuals frequently suggest very different responses in terms of weight gain/loss. This, however, when examined in controlled studies, is usually resultant from poor reporting of actual food intake and misconceptions or self-deceptions regarding portion size and meal and snack frequency (Sims et al., 1968, Ward et al., 2007, Scagliusi et al., 2009).
Notwithstanding this, there are clearly a significant number of individuals including those with sedentary lifestyles who do not become overweight in the face of the ‘obesity epidemic’ (Leibel, 1995).

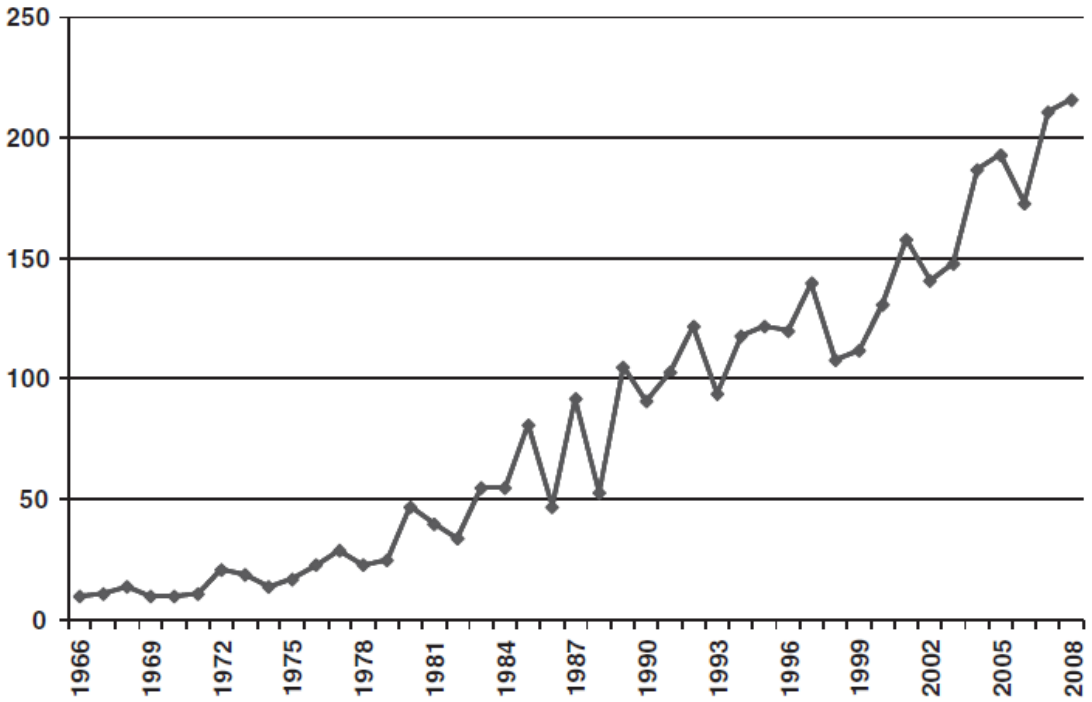
What is unclear is which mechanisms underpin successful defence of a 'healthy' set point for body fat accumulation. In addition, relatively little is known about the interplay between environmental challenges (sedentary lifestyle and affordable energy dense food for much of the population) and activation of genes, which may inhibit ingestive behaviour or increase energy expenditure. Given that most of the chronic health conditions associated with excess adiposity tend to be found beyond average reproductive age, there is clearly little impact of metabolic evolution to select for a lean phenotype. This contrasts the situation in which calorie retention would have been highly favoured in evolving man. Therefore, while the search for 'obesity genes' continues, it appears unlikely that finding the genes that probably predisposed our ancestors to survive will be particularly helpful in curbing a trend for rising levels of obesity. Additionally, this is likely to be a complex polygenic configuration involving many hundreds of genes rather than one or two. The niche, in which many people now find themselves, being that of relative calorie surfeit, might reasonably be expected to produce an increase in the number of obese individuals as the energy balance variable shifts inexorably towards the positive. As the obesity pandemic is on the rise, in light of the failure of current available treatments of excess adiposity, understanding better the biologic components of fatness may lead to alternative therapeutics to reduce or delay the signs and symptoms of chronic metabolic syndrome diseases.

Morbidity and mortality associated with obesity are substantial, but can be effectively reduced following weight loss (Williamson, 1997). As early as 1975, a multi-factorial approach was used for the treatment of obesity, including dietary modification, exercise, psychotherapy and medication (Mazansky, 1975). Over the past two decades, numerous intervention studies have examined strategies to ameliorate the obesity epidemic. Dietary modifications and exercise are still the only non-invasive interventions for both men and women that cause changes in the energy balance both in the short and long-term. An abundance of studies provide evidence of successful body weight reductions following dietary restriction and physical activity (Saris et al., 2000, Glass et al., 2002, Meckling et al., 2002, Poppitt et al., 2002). However, the majority of people are not able to maintain the achieved

weight losses, and over the long-term, weight regain is usually the case (Borg et al., 2002, Leslie et al., 2002, Byrne et al., 2003). In the recent years, bariatric surgery has been used to help the morbidly obese to reduce their body weight. Studies show that bariatric surgery is successful in inducing short-term body weight loss and health improvements (Courcoulas et al., 2013). Little is still known, however about long-term effects of bariatric surgery on weight loss and health. Bariatric surgery is also only offered to the morbidly obese, is expensive and may result in post-operative health complications including nausea and vomiting, leaks from staple lines, abdominal hernia, chest pain, collapsed lung, injury to stomach and surrounding areas and many more (Kriwanek et al., 1995).

At the same time, extensive research into calorie restriction diets and its effects on health and longevity has advanced in the last couple of decades. Anti-aging inquiries by modern investigators continue to unravel the quest of the Spanish explorer Ponce de Leon, who was in a pursuit for the ‘‘Fountain of Youth’’ on the coast of Florida in the early 1500s. Basic and clinical research is carried out with the purpose to delay and possibly alleviate the constellation of disorders associated with the aging process. Both calorie restriction and alternate day fasting have a robust and well documented multiple benefits across a broad range of species, including prolonging lifespan (Pugh et al., 1999, Johnson et al., 2006, Antosh et al., 2011, Rizza et al., 2013), improvement in insulin sensitivity (Huffman et al., 2012, Sequea et al., 2012), lowering blood lipids (Malandrucco et al., 2012), and beneficial effects on low grade systemic inflammation in obese humans (Schulte et al., 2012). The calorie restriction lifespan connection was first identified in a multicellular organism i.e. a rat (McCay et al., 1989). Simpler systems including eukaryotic (Roux et al., 2010) and prokaryotic (Houthoofd and Vanfleteren, 2006) organisms were later adapted to study the effects of calorie restriction due to short life span of these organisms, as well as considerable genetic plasticity and the ease of diet manipulation. Ironically, whilst research scientists continue to unfold the mysteries of prolonging lifespan and research into anti-aging continues to grow (see Figure 1), the current economic and societal environment is saturated with conveniently available calorie dense and high sugar food coupled with a strong tendency towards a more sedentary lifestyle.

Figure 1: Calorie restriction citations per year.



Source: (Redman and Ravussin, 2011) The chart shows the number of studies as found in ISI Web of Knowledge-MedLine from 1966 until 2008. Interestingly, as obesity was rising at the same time the numbers of published studies on caloric restriction have increased almost exponentially topping more than 200 citations per year for the last 2 years between 2006-08.

1.1.2 The biology of adipose tissue

In evolutionary terms, the biology of fat tissue dates back to the time where organisms had to develop strategies to cope with changeable and sporadic supplies of nutrients. Whilst the need to store and utilize energy most efficiently was the key surviving factor in omnivorous mammals, *Homo sapiens's* feeding routines evolved to a more complex societal level and revolved around shared meals (Collins, 2005). Therefore, the social status was associated with the acquisition of food sources, and to be well-endowed with a layer of fat was a clear indication of fortune, wealth, and social eminence. However, the portrayal of a human body has changed and in times that are more recent it is desirable to possess the least amount of body fat. This contemporary idealism of leanness reflects the new prototype of the species, adorned by fashion, media, and art. At the same time, medical research also

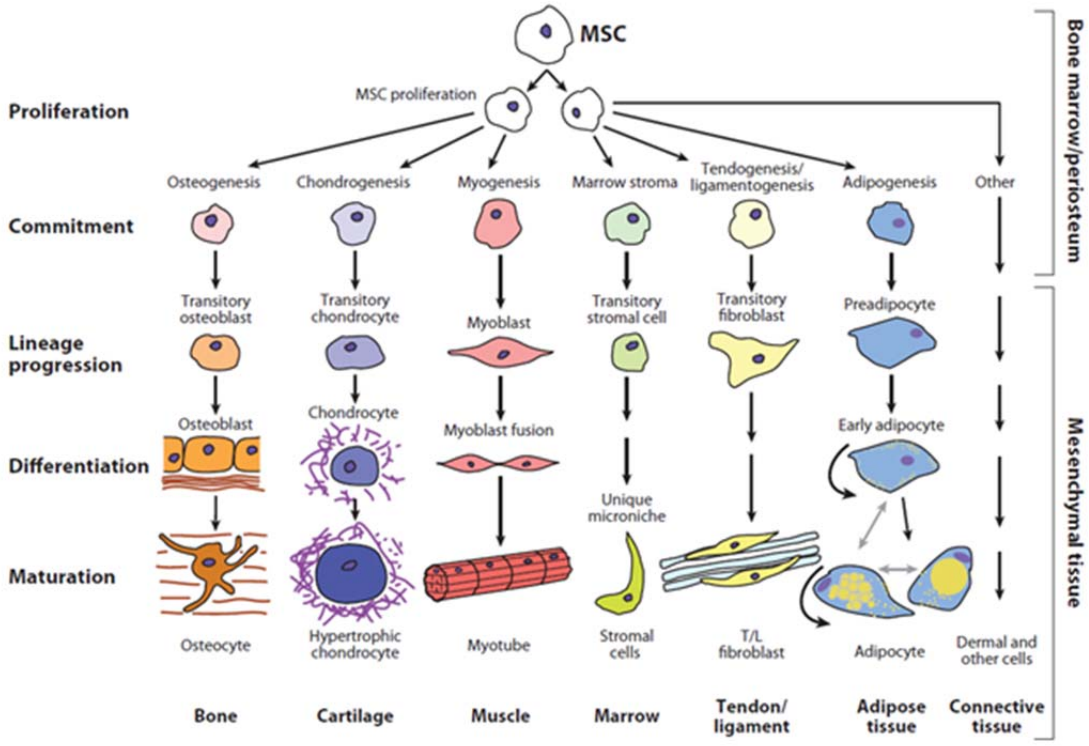
emphasizes the importance of a leaner physique in general well-being, vitality, and longevity.

Until the 1990s, adipose tissue initially was thought to only play a role in energy homeostasis, insulation and thermoregulation and it was not until a decade ago that the extraordinarily dynamic role of adipose tissue was revealed including reproduction (Bohler et al., 2010), apoptosis (Markelic et al., 2011, Kurokawa et al., 2011), inflammation (Shimizu et al., 2012), angiogenesis, blood pressure, atherosclerosis, coagulation, fibrinolysis (Juhan-Vague and Alessi, 1999), and immunity (Schaffler and Scholmerich, 2010). The pleiotropic role of adipose tissue depends on its ability to synthesize and secrete a variety of molecules collectively known as adipokines including adipose tissue specific hormones, a range of enzymes, pro- and anti-inflammatory mediators, growth factors, various cell membrane proteins, and complement factors. There are two types of adipose tissue, namely the brown adipose tissue (BAT) and white adipose tissue (WAT) (Garruti et al., 2008) and both originate from the mesenchymal stem cells (MSC) as shown in Figure 2.

1.1.2.1 Brown adipose tissue structure

Scientifically, BAT is a relatively new structure. First documents describing BAT date back to 1551 when the Swiss researcher Konrad Gessner found a tissue in the interscapular area of the Alpine marmot *(Marmota marmot)* referring to it being 'neither fat not flesh' (nec pinguitudo, nec caro)(Tews and Wabitsch, 2011). However, it was not until much later that its role in non-shivering thermogenesis (NST) was discovered (Dawkins and Hull, 1964a, Smith and Roberts, 1964). NST is an adaptive response to generate heat in a cold environment during a metabolic energy transformation process that does not recruit contraction of muscle fibres (Ouellet et al., 2012). BAT has a high metabolic rate, where just 50 grams of the fully activated tissue can account for 20 % of total energy expenditure in humans (Cypess et al., 2009). Consequently, modern scientists have been investigating possible mechanisms that can recruit and activate BAT differentiation as a potential treatment of obesity.

Figure 2: Adipocyte differentiation from the precursor cells of mesenchymal origin.



Source: (Singer and Caplan, 2011) MSC, mesenchymal cells. Embryonic stem cells are pluripotent and can give rise to cells of all three embryonic germ layers. Ectoderm, which differentiates into brain, spinal cord, nerve cells, skin and teeth; mesoderm, which differentiates into muscle, adipose tissue, blood, blood vessels, connective tissue and the heart; and endoderm, which differentiates into the gut, lungs, bladder and germ cells. Mature adipocytes are derived preadipocytes during the process of adipogenesis.

The role of adipose tissue in early life is distinguished greatly from that at any other subsequent stages of life when its function predominantly is of effective production of heat, rather than as merely storage organ for surplus energy intake (Symonds et al., 2003). NST is generally significantly up-regulated during parturition (child birth) via the action of uncoupling protein (UCP) 1 as a result of the subsequent rise in secretion of an array of endocrine stimulatory mediators (i.e. cortisol and prolactin) (Symonds et al., 1995). Cortisol and prolactin are partly responsible for the initial appearance of UCP-1 in the adipose tissue of a new born following through during gestation (Stephenson et al., 2001). However, a steady decline is observed in both the endocrine mediators and the abundance of UCP-1 after birth (Pearce et al., 2003).

a) Anatomical distribution

BAT is often difficult to distinguish from white adipose tissue by the naked eye. Brown adipocytes when filled with fat have a white appearance. Adipocytes that are partly depleted of fat change the colour to a characteristic yellow-brown. Brown adipose cell totally depleted of fat develops a red-brown colour and it resembles in appearance, the liver. The red-brown colour is due to the high content of mitochondrial cytochromes (Johansson, 1959). The tissue is characterized by a fine lobulated surface, and a very rich nerve and blood supply. It lies around and between the muscles of the neck and back, as well as in the axillae and groins, and in islands of tissue around the thoracic and abdominal viscera whilst the largest deposits are found around the kidney and adrenal gland (see Figure 3) (Aherne and Hull, 1964, Dawkins and Hull, 1964b).

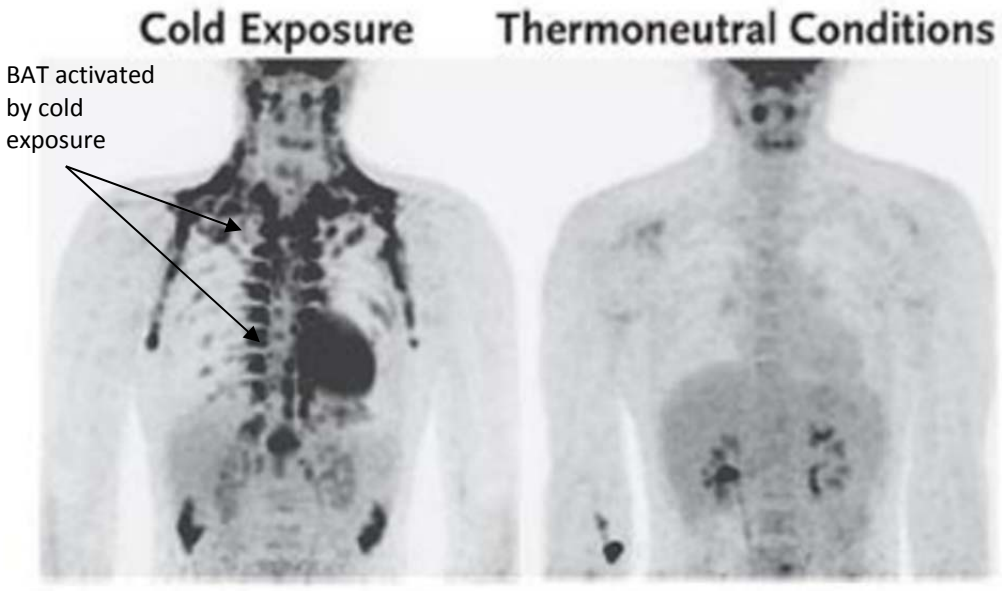
The level and distribution of BAT differs according to species. For example, in the newborn mouse (*mus*), rat (*rattus*) and rabbit (*lepus*) the main deposits are found around the neck and on the back between the scapulae. The newborn coypu (river rat) (*myocastor coypus*) has large interscapular pad and large amounts in the axillae. Whilst, in the lamb (*agnus*), kitten (*cattus*) and human (*homo sapiens*) new-born, BAT is widely distributed about the human body around the neck, on the back between the scapulae and in the axillae (Dawkins and Hull, 1964a). Until recently, the presence of BAT was thought to be relevant only in small mammals and infants, with negligible physiologic relevance in humans. In recent years, PET and computed tomography (CT) were used to examine the presence, distribution, and activity of BAT in lean and obese humans (van Marken Lichtenbelt et al., 2009). The activity of BAT is lower in the overweight or obese subjects than in the lean subjects (Vijgen et al., 2012).

b) Microscopic appearance

The brown adipose cell consists of a round nucleus, a granular cytoplasm and often contains many small vacuoles of fat as shown in Figure 4. The cytoplasm is granular in appearance and this is due to many mitochondria, which are large and complex and lie adjacent to the fat vacuoles (Napolitano and Fawcett, 1958). The multiple

mitochondria can clearly be seen in the example of electron micrograph of brown tissue Figure 5. Exposure to cool environment (30 °C) increases the number of mitochondria that fill the cytoplasm and decreases the vacuoles of fat (see Figure 6). BAT is important in the regulation of body weight as it is involved in adaptive thermogenesis, defined as the facultative heat produced in response to cold and diet (Lowell and Spiegelman, 2000). The many mitochondria found in BAT in a close proximity of fat vacuoles enable its function in oxidative metabolism and the rapid oxidation of fat.

Figure 3: Brown-adipose-tissue activity as assessed by PET–CT with 18F-fluorodeoxyglucose (18F-FDG).



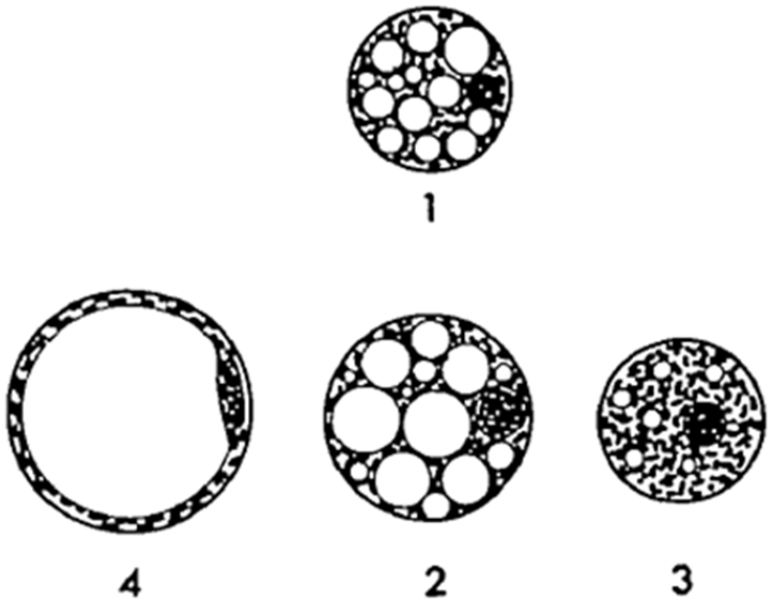
Source: Adapted from (van Marken Lichtenbelt et al., 2009) Comparative PET–CT scans showing the patterns of 18F-FDG uptakes in the same subject after exposure to cold and under thermoneutral conditions. The PET-CT scan shows that exposure to cold activates BAT as shown in the figure on the left marked by visible dark spots around the sternum and upper chest, which are not shown in the figure on the right under thermoneutral conditions.

1.1.2.2 Brown adipose tissue function

BAT is a site of heat production during exposure to a cold environment in most newborn mammals. Cold exposure increases O_2 consumption and therefore in turn non-shivering heat production by BAT (Cypess et al., 2009). BAT is characterized by a rich blood supply for O_2 delivery, as heat production is dependent on adequate supply of O_2. Angiogenesis is four to six times higher in BAT when compared to WAT

(Asano et al., 1999). Additionally, the tissue is also highly innervated, predominantly by sympathetic nerve fibres. The inner membrane of the mitochondria contains

Figure 4: The cytological features of BAT.

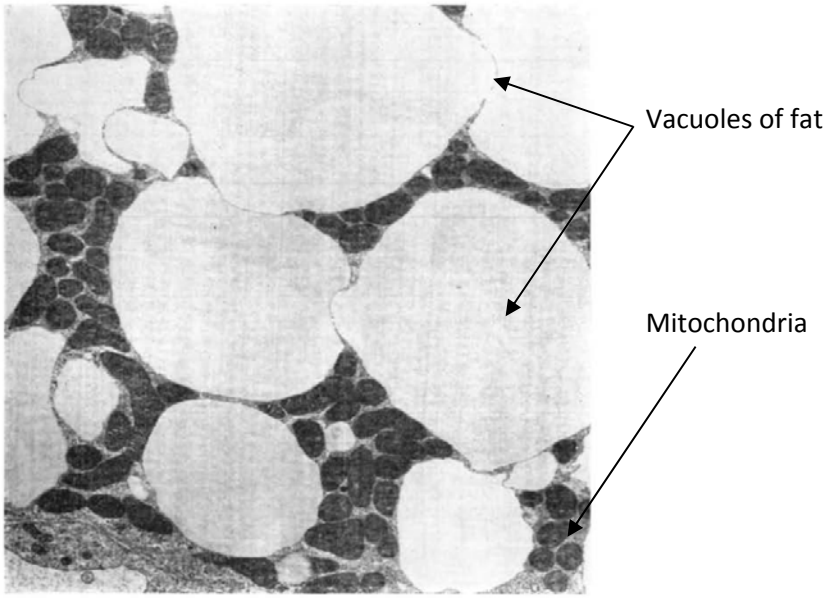


Source: (Hull, 1966). Brown adipose tissue, 1: a developing cell. The cytoplasm contains a round nucleus, large and numerous mitochondria and many fat vacuoles; 2: a mature cell. The cell contains more cytoplasm and more fat than the developing cell; 3: a fat-depleted cell. The cytoplasm is filled with mitochondria and a few small fat vacuoles; 4: a unilocular brown adipose cell. The fat vacuoles have fused to form a single vacuole.

UCP-1, which constitutes 5 % of total mitochondrial protein (Luevano-Martinez, 2012). Induction of UCP-1 is coupled to triglyceride break down and is activated by fatty acids in BAT. Purine nucleotides, in turn, inhibit the action of UCP-1. Lipolysis is a complex process affected by the environment and hormonal influence e.g. in cold conditions, the sympathetic nerve endings secrete noradrenaline which binds to β_3-adrenergic receptors located on brown adipocytes.

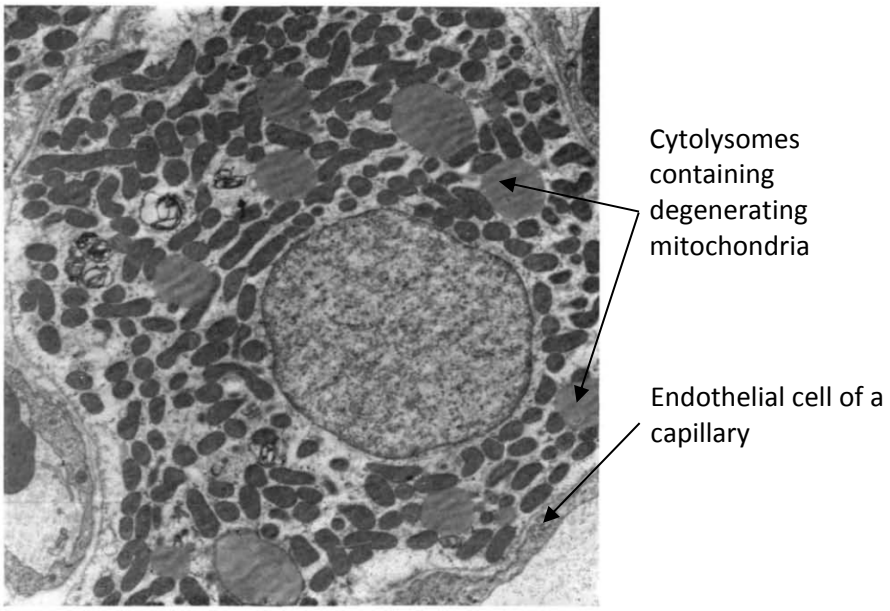
The action of noradrenaline switches on adenylyl cyclase and leads to an increase in cyclic adenosine monophosphate (cAMP) concentration. Following cAMP stimulation, protein kinase A activity is increased, which further mobilizes lipolysis by hormone-sensitive lipase (Tews and Wabitsch, 2011).

Figure 5: Electron micrograph of brown adipose tissue from a new-born rabbit killed soon after birth.



Source: (Hull, 1966) Large mitochondria surround the numerous vacuoles of fat. BAT is characterised with a greater number of mitochondria located closely to vacuoles of fat enabling mobilisation of fat for cellular respiration within the mitochondria. Only a part of one cell is shown (magnification X 5,625).

Figure 6: Electron micrograph of brown adipose tissue from a fasting new-born rabbit after exposure to a cool environment (30 °C) for 48 hours.

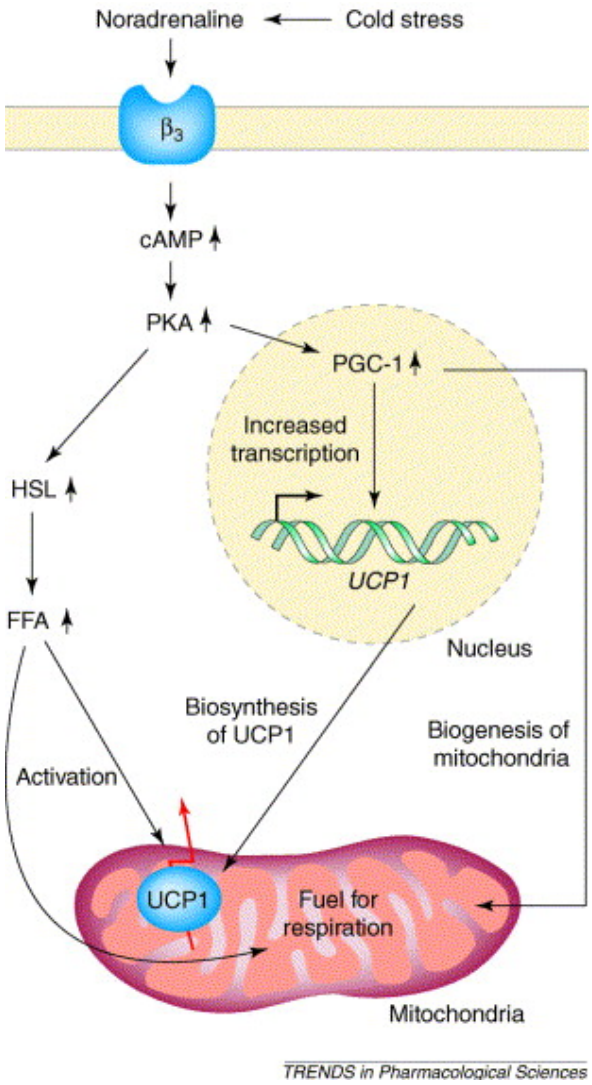


Source: (Hull, 1966) The fat vacuoles are significantly smaller due to fasting; the cytoplasm is largely filled with mitochondria. Staining bodies are probably cytolysomes and may contain degenerating mitochondria (magnification x 5,625).

1.1.2.3 Endocrine regulation of brown adipose tissue.

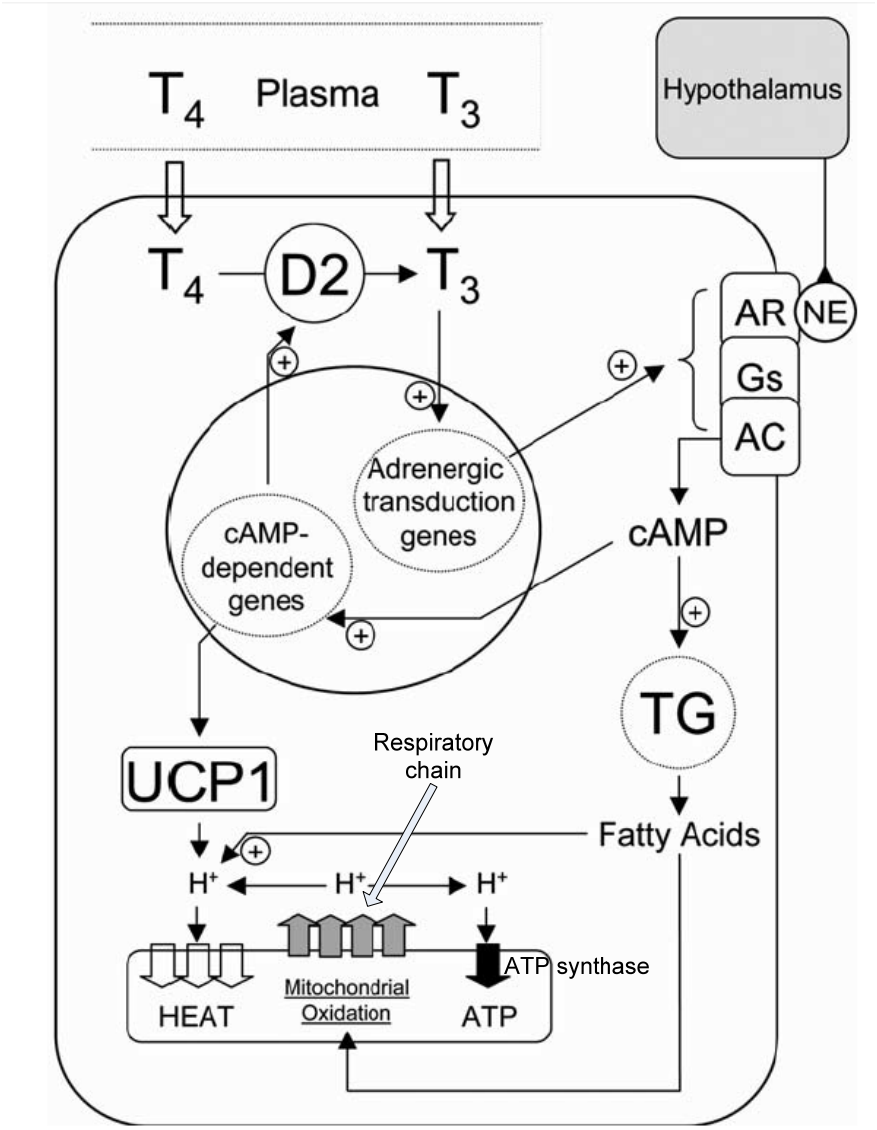
The thermogenic function of BAT is also stimulated via up-regulation of UCP-1 expression by catecholamines and thyroid hormones. Thyroxine hormone is converted into its active form triiodothyronine (T_3) via the action of protein kinase A which phosphorylates deiodinases. Deiodinases are found in abundance in BAT and their role is in the modulation of thyroid hormones. Active T_3 potentiates catecholamine-induced UCP-1 expression by adhering to its intracellular receptors and to thyroid hormone-sensitive elements in the UCP-1 promoter (Cannon and Nedergaard, 2010). The biomechanics of brown fat mitochondria as the site of heat production is currently well defined (Flatmark and Pedersen, 1975, Ricquier, 2011). The mitochondrial membrane of brown fat mitochondrion is permeable to protons enabling its unique function in a controlled form of uncoupling during oxidative phosphorylation (Himms-Hagen, 1979). During normal respiration the energy currency molecule, adenosine triphosphate (ATP) is formed, whilst the uncoupled pathway in brown fat produces heat instead. The heat production is regulated by opening or closing of the uncoupled pathway, with the latter having a slow-down effect and a reduction in the amount of heat that is yielded (Figure 7). Cold-induced non-shivering thermogenesis was found to be defective in the genetically obese mouse (*ob/ob*) and brown fat mitochondrial function is very limited in the brown adipocytes of the obese mouse (Kates and Himms-Hagen, 1990). Scientists speculated that BAT of the obese mice has an impaired thermogenic function (Himms-Hagen, 1979) since the level and distribution of brown fat is similar in both lean and obese mice, however their capacity to oxidise food is expressed differently with the latter having a lower efficiency of food energy utilization as heat especially in a cool environment (about 23 °C) (Thurlby and Trayhurn, 1979, Wijers et al., 2010). In accordance with these facts, it is plausible to hypothesise that excess calories from food in lean mice dissipate as heat generated by BAT. This indeed was demonstrated in an animal model of the cafeteria-fed rat (Rothwell et al., 1982). For this reason, a malfunctioning of BAT may lead to decreased capacity of the adipocytes mitochondria to use food energy to produce heat in a cold environment, and therefore fail to expand extra calories from excess consumption of food. Although humans often adopt behavioural approach to compensate for the low

Figure 7: Regulation of UCP1 and its activity in brown adipose tissue.



Source: (Chiesi et al., 2001) BAT generates heat on activation by cold exposure via the action of noradrenaline. Noradrenaline activates β3-adrenoceptors, which in turn induce the mitochondrial protein UCP-1. UCP-1 is exclusively located in the inner mitochondrial membrane of brown adipocytes where it dissipates the proton gradient created by the oxidation of metabolic products and therefore generating heat (as indicated by the red arrow). The cold exposure activates the protein kinase A (PKA) and hormone sensitive lipase (HSL), which results in increased free fatty acids (FFA) and simultaneous activation of the protein. The metabolic and hormonal signals lead to an up-regulation in transcription of the gene encoding UCP-1. This process is up-stream of the expression of peroxisome proliferator-activated receptor gamma (PPAR γ) co-activator-1 (PGC-1) and it requires a prior activation of cAMP.

Figure 8: Heat generation in BAT via D2-thyroid mechanism.



Source: (Bianco et al., 2005) Brown adipocytes are stimulated by noradrenaline (NE) released from sympathetic terminals within the BAT. NE interacts with adrenergic receptors (AR) and activates adenylate cyclase (AC) through stimulatory G-protein (Gs), increasing intracellular cAMP levels. This activates triglyceride (TG) breakdown (lipolysis) and the expression of a series of cAMP dependent genes, namely UCP-1, D2, and genes encoding proteins involved in the adrenergic signalling pathway. Intracellular T3 concentration increases because of D2 activation, and there is strong synergism between T3-dependent actions with cAMP-dependent actions towards an overall increase in energy expenditure and thermogenesis.

temperature of the environment such as wearing extra clothes and cold-induced non shivering thermogenesis is not therefore significantly induced, it may however, help explain why certain individuals are more successful at controlling their set-point

body weight and others are not as inter-individual differences in the capacity to burn off extra energy from food are well documented (Leibel et al., 1995).

It is well established that thyroid hormones play a key role in the regulation of UCP-1 (Cannon and Nedergaard, 2010) which extends into early life programming during which time the increase in triiodothyronine (T_3) alongside the rise in cortisol level promote the abundant distribution of UCP-1 (Mostyn et al., 2003). A normal function of BAT is dependent on the enzyme 2,5'monodeiodinase (D2), which if blocked, leads to an impairment of both differentiation and oxidative capacity of BAT in the mouse fetus (Hall et al., 2010). D2 is expressed in abundance in BAT and its role is in amplifying thyroid hormone signalling (Figure 8). Organisms with inactivation of the D2 pathway exhibit significantly impaired thermogenesis in BAT, leading to hypothermia during cold exposure and a greater susceptibility to diet-induced obesity (Marsili et al., 2011). D2 controlled thyroid hormone signalling in BAT has important metabolic repercussions for energy homeostasis in adulthood (Castillo et al., 2011).

1.1.2.4 White adipose tissue structure

WAT was demonstrated to have a capacity to change its morphological structure depending on the requirements of an organism. Predominantly, in homeotherms, WAT develops extensively in proportion to body weight, varying greatly between species. Adipocytes originate from stellate or fusiform precursor cells of mesenchymal origin (Figure 2). In human adipose tissue, formation takes place in the prenatal period around the 14th and 16th week during which gland-like aggregations of epitheloid precursor cells, called lipoblasts, or preadipocytes begin to form.

Further fat formation takes place after 23rd week of gestation and subsequently, during the early postnatal period. The number of adipocytes is thought to remain constant and a developmental hypertrophy of fat lobules is accompanied during growth. At birth, WAT accounts for 16 % of total body weight (with BAT constituting 2 to 5 %). The adipocyte development also called adipogenesis varies according to sex and age. During adulthood, a normal healthy level of total body fat % is up to 23 and 33 in males and females, respectively. Exposure of young cats (*felis domestica*)

intermittently to a temperature of minus 30 °C for two periods of 1 hour per day, decreased the diameter of adipose cells, increased WAT capillarisation and significantly increased the number, volume and density of mitochondria in brown adipose tissue (Loncar et al., 1986).

a) Anatomical distribution

Adipose tissue is essential for the regulation of energy balance and therefore is present in every mammal. WAT has a white/yellowish appearance. The subcutaneous tissue is spanned with a continuous layer of WAT called *panniculus adiposus*. Subcutaneous WAT is located in abundance especially in the lower abdomen and buttocks, particularly in females. The other WAT depots include that found surrounding the organs (omental), in the breasts, mesentery and retroperitoneal/perirenal regions. WAT is also deposited in the axilla, pericardium, bone marrow, orbits, hands, and soles of the feet (Collins, 2005). The variation in sexual dimorphism of body shape in humans is largely accounted for by differences in subcutaneous WAT (Bjorntorp, 1996). Females are characterized by larger WAT proportion in the breasts and the hypodermis of the lower abdomen, buttocks and thighs than males. These distinct differences are largely a consequence of the effect of sex hormones and a greater parity, as the female (gynoid) pattern of fat distribution tends to develop in a central (gynoid) location at menopause (Wells, 2007). The amount of WAT can increase drastically with obesity and decrease with anorexia nervosa (Frisch and McArthur, 1974), cancer and other wasting diseases. By weight, WAT comprises between 70 % and 90 % lipid, 5 % to 30 % water, and 2 % to 3 % protein. Most of fat content is in the form of TAGs (> 90 %) and in small amounts as diglyceride, cholesterol, and phospholipid.

b) Microscopic distribution

Adipose tissue is characterised by unilocular (single large lipid inclusion) white adipocytes found in many areas of connective tissue that is partitioned into lobules and is supported by a matrix of collagen fibres together with a network of blood supply. The adipose lineage originates from multipotent mesenchymal stem cells that develop into adipoblasts. These adipoblasts are precursors to preadipocytes, cells that express early markers but have yet to develop into mature adipocytes.

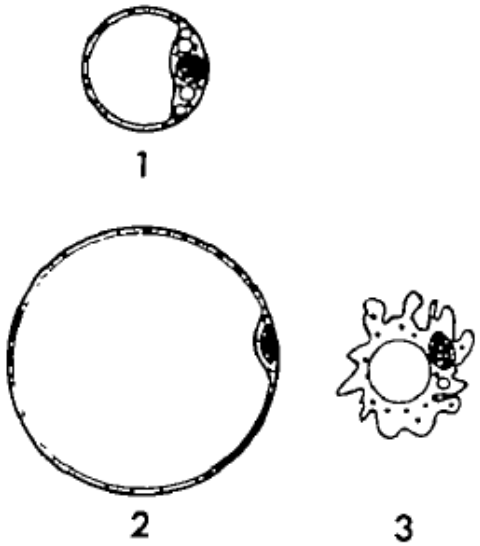
When fat tissue is processed for microscopic examination with xylene or other fat solvents, lipid is leached from the cells, so that the specimen resembles a meshwork of polygonal or ovoid structures (20 to 200 µm diameter) when observed under the microscope (Figure 9). The morphology of WAT is very much influenced by the nutritional status. Excess energy consumption leads to obesity and an increase in WAT due to hypertrophy and hyperplasia of adipocytes (Bjorntorp, 1974, Stern and Greenwood, 1974, Carpentier et al., 1977). Fat droplet formation is a process during which abundant micropinocytotic invaginations and vesicles are shaped and coalesce into multilocular and then finally unilocular lipid inclusions (Cushman and Rizack, 1970, Cushman, 1970). Conversely, fasting leads to a reduction in the size of the fat droplet, an irregularity of the plasma membrane characterized by a number of micropinocytotic invaginations and vesicles, and a noticeable smooth endoplasmic reticulum (Carpentier et al., 1977). Long-term fasting causes white fat cells to transform into spindle-shaped fibroblast-like cells containing a small number of lipid inclusions (Figure 10).

1.1.2.5 White adipose tissue function

The study of the adipocyte as an endocrine cell is new and the understanding of its function is defined as the ability to secrete and regulate molecules involved in appetite control and long-term energy equilibrium (Trayhurn et al., 2008b). It is known that the function of the adipocyte is suppressed during obesity and it is linked with the enlargement of the adipocytes (hypertrophy), imbalance between lipogenesis and lipolysis, blunting of transcriptional regulation during adipogenesis, and loss of sensitivity to external signals, as well as diminished signal transduction process (Lafontan, 2008).

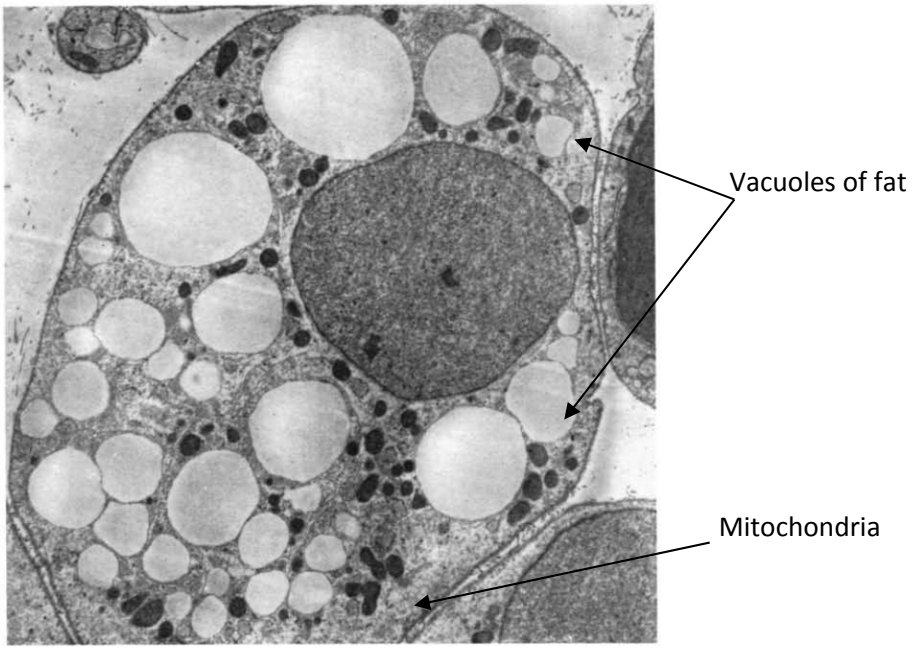
The main function of adipose tissue is to store energy in the form of TAGs also known as triglycerides, in the adipocytes. TAGs are made available through the lipolysis process to provide fuel to other organs and to deliver substrates to the liver for gluconeogenesis (glycerol) and lipoprotein synthesis (free fatty acids)(Large and Arner, 1998). Scientists thought that adipose tissue's main function is to store energy

Figure 9: Cytological features of white adipose tissue.



Source: (Hull, 1966) White adipose tissue, 1: a developing cell. The nucleus is round and the cytoplasm contains a number of small fat vacuoles and one large one; 2: a mature cell. The nucleus is compressed against the cell wall; the mitochondria are few, small and round; 3: a fat-depleted cell.

Figure 10: Electron micrograph of developing white adipose tissue from the groin of the newborn rabbit.



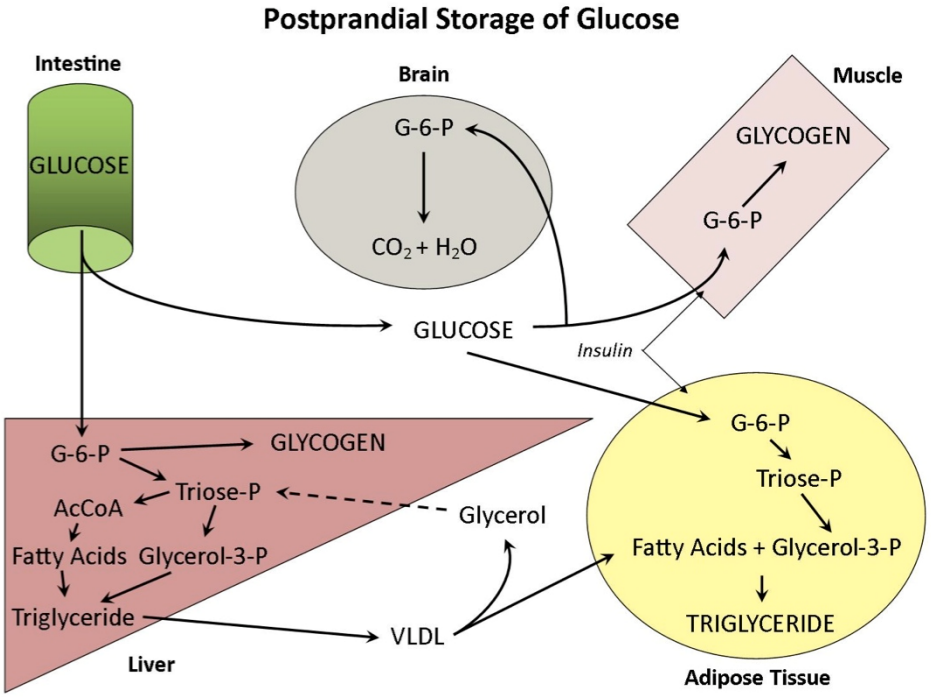
Source: (Hull, 1966) The cell contains many small vacuoles of fat, but the mitochondria, unlike the mitochondria of multilocular cells of brown adipose tissue, are few and small and they do not lie adjacent to the fat vacuoles (magnification x 5,625).

during the times of plenty and during fasting periods to release energy. Following the discovery of leptin (Zhang et al., 1994), a hormone released by adipocytes, much attention has been paid to the function of WAT as an endocrine organ. Since then, many hormones including adiponectin, visfatin, resistin, and inflammatory markers and essential proteins that alter appetite and satiety, glucose and lipid metabolism, blood pressure regulation, inflammation and immune function have been discovered to be synthesized by the adipocytes (Redinger, 2009, Koleva et al., 2013).

Excess energy is stored in WAT in the form of TAGs that are synthesized from fatty acids found in the blood postprandially. Fatty acids can also be synthesized *de novo* from glucose in the liver and in the adipocytes in a process known as lipogenesis (Lafontan, 2008). Figure 11 shows a summary of TAG formation in adipocytes. During the fasting state, low levels of insulin inhibit TAG synthesis and lipolysis is stimulated by glucagon in the adipose tissue (Figure 12). Free fatty acids catabolised from TAGs circulate in the plasma and are carried by albumin to the liver where they are oxidized for the formation of ATP via acetyl CoA (Groff, 2000).

Fatty acids are the main molecules of fuel storage; moreover fatty acids are essential for the synthesis of vital structural molecules, such as phospholipids, hormone-like substances (i.e. prostaglandins), and signalling molecules, such as inositol phosphates. Most importantly, dietary fat represents the major pool of stored nutrients when compared with protein and carbohydrate (Flatt, 1998). On average, a daily intake of dietary carbohydrate equals to 150 to 300 grams whilst only 400 to 800 grams can be stored as glycogen mainly in muscle. Daily protein consumption is around 60 to 100 grams, however there is no storage pool of protein in the body because proteins play a critical role in virtually all body functions having a daily turnover of 300 to 400 grams. For comparison, daily fat intake is quite low ranging from 60 to 90 grams but due to its high energy density (9 kcal/grams) it yields twice as much energy as protein or carbohydrate (both 4 kcal/grams) (Bessesen et al., 2008).

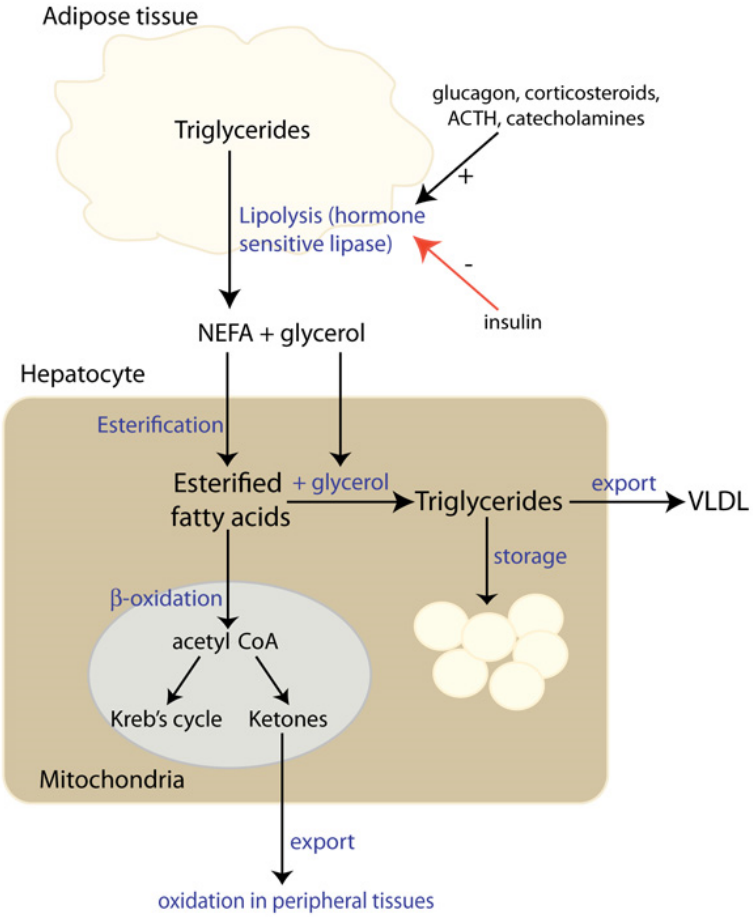
Figure 11: Lipogenesis.



Source: (Adapted from Groff, 2000) TAG formation in adipose tissue. G-6-P, glucose-6-phosphate; CO^2, carbon dioxide; H_20, water; P, phosphate; AcCoA, acetyl coenzyme A; VLDL, very low density lipoprotein. Circulating blood glucose has three main fates: 1) It is used as fuel for the function of the brain, 2) a limited amount can be stored as glycogen in the liver and skeletal muscles, 3) and further excess glucose is converted into TAGs via the process of lipogenesis in the liver and adipocytes. The adipose tissue acts as the main storage tissue of excess glucose in the form of TAGs.

Adipose tissue has a large capacity for storing fat, which is slowly absorbed during the post-meal excursion of triglyceride. To put this in to perspective, a 70 kg individual with a 25 % body fat has 17.5 kilograms of lipids stored as TAGs in adipose tissue and around 400 to 800 grams of glycogen that can be used to produce energy in the form of ATP. The production of fatty acids and glycerol can be measured to investigate adipocyte metabolism during adipogenesis under different conditions.
It is recognized that adipose tissue exerts an effect on whole-body metabolism (Yamada et al., 2013). Adipose tissue not only stores and releases energy but also secretes hormones and inflammatory molecules that alter appetite, regulate energy

Figure 12: Lipolysis of triacylglycerols in the adipose tissue.



Source: (Adapted from Groff, 2000). Lipolysis of TAGs and β-oxidation of fatty acids. ACTH, adrenocorticotropic hormone, NEFA, non-esterified fatty acids; VLDL, very low density lipoprotein; acetyl CoA, acetyl Coenzyme A. During e.g. fasting or exercise hormones such as glucagon, corticosteroids, ACTH and catecholamies activate the hormone sensitive lipase. Hormones sensitive lipase mobilises TAGs from adipose tissue into hepatocytes. TAGs undergo esterification into fatty acids and glycerol. Fatty acids can enter the Kreb's cycle within the mitochondria and be oxidised in peripheral tissues.

expenditure and are involved in other metabolic pathways in autocrine and paracrine manner.

Research in the last two decades further demonstrated that adipose tissue is not just a storage tissue. We now know that adipose tissue is very active and adipocytes secrete a range of molecules including adipokines and inflammatory markers (Wang et al., 2008, Cao, 2014). WAT is now considered to be a major endocrine organ and signalling tissue, which communicates extensively with other organs to influence

long-term energy balance and physiological and metabolic control (Trayhurn, 2005). There is a need for further research into understanding the relationship between adipose tissue, adipocytokine levels, and disease risk found in the obese.

1.1.2.6 Endocrine regulation of white adipose tissue

The study of adipose tissue as an endocrine organ is relatively new and dates back to the past two decades during which several key hormones and receptors regulating short and long-term energy homoeostasis have been identified. Namely, leptin was discovered in 1994 as the first hormone to be only secreted by the adipose tissue (Halaas et al., 1995).

It was known before that the hypothalamus plays a key role in the long-term energy balance (i.e. adipose tissue mass) and coordinated control of food intake. In 1940, Hetherington and Ranson, (1940) demonstrated that damage to the medial basal hypothalamus without the injury of the pituitary gland can lead to morbid obesity and neuroendocrine disruptions (Hetherington, 1940). Further studies, firmly established that a normal function of the energy and glucose homeostasis is dependent upon undamaged hypothalamus (Stevenson, 1969, Elmquist et al., 1999). Within the hypothalamus, it was suggested that a feeding centre was located in the lateral part, and a satiety centre in the ventromedial area based on studies showing that damage to the hypothalamus can cause obesity and lesions in the lateral part of the hypothalamus result in leanness (Stellar, 1954). It is now well established that humans and other mammals have regulatory mechanisms that match food intake with energy expenditure, therefore sustaining a stable body weight over long periods. In 1953 Kennedy introduced a mechanism of energy balance regulation in which signals from fat depot bring about compensatory adjustments in calorie intake and energy expenditure, to maintain the level of adiposity at a presumed set point (Kennedy, 1953). His theory was supported by findings in rodents demonstrating that an increase in body weight because of forced feeding led to a compensatory decrease in voluntary consumption of food; increased physical activity; and gradual restoration of body weight to the baseline level. Whilst starvation and lipectomy brought about a decrease in energy expenditure and an increase in feeding to

restore body weight and adipose tissue mass to a previous 'set point' (Harris et al., 1986, Harris, 1990, Faust et al., 1977). However, the central nervous system (CNS) roadmap with its key signals mediating the interaction between fat mass and the brain was not known until later. Investigations by Harvey gave important insights into the regulation of energy expenditure and long-term energy balance (Hervey, 1959, Parameswaran et al., 1977). He showed that parabiosis (parabiosis is a surgical process that joins two organisms together and it enables them to share the blood circulation) between obese rats with ventromedial hypothalamic (VMH) lesions and normal (non-lesioned) rats reduced food consumption and therefore, body mass in the non-lesioned rats (Hervey, 1959). Findings of this study led to conclusions that obese VMH-lesioned rats secreted some type of ''satiety factor'' resulting in inhibition of feeding in non-lesioned parabiotic rats. The finding of the lack of response in VMH-lesioned rats was previously demonstrated suggesting the existence of a satiety centre (Stellar, 1954). This assumption was further supported following the development in molecular biology of recessive mutations in mice, *ob* and *db*, leptin and its receptor, respectively, both of which led to hyperphagia (over eating), decreased physical activity, and morbid obesity (Ingalls et al., 1950). In the late 1990's the *ob* (leptin) and *db* (leptin receptor) genes were cloned, and the hormone product of the *ob* gene was termed ''leptin'' (from Greek root *leptos*, meaning ''thin'') as the studies suggested that injection with leptin into leptin-deficient mice inhibited feeding, decreased body weight and body fat mass (Potempa et al., 2009, Campfield et al., 1995, Halaas et al., 1997, Halaas et al., 1995). Aside from the obese and diabetic mouse strains, the lethal yellow agouti (A^{Y}) mouse was also shown to express an obesity syndrome (Miller et al., 1993). The cloning of the agouti gene led to an important discovery of the CNS circuits involved in the homeostasis of energy balance, the central melanocortin system (Bultman et al., 1992, Lu et al., 1994). These research findings over the past two decades, have established the beginnings of a cellular and molecular basis for the neuroendocrine control of energy balance (Figure 13).

1.1.3 Control of feeding and energy stores

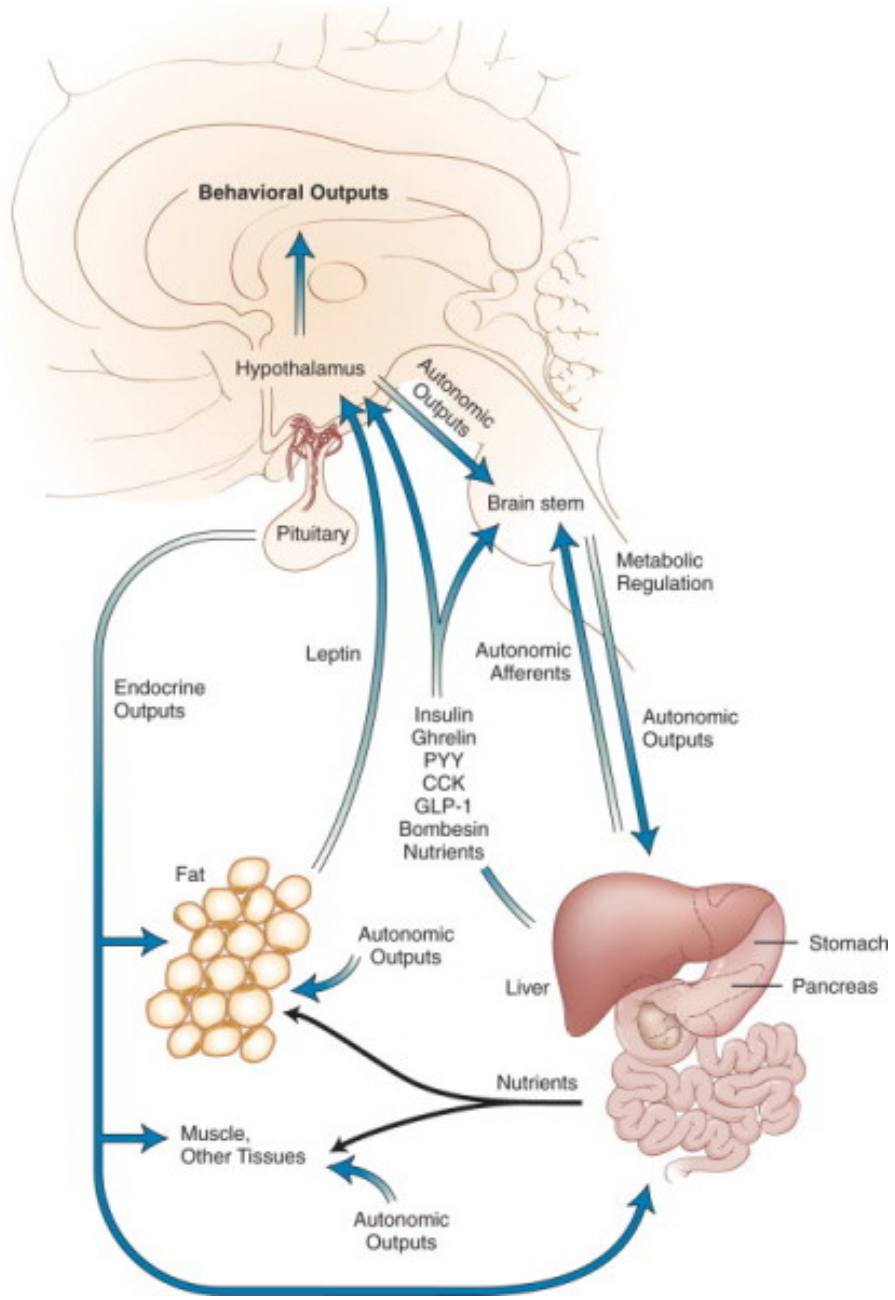
The main circuits involved in energy balance and calorie intake arise in the hypothalamus and brainstem (Figure 14). The hypothalamus is the foremost and highly conserved region of the brain that controls short and long-term energy homeostasis in mammals. The destruction of the hypothalamus would lead to death making it an essential part of the brain (Saper, 1995). The hypothalamus is involved in processing sensory outputs and information reflecting the internal environment. Hormones released from adipose tissue and the gut, and several key hormones released from the endocrine system directly exert an effect on neurons in the hypothalamus. These include e.g. glucocorticoids, oestrogen, leptin, ghrelin, and adrenalin.

The hypothalamus integrates information from the afferent sensory inputs from the external and internal environment and provides motor outputs to target regulatory sites including the anterior and posterior pituitary gland, the motor neurons in the brainstem and spinal cord, and autonomic (parasympathetic and sympathetic) preganglionic neurons (Cone, 2008). Several hypothalamic sites are considered to be involved in the neuroendocrine control of energy stores. The medial hypothalamus comprises of arcuate nucleus, the ventral medial nucleus, the dorsal medial nucleus, and the paraventricular nucleus. The lateral hypothalamus area and perifornical hypothalamus are the main components of the hypothalamus involved in energy balance (Figure 14). The arcuate nucleus has been well established as the key node of hypothalamic regulation of feeding behaviour and fat mass. Its role involves conferring the function of metabolic signals such as leptin, insulin, and ghrelin. More specifically, pro-opiomelanocortin (POMC) and neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons within the arcuate nucleus are the main modulators of hunger and satiety, feeding and glucose equilibrium (Figure 14).

The arcuate nucleus is therefore, the best-defined hypothalamic nucleus thought to play a key role in the regulation of energy balance. The arcuate nucleus coordinates metabolic signals exerted by the actions of leptin, insulin, and ghrelin. It is well established that the feeding and satiety centre, the POMC and NPY/AgRP neurons

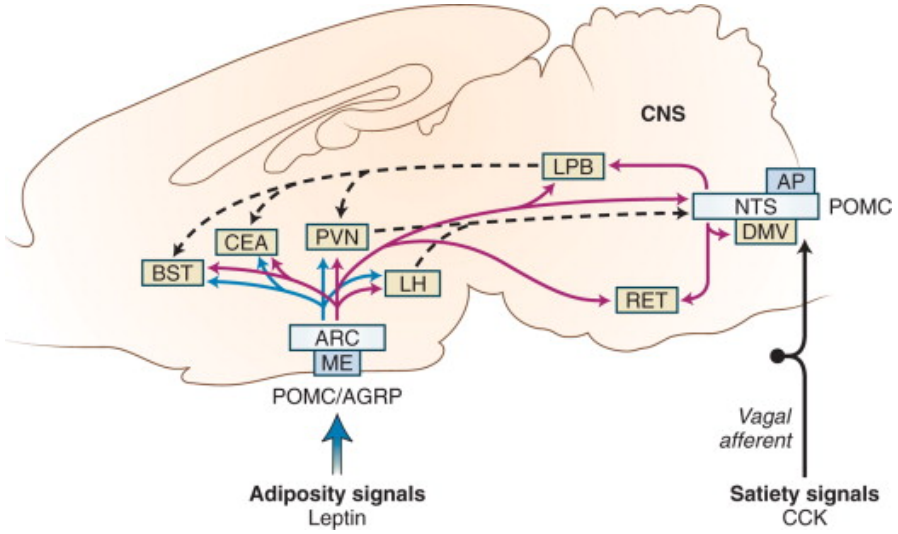
located within the arcuate nucleus exert anorexic and orexigenic effects, respectively. POMC is a pleiotropic propeptide that is a precursor protein to a variety of key peptides (Figure 15). For example, melanocortin peptides, more specifically α-

Figure 13: Regulation of energy homeostasis by the brain-gut-adipose axis.



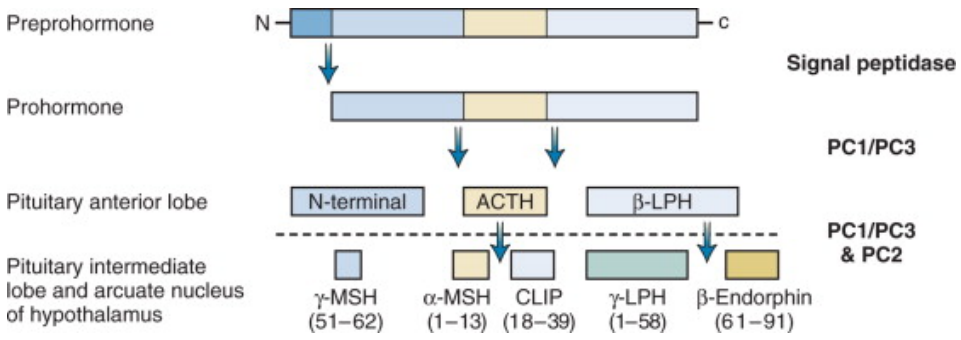
Source: (Cone, 2008) The hypothalamus receives sensory signals from the gut and the adipose tissue. An array of adipose tissue and gut specific hormones are involved in the relay of autonomic outputs. Both the brain stem and parts of the hypothalamus are involved in processing of the neuroendocrine information with an adequate behavioural output consequently. CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; PYY, peptide YY.

Figure 14: Schematic view of brain structures involved in energy balance.



Source: (Fan et al., 2004) Schematic representation of adipostatic signals and acute satiety signals in the brain. Blue: leptin-responsive neurons; yellow: nuclei containing MC_4 neurons that may serve to integrate adipostatic and satiety signals; pink: some circumventricular organs involved in energy homeostasis; red arrows: POMC projections; blue arrows: AgRP projections. BST, Bed nucleus of the stria terminalus; CEA, central nucleus of the amygdala; PVN, paraventricular nucleus of the hypothalamus; LH, lateral hypothalamic area; LPB, lateral parabrachial nucleus; AP, area postrema; DMV, dorsal motor nucleus of the vagus.

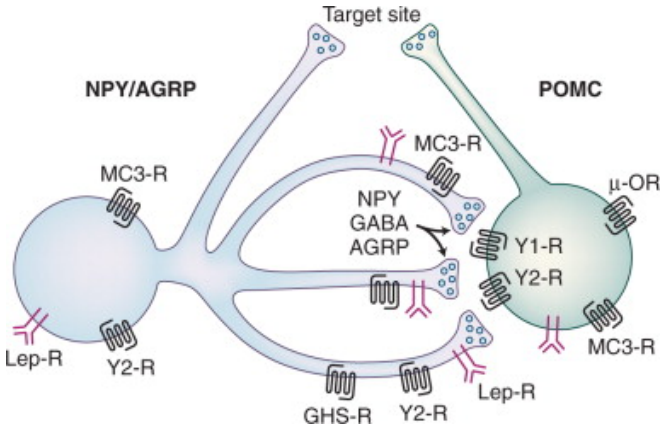
Figure 15: Schematic representation of proopiomelanocortin (POMC).



Source: (Cone, 2008) POMC is a precursor hormone of corticotropin (ACTH), β-lipotropin (β-LPH), corticotropin-like intermediate lobe peptide (CLIP), β-endorphin, prohormone convertase (PC), α melanocyte stimulating hormone (α-MSH) and insulin.

melanocyte stimulating hormone (α-MSH) is one of the key products involved in feeding and energy balance regulation within the brain. α-MSH is also an agonist for MC_4 , which modulates glucose balance, energy homeostasis and feeding behaviour in both rodents and humans (Cone, 2005, Barsh et al., 2000).

Figure 16: Regulation of the arcuate nucleus of the hypothalamus by various hormones and neuropeptides.



Source: (Cowley et al., 2001) The arcuate nucleus consists of two major neurons, namely, neuropeptide Y (NPY)/Agouti related peptide (AgRP) and proopiomelanocortin (POMC) which provide a framework where dense NPY/AgRP fibres protrude to POMC cell bodies. A wide range of receptors for the multi hormonal and neuropeptide system involved in the modulation of the network are also shown here. Abbreviations: melanocortin-3-receptor (MC_3), gamma-aminobutyric acid (GABA), leptin-receptor (lep - R), growth hormone secretagogue-receptor (GHS-R), neuropeptide Y – receptor 2 (Y2-R).

AgRP is co-expressed with neuropeptide Y (NPY) in the neuronal parts of arcuate nucleus and acts as an endogenous MC_4 antagonist. Starvation, which is known to decrease leptin concentration in the plasma, and leptin deficiency, both down-regulate the expression of POMC and induce AgRP and NPY expression (Schwartz et al., 1997, Stephens et al., 1995). It has been shown that POMC and NPY/AgRP are directly neuromodulated by the action of leptin, ghrelin, glucose, and other key metabolic signals. For example, leptin directly depolarizes (switches on) the POMC centre and hyperpolarizes (switches off) the AgRP/NPY centre (Cowley et al., 2001, Spanswick et al., 1997) whilst lesions to leptin receptors in the POMC centre result in mild obesity in mice (Balthasar et al., 2004). The gastrointestinal peptide hormone, ghrelin exerts a direct depolarizing effect on AgRP/NPY neurons (Cowley et al., 2003). Moreover, it was demonstrated that serotonin also directly switches on POMC neurons (Heisler et al., 2002) and turns off NPY/AgRP neurons (Heisler et al., 2006). Fenfluramine is an amphetamine derivative and its function partly depends on MC_4's (Heisler et al., 2006). Interestingly, fenfluramine was used in combination with phenteramine (Fen/Phen) to significantly reduce appetite and body weight in

obese humans, however due its cardiovascular complications (heart valve disorders and pulmonary hypertension) and side effects was removed from the market (Surapaneni et al., 2011). Both, the POMC neurons and the AgRP/NPY neurons work in a coordinated way to regulate energy homeostasis via the actions of a range of hormones, drugs, and nutrients (Figure 16).

1.1.4 Adipose tissue specific hormones

1.1.4.1 Pro-inflammatory mediators

1.1.4.1.1 Leptin

Leptin, a peptide hormone, comprises of 167 amino acids (16 kd) and is the product of the *ob* gene, is synthesized in WAT and modulates food intake, energy expenditure and neuroendocrine status (Zhang et al., 1994, Tartaglia et al., 1995). The discovery of leptin hormone in the early 1990s led to further research and increased understanding of WAT not merely as a storage depot but a *bona fide* organ. Comparisons of the leptin gene showed 84 % homology demonstrating a highly conserved protein. The protein is secreted into the blood stream proportionally to the amount of, chiefly, sub-cutaneous rather than visceral fat depots (Van Harmelen et al., 1998). The normal concentration levels of this hormone range from 5 to 10 ng/ml in healthy individuals and 40 to 100 ng/ml in individuals with obesity/metabolic syndrome (Howard et al., 2010).

Leptin circulates in the blood both in the free form as well as bound to its receptor (Ob-Re). Leptin is secreted largely from the sub-cutaneous adipose tissue, but low levels of leptin also were noted in the skeletal muscle, placenta, and the stomach (Zhang et al., 1994, Wang et al., 1998).

A complete lack of leptin or impaired leptin signalling, both in rodents and humans results in morbid obesity that is associated with panoply of neuroendocrine complications. Exogenous administration of leptin restores these anomalies (Pelleymounter et al., 1995, Campfield et al., 1995, Halaas et al., 1995, Farooqi et al., 1999). During periods of starvation and decreased body fat stores, plasma leptin concentration fall and at the same time there are significant changes at the

neuroendocrine level, including an induction of the hypothalamic-pituitary-adrenal (HPA) axis, decreased synthesis of the growth hormone, and inhibition of the thyroid axes and reproductive function (Ahima et al., 1996, Spiegelman and Flier, 2001, Frederich et al., 1995). Because administration of exogenous leptin seem to bring about changes in these starvation-induced adaptations i.e. it blunts the effects of starvation, these observations have led to the suggestion that plasma leptin has an adipostatic role in sending signals to the brain with regards to the amount of energy stores.

Leptin is transported through the blood-brain barrier and binds to its receptors in the hypothalamus and brainstem (Lee et al., 1996, Tartaglia et al., 1995, Tartaglia, 1997). The Ob-Rb leptin receptor belongs to the cytokine-receptor superfamily and it binds janus kinases (JAK), tyrosine kinases that take part in intracellular cytokine signalling. Induction of JAK precedes phosphorylation of members of the signal transduction and transcription (STAT) family of proteins. Successively, STAT proteins stimulate transcription of leptin target genes. There are other leptin receptors including a range from Ob-Ra to Ob-Rf but only Ob-Rb leptin receptor is involved in the STAT family of proteins. Meanwhile, the function of others remains elusive.

It was demonstrated that the Ob-Rb leptin receptor is involved in normal energy balance as deletions or modifications of this gene purport to the obese phenotype of the *db/db* mouse and the Zucker rat (White et al., 1997, Chen et al., 1996, Lee et al., 1996). Leptin receptors are located in abundance across a number of hypothalamic nuclei within the medial basal hypothalamus. This comprises of the arcuate, dorsomedial, ventromedial, and ventral premamillary nuclei (Mercer et al., 1996a, Mercer et al., 1996b, Fei et al., 1997, Schwartz et al., 1996, Elmquist et al., 1998, Cheung et al., 1997).

Leptin deficiency (Montague et al., 1997) and leptin receptor defects (Clement et al., 1998) in humans are exceptionally infrequent, however, are representative of the metabolic importance of this hormone. Serum leptin levels are generally corresponding to the level of fat tissue mass (Considine et al., 1996, Frederich et al.,

1995). Increased level of leptin in the obese is associated with the development of obesity-related hypertension (Canale et al., 2013). Therefore, the preponderance of obese humans is thought to be leptin-resistant rather than deficient in leptin (Barsh et al., 2000, Spiegelman and Flier, 2001, Considine et al., 1996). This phenomenon of leptin resistance is currently not well understood despite the developments in molecular genetics and obesity. Currently, there is no consensus on the effect of ageing on leptin and the effect of leptin on long-term age related chronic conditions. Some studies suggest, however, that leptin resistance and/or loss of function of leptin receptors increases with ageing for this hormone (Gulcelik et al., 2013).

1.1.4.1.2 Visfatin

Visfatin protein of 52 kDa, is also known as pre-B cell enhancing factor (PBEF) (Ray, 2012). The name, visfatin, was derived to designate its preferential expression in visceral fat (Fukuhara et al., 2005). Originally, the protein was first discovered in 1994, as a clone from a human peripheral blood lymphocyte cDNA library and named pre-B-cell colony-enhancing factor (PBEF), for its ability to stimulate the action of IL-7 and Sertoli cell factor (SCF) on pre-B-cell colony formation (Ognjanovic et al., 2001, Samal et al., 1994). PBEF/visfatin is found in various tissues, but mainly is contained intracellularly (Rongvaux et al., 2002). PBEF/visfatin acts as a nicotinamide phosphoribosyltransferase, catalyzing the rate-limiting step in the biosynthesis of nicotinamide adenine dinucleotide+ (NAD) +. To date, it has been shown to have a function in programmed cell death, regulation of cell metabolism during stress responses and the stimulation of the immune system (Rongvaux et al., 2002, Samal et al., 1994). It is not clear yet how visfatin mediates metabolism extracellularly or which signalling sequence is involved in its mode of secretion and action. It was demonstrated that visfatin when secreted in the visceral fat can lower plasma glucose, possibly via its ability to bind to the insulin receptor (Fukuhara et al., 2005). Visfatin is a non-competitive insulin mimetic and it can act to induce insulin sensitivity, however its concentration levels are much lower than insulin (Sethi and Vidal-Puig, 2005, Tilg and Moschen, 2006). Circulating plasma visfatin concentrations are directly induced by hyperglycaemia (Alexiadou et al., 2012) and this effect is reversed by exogenous hyperinsulinaemia or somatostatin infusion (Haider et al.,

2006). The capillary network of visceral fat is unusual in that it channels into the hepatic portal vein. Thereupon, hormones and proteins derived from the visceral fat may have a more direct effect on the liver metabolism than those derived from sub-cutaneous fat (Arner, 2006). However, human based clinical studies do not point to a clear link between high circulating levels of visfatin, diabetes mellitus, and atherosclerosis (Sethi and Vidal-Puig, 2005, Yun et al., 2013). There is some evidence showing that chronic degenerative disease states and progressive deterioration of beta cells are linked with more accentuated visfatin increases (Chen et al., 2006b, Lopez-Bermejo et al., 2006). Visfatin mRNA is also up regulated in adipocytes in hypoxia possibly via the involvement of the transcription factor HIF-1α (Segawa et al., 2006). The source of visfatin in visceral WAT was demonstrated to also be of macrophage origin as the infiltration of these leukocytes is increased in visceral WAT of the obese (Curat et al., 2006). Other study suggests that endothelial cells can also be a source of visfatin, where if synthesised, visfatin can induce inflammation in vascular cells (Romacho et al., 2013).

1.1.4.1.3 Resistin

Resistin belongs to a family of proteins called the resistin-like molecules (RELMs) and is also known as adipocyte secreted factor (ADSF). It has been named after its association between type 2 diabetes and obesity and therefore potential function in insulin resistance (Steppan et al., 2001a). Resistin is a 12.5 kDa polypeptide hormone made up of 114 amino acids consisting of a putative N-terminal signal sequence and a motif of 11 cysteine residues, 10 of which belong to the RELM family (Steppan et al., 2001a, Steppan et al., 2001b, Gerstmayer et al., 2003). Resistin is secreted as a dimer. Resistin receptor has been unknown until recently two possible candidates have been identified including an isoform of decorin - unreported cleavage product of decorin (DCN) (named ΔDCN) in 3T3-L1 adipose progenitor cells (Daquinag et al., 2011) and a mouse receptor tyrosine kinase-like orphan receptor (ROR) 1 in 3T3-L1 cells (Sanchez-Solana et al., 2012). Decorin is type of proteoglycan enriched with the leucine amino acid and it is recognized for its function in cell proliferation and migration in a range of tumor cell lines (Liang et al., 2013).

There is only 53 % homology between rodent and human resistin at the aa level (Ghosh et al., 2003, Yang et al., 2003). Expression of resistin is predominantly found in the adipose tissue in mice (Steppan et al., 2001a). In humans, there is some expression of resistin in the adipocytes, however, the major source is thought to be from blood mononuclear cells (McTernan et al., 2002, Nagaev and Smith, 2001, Savage et al., 2001, Al-Suhaimi and Shehzad, 2013). Moreover, the expression of this hormone is greater in WAT than in BAT (Nohira et al., 2004). Plasma resistin concentration is increased in obese humans and mice with diet-induced and genetic forms of obesity and type 2 diabetes. Exogenous administration of recombinant resistin to normal mice blunted the function of insulin and the metabolism of glucose. Neutralization of resistin decreased hyperglycaemia in obese, insulin-resistant mice, partly by enhancing insulin sensitivity. Resistin, therefore has been implicated in the link between excess fat mass, type 2 diabetes and cardiovascular complications via its blunting action of insulin function (Park and Ahima, 2013).

1.1.4.2 Anti-inflammatory mediators

1.1.4.2.1 Adiponectin

The complimentary deoxyribonucleic acid (cDNA) gene encoding adiponectin was first discovered in 1995 in mice 3T3-L1 adipocytes (Scherer et al., 1995). The gene transcript of the protein is 29 kDa and contains a putative N-terminal sequence. Adiponectin, was also alternatively named as adipocytes complement-related protein of 30 kDa (Acrp30), adipoQ and adipose most abundant gene transcript 1 (apM1) (Berg et al., 2002). The name, Acrp30, was derived from the protein's closest homolog, complement factor C1q, and from its size as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Combs et al., 2002). Normal plasma levels of adiponectin range from 1.9 to 17.0 mg/ml in healthy volunteers and accounts for approximately 0.01 % of total plasma protein (Scherer et al., 1995, Fruebis et al., 2001, Arita et al., 1999). Blood concentration of adiponectin is significantly reduced during obesity, insulin resistance and type 2 diabetes (Kishore and Reid, 2000, Stefan et al., 2002, Matsubara et al., 2002) and adiponectin levels increase concomitantly with weight loss and improved insulin sensitivity (Faraj et al., 2003, Yang et al., 2001). Hypoadiponectinemia is closely correlated with type 2

diabetes, hyperinsulinemia (Weyer et al., 2001) and coronary heart disease (Bidulescu et al., 2013). Administration of thiazolidinediones to enhance insulin sensitivity also induces adipose tissue adiponectin gene expression and plasma concentrations (Yu et al., 2002). Additionally, treatment with recombinant adiponectin has hypoglyceamic effects and promotes insulin sensitivity in mouse models of obesity or diabetes (Berg et al., 2002).

Adiponectin is significantly increased during adipocyte maturation and its production is regulated by insulin (Hu et al., 1996, Scherer et al., 1995). There are two receptors for adiponectin, named AdipoR1 and AdipoR2, which have been identified and cloned (Yamauchi et al., 2003). The former is predominantly expressed in skeletal muscle, the latter in the liver. Stimulation with adiponectin has a glucose lowering effect and improves insulin resistance in mice (Berg et al., 2001). On the contrary, adiponectin-deficient mice develop insulin resistance and diabetes (Kubota et al., 2002, Maeda et al., 2002). The insulin sensitizing effect of adiponectin has been linked with the induction of AMP kinase and PPARα and increased fatty-acid utilization (Fruebis et al., 2001).

Adiponectin is the only adipose tissue specific hormone shown to play a protective role in atherosclerosis (Koleva et al., 2013). A study by Hotta et al., (Hotta et al., 2000) demonstrated a significant correlation between hypoadiponectinemia and the risk of coronary artery disease (CAD) in human patients. They also found that adiponectin influenced monocyte adhesion to endothelium, myeloid differentiation and macrophage cytokine production and phagocytosis (Ouchi et al., 1999). Moreover, they showed that adiponectin had a direct effect on monocytes linking it with foam cell development. All of the above processes are related to atherosclerotic plague formation and therefore suggesting adiponectin's role in CAD (Amirzadegan et al., 2013). In addition, it was shown that adiponectin reduced lipid accumulation and decreased the uptake of acetylated low-density lipoprotein (LDL) in cultured macrophages (Ouchi et al., 2001). In summary, adiponectin has been shown to have insulin sensitizing, anti-inflammatory, and antiatherogenic properties (Gulcelik et al.,

2013). It has also been linked with extreme longevity in a study where centenarians expressed higher plasma adiponectin levels (Roszkowska-Gancarz et al., 2012).

1.1.5 Endogenous mediators of inflammation

1.1.5.1 Pro-inflammatory mediators

1.1.5.1.1 C reactive protein (CRP)

CRP is the prototypical acute phase protein in humans and is an important modulator of immune host defence (Black et al., 2004, Pepys and Hirschfield, 2003). CRP was discovered in the early 30's in Oswald Avery's laboratory during an investigation of *Streptococcus pneumonia* infection in individuals acutely ill with lobar pneumonia (Tillett and Francis, 1930). Sera obtained from these patients during the early stage of the illness contained a protein that could precipitate the C polysaccharide derived from the pneumococcal cell wall. The first specific ligand for CRP in the pneumococcal C polysaccharide was later identified as phosphocholine (Volanakis and Kaplan, 1971). Phosphocholine is a constituent of many bacterial and fungal cell walls, and is the principal ligand of CRP (Thompson et al., 1999). The CRP gene comprises of two exons and one intron in humans and is located on the short arm of chromosome 1 (Walsh et al., 1996). CRP consists of 206 amino acid polypeptides and secreted as a ~23 kDa, non-glycosylated monomer. The monomer, non-covalently associates to form the homopentameric ring structure characteristic of the pentraxin family (Thompson et al., 1999). The term pentraxin has been used to describe the family of proteins that are arranged symmetrically around a central pore. The liver produces CRP at large, as a response to pro-inflammatory stimuli. Normal baseline concentration of circulating CRP is low, increasing up to 10,000-fold just within hours of inflammation caused by either injury or invasion of pathogens (Shrive et al., 1996). CRP up-regulation is a part of a larger picture of reorchestration of liver gene expression during an inflammatory process also referred to as the acute phase response, during which production of an array of plasma proteins (e.g. clotting proteins, complement factors, anti-proteases and transport proteins) is induced whilst, notably plasma albumin is decreased (Black et al., 2004). The induction of CRP gene in hepatocytes is mainly controlled at the transcriptional level by the cytokine IL-6, an effect, which can be further enhanced by IL-1β (Kushner et al., 1995). In turn,

the expression of many acute phase protein genes is regulated by IL-6 and IL-1β via activation of the transcription factors STAT3, C/EBP family members and Rel proteins (NF-κB) (Jialal et al., 2004). CRP has increasingly been used as a biomarker of diseases characterized by a chronic inflammation found in the systemic blood including cardiovascular disease, type 2 diabetes and stroke (Pepys and Hirschfield, 2003, Black et al., 2004, Frank and Hargreaves, 2003, Richard et al., 2013, Kuba et al., 2013).

1.1.5.1.2 Interleukin- 6 (IL-6)

In the late 60s, investigations into the mediators of immunity identified a connection between T- and B-cells in the antibody signalling and production (Miller and Mitchell, 1968, Claman et al., 1968). In the 70s and 80s, a wide range of antigen-specific factors and cytokines were isolated. IL-6 was isolated from B-cells and its cDNA cloned and initially named B-cell differentiation factor (BCDF)/B-cell stimulatory factor (BSF)-2 (Hirano et al., 1986). IL-6 is multifunctional cytokine involved in the regulation of many biological processes including immune system function, inflammation, haematopoiesis and tumourigenesis. Its activities are closely linked with the actions of other cytokines, namely, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and oncostatin M (Kishimoto, 2005). Secretion of IL-6 stimulates production of liver acute phase proteins, such as CRP, fibrinogen, serum amyloid protein, and haptoglobin (Gauldie et al., 1987, Andus et al., 1987). Further studies into the effects of IL-6 on immune responses showed that it is a key mediator in antiviral antibody responses as well as for the stimulation of acute phase reaction (Kopf et al., 1994). IL-6 was studied in T-cell function where it has a role in inducing T-cell growth and cytotoxic T-cell differentiation (Noma et al., 1987, Okada et al., 1988, Le et al., 1988) via the actions of IL-2 receptor (Noma et al., 1987, Garman et al., 1987). IL-6 performs its action synergistically with IL-3 to modulate the differentiation of multilineage blast cell colonies during haematopoiesis (Leary et al., 1988, Ikebuchi et al., 1987, Ogawa, 1993). IL-6 also is involved in macrophage (Nicola et al., 1983) and megakaryocyte (Ishibashi et al., 1989) differentiation.

IL-6 has a unique receptor system comprising of IL-6R of 80-kDa and a cell surface glycoprotein, gp130 of 130-kDa (Taga et al., 1989). The gp130 protein is responsible

for signal transduction. As gp130 is expressed ubiquitously in all tissues suggests that this unique receptor functions as a common transducer for other cytokines (Hibi et al., 1990).

A critical point in the resolution of an inflammatory event is the switch between innate to acquired immunity. Inadequate regulation of this 'immunological switch' from innate to acquired immunity may cause tissue damage and/or chronic inflammation (Jones, 2005). IL-6 is involved in leukocyte recruitment, activation, and apoptosis and therefore, has emerged as a regulator of this immunological switch (Jones, 2005). Patients with CAD have a significantly increased level of serum IL-6 suggesting that IL-6 is involved in the aetiology of cardiovascular complications (Su et al., 2013). Data also shows that the pro-inflammatory action of IL-6 may be linked with increased risk for cancer in obese individuals (Ghosh and Ashcraft, 2013). Both inflammatory markers, IL-6 and CRP are strongly correlated with type 2 diabetes and are likely to predict the risks of glucose intolerance as identified in the plasma of obese Caucasians (Gimeno and Klaman, 2005, Yudkin et al., 1999).

1.1.5.1.3 Tumour necrosis factor alpha (TNF α)

At the end of the 19th century (1893) Coley (Coley, 1893) hypothesized that human cancer patients could be treated with bacterial toxins to regress tumour progression. Almost a century later (1985), Old (Old, 1985) demonstrated that a protein in the serum of endotoxin-treated rabbits caused the haemorrhagic necrosis of tumours. The protein, therefore, was named tumour necrosis factor (TNF). Around the same time another group discovered what turned out to be the same protein but they named it, cachectin, due to its involvement in the development of muscle wasting (cachexia)(Beutler and Cerami, 1986). TNF-α is in total a 26 kDa type II transmembrane protein and it is made up of a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane segment, and a 177 aa extracellular domain (Pennica et al., 1984, Wang et al., 1985). TNF-α is widely expressed in a range of immune (i.e. macrophages), epithelial, endothelial and tumour cells sharing 97 % aa sequence homology with rhesus monkey, and 71 – 92 % with bovine, canine and rodents. TNF-α exerts its biological function via binding to the ubiquitous receptors:

type I (TNF-RI) of 55 - 60 kDa (Loetscher et al., 1990, Dembic et al., 1990) and type II (TNF-RII) of 78080 kDa (Smith et al., 1990), which can be found on the membrane of all cell types with the exception of erythrocytes. The protein has pleiotropic actions during inflammation, the growth of immune system, cell death and fat metabolism (Salek-Ardakani and Croft, 2010, Van Herreweghe et al., 2010, Chen et al., 2009). The function of TNF-α has also been implicated in a range of pathological conditions including asthma, Crohn's disease, rheumatoid arthritis, excess body fat, insulin resistance, autoimmunity and cancer (Hehlgans and Pfeffer, 2005, Berry et al., 2007, D'Haens, 2003, Feldmann and Maini, 2001, Tzanavari et al., 2010). In the early 1990s, research into the adipose tissue inflammation identified that TNF-α expression was induced in the WAT of obese mice and humans (Hotamisligil et al., 1993, Kern et al., 1995). The cytokine is expressed in and secreted by the adipose tissue, and its plasma concentration levels have been found to correlate with the level of excess body fat and the associated impaired glucose tolerance (Tzanavari et al., 2010). Administration of TNF-α decreased activity of lipoprotein lipase (LPL), the enzyme involved in the hydrolysis of triacylglycerols and the transport of fatty acids in the 3T3-L1 adipocyte cells (Price et al., 1986). *In vitro* study of human adipose tissue also demonstrated a significant dose-dependent suppression of LPL activity on TNF-α stimulation (Fried and Zechner, 1989). Further studies of exogenous TNF-α delivery confirmed a suppression of LPL activity in the adipose tissue of the rat, mouse, and guinea pig (Cornelius et al., 1988, Fried and Zechner, 1989). The effect of TNF-α and the associated decreased activity of LPL has been shown to influence the uptake of exogenous [^{14}C) lipid (triolein (glycerol tri [$^{1\text{-}14}$C] oleate) by adipocytes and lead to an increase in circulating TAGs in the rodent (Evans and Williamson, 1988).

Along with other proinflammatory cytokines, chemokines and a range of immune cells, TNF-α has been implicated in the development of atherosclerotic lesions by inducing the expression of adhesion molecules on endothelial cells, the influx and activation of inflammatory cells, the start of the inflammatory cascade within the arterial wall i.e. plaque rupture (Ross, 1999, Skoog et al., 2002, Frostegard, 2013). As well as *in vitro* studies showing that TNF-α directly impairs the metabolic pathway of TAGs and cholesterol (Feingold et al., 1998, Grunfeld and Feingold, 1991, Memon et

al., 1993), also there is clinical evidence linking increased TNF-α concentration, and high circulating TAGs in patients with various inflammatory disorders including sepsis, cancer and chronic infections (i.e. acquired immunodeficiency syndrome) (Lind and Lithell, 1994, Gabay and Kushner, 1999, Rossi Fanelli et al., 1995). The resultant high circulating plasma TAGs are due to TNF-α's effect on the release of FFA both from the adipose tissue and the liver. The process has been shown to be predominantly mediated via TNF-RI (Sethi et al., 2000).

TNF-α secreted from the adipose tissue exerts its effects in an autocrine and paracrine manner and it is closely associated with the development of insulin resistance and glucose intolerance in obesity (Hotamisligil and Spiegelman, 1994, Hotamisligil et al., 1995, Gimeno and Klaman, 2005, Frostegard, 2013)). TNF-α and TNF-RI knockout mice have lower body weights than wild-type mice and their tolerance to glucose metabolism is increased (Uysal et al., 1997, Hofmann et al., 1994). Exogenous administration of TNF-α in the cultures of neonatal subcutaneous adipose tissue of pigs reduced the mRNA abundance, and induced monocyte chemotactic gene expression. On stimulation with insulin and TNF-α there was also a reduction in the lipogenesis suggesting that TNF-α can act to suppress lipid accumulation within adipose tissue (Ramsay et al., 2013).

1.1.5.2 Insulin

The investigation into the effects of a pancreatic extract by Banting in 1921 was a break through event in the biological sciences (Best, 1942). Further studies into insulin's function and its ability to restore health led to dramatic descriptions of insulin's clinical pharmacology in almost biblical terms, such as ''the rising of the dead''. Plasma insulin is a polypeptide hormone, of 58 kDa molecular weight, and is synthesized and secreted by the β-cells of the islets of Langerhans in the pancreas. Insulin is the principal hormone involved in the regulation of glucose metabolism. It is firstly synthesized as a precursor, proinsulin, which is converted into C-peptide and insulin (Flier et al., 1979). The insulin molecule consists of two polypeptide chains (linked by disulphide bridges), the A chain and B chain comprising of 21 and 30 amino acids, respectively (Brange and Langkjoer, 1993). Insulin secretion from the

pancreas is predominantly stimulated by plasma glucose concentration (hyperglycaemia) and the hormone has a wide range of metabolic functions, namely, it regulates hepatic output inhibition and stimulates peripheral glucose uptake and utilization in peripheral tissues via the action of glucose transporters e.g. GLUT 1 (Judzewitsch et al., 1982, Gould and Holman, 1993). Insulin action has hypoglycaemic effects through the inhibition of gluconeogenesis and glycogenolysis and its function is opposed by the counter-regulatory hormones (glucagon, growth hormone, cortisol and adrenaline)(Frier et al., 1981). Pancreatic output of insulin secretion is severely reduced in insulin-dependent diabetes mellitus (Juvenile or type 1) and some other conditions such as hypopituitarism. Type 1 diabetes is characterized predominantly by a dysfunction of the beta cells resulting in an absolute lack of insulin secretion. In type 2 diabetes also known as non-insulin dependent, maturity onset, insulin resistance associated with obesity plays an important role. Type 2 diabetes is distinguished by high circulating levels of insulin; however, the hormone does not function properly, therefore, is referred to as tissue resistant. As type 2 diabetes progresses, beta cell failure may develop, leading to a relative insulinapenia requiring, in some cases, insulin administration. The assessment of plasma insulin levels is an important parameter in the diagnosis of hypoglycaemia. Insulin concentration can be determined either in the fasting state or during dynamic test such as the oral glucose tolerance test or the inhibition test, which involves somatostatin infusion in a fasted state (Sherwin et al., 1977).

1.1.5.3 Cortisol

Cortisol, also referred to as hydrocortisone or compound F, is the main human glucocorticoid synthesized by the adrenal cortex and it was discovered by Kendall and Reichstein in the late 1940s as the ‘active principle of adrenal glands’ (Hench et al., 1949). Cortisol is a pleiotropic hormone, and it is predominantly associated with its regulatory function in immunity and cell metabolism. It is a by-product of cholesterol metabolism and it also serves as a precursor for a variety of other steroid based hormones including mineralocorticoids, oestrogens, androgens and progestins (Lisurek and Bernhardt, 2004).

Cortisol homeostasis is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Figure 17) (Chrousos, 1995). The anterior pituitary gland secretes adrenocorticotropic hormone (ACTH) which in turn stimulates the secretion of cortisol from the adrenal cortex. Further, various peptides such as e.g. arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH) can influence the production of cortisol. The free form of cortisol mediates most of the physiological effects, but there is some evidence that the corticosteroid-binding globulin (CBG/transcortin) of which around 90 % of cortisol is bound to, has a function in mediating cortisol's action as well (Breuner and Orchinik, 2002, Nakhla et al., 1988, Strel'chyonok and Avvakumov, 1991). Increased levels of cortisol inhibit the synthesis of CRH via a negative feedback loop (Kirschbaum and Hellhammer, 1994).

Cortisol is secreted in a rhythmic fashion and has a diurnal pattern, with highest plasma levels minutes after waking in the morning and lowest plasma levels in the evening (Young et al., 2004, Goodyer et al., 1996). Glucocorticoid hormones are involved in a variety of physiological processes. As the name implies, they regulate glucose metabolism via a number of mechanisms including induction of gluconeogenesis, glycogenolysis, and insulin resistance (Lecavalier et al., 1990, Reinehr and Andler, 2004, Rizza et al., 1982).

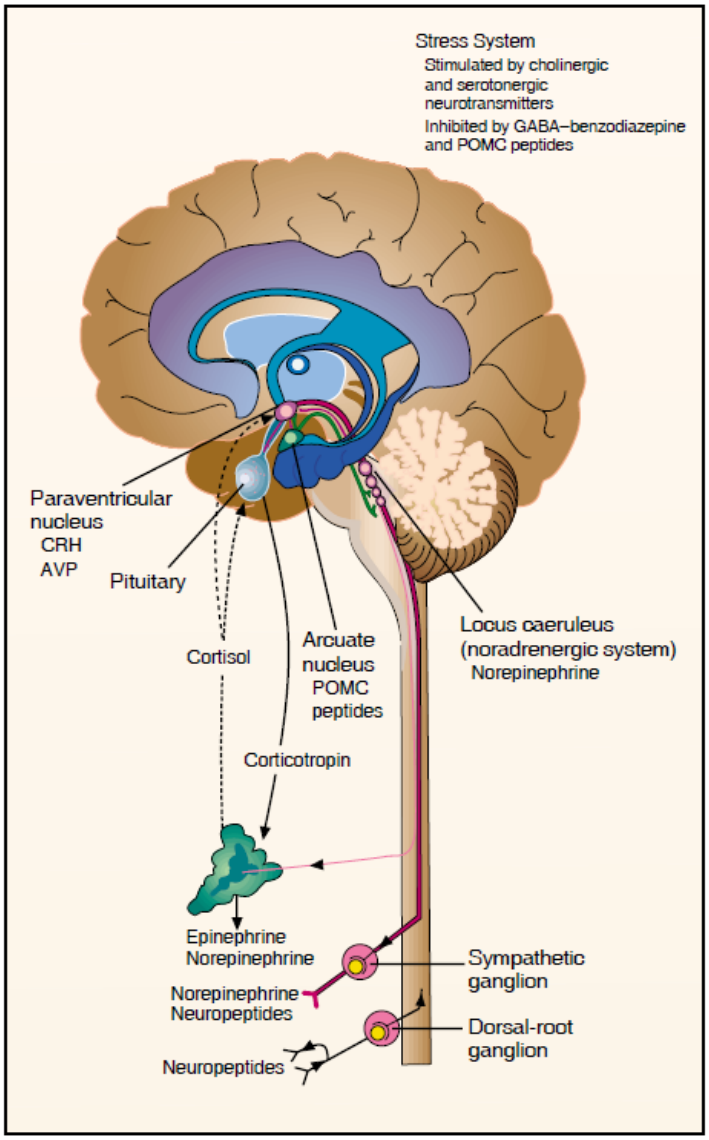
Glucocorticoids also have a role in immunity and can act both to prevent or suppress inflammation (Boumpas et al., 1993). Amongst other pleiotropic functions glucocorticoids have an effect on cardiovascular system, cognitive processing, emotional health, memory, and growth (Goodyer et al., 1996, Buchanan and Lovallo, 2001, McEwen, 1999, Whitworth et al., 2000). Supra-physiological levels of cortisol, as found in Cushing's disease, are associated with a plethora of metabolic events including high blood pressure, obesity, muscle atrophy, and insulin resistance (Arnaldi et al., 2003). Extensive studies confirmed that there is an association between android central deposition of fat and increased circulating serum and urinary free cortisol (Bjorntorp, 2000). This increased level of cortisol is thought to stimulate hepatic gluconeogenesis and therefore, an imbalance in glucose homeostasis.

It is also common to find that individuals with android obesity (preferential visceral fat deposition) have high blood glucose concentration and impaired glucose metabolism (Levitt et al., 2000). Adrenal insufficiency including Addison's disease (the adrenal glands do not produce enough steroid hormones i.e. cortisol) is characterized with low blood glucose levels (hypoglycaemia). It is plausible to postulate that the hypoglycaemia is due to insufficient gluconeogenesis, glycogenolysis or both. Furthermore, it has been demonstrated that exogenous administration of cortisol increases glucose production via the process of gluconeogenesis (Khani and Tayek, 2001). A recent study demonstrated that android obesity with high cortisol levels was most closely correlated with high total and added sugar consumption in the youth (Gyllenhammer et al., 2013).

The has been an extensive body of literature published with regard to the process of inflammation in the past decade, resulting in a discovery of endogenous pro- and anti-inflammatory pathways (Nathan, 2002). Scientists have pioneered a hypothesis regarding the process of inflammatory response where under ideal conditions, a normal distribution curve is observed i.e. an inflammatory stage is followed by a pro-resolution phase with attenuation of the response (Perretti, 1997, Serhan and Savill, 2005, Serhan et al., 2007). This process is achieved through a balanced and active action of inhibitory molecules and processes (Serhan et al., 2007). Figure 18 represents the necessary balance between pro-inflammation and anti-inflammation needed to bring about immune system homeostasis in a tissue that is inflamed.

Glucocorticoids are now commonly employed in the treatment of inflammatory diseases. Glucocorticoids are the first class of endogenous anti-inflammatory mediators that have been successfully used for therapeutic purposes (Perretti and D'Acquisto, 2009). The inflammation process is characterized by an initial rise of the levels of cortisol to dampen local and systemic inflammatory pathways, thereby promoting adequate resolution of the inflammatory response (Hardy et al., 2012). These axiomatic pathophysiological actions of glucocorticoids are mediated via complex molecular mechanisms. For example, melanocortin receptor 2 (MC_2), also sometimes referred to as the adrenocorticotropic hormone receptor gene (ACTHR),

Figure 17: Schematic representation of the central and peripheral stress systems.



Source: (Chrousos, 1995).The central and peripheral stress system components are shown which comprise of the paraventricular nucleus and the locus caeruleus together with the HPA axis, and the adrenomedullary and systemic sympathetic systems. Corticotropin-releasing hormone (CRH) and the noradrenergic neurons play a role in innervation and activation whilst arginine vasopressin (AVP) secreted from the paraventricular nucleus works synergistically with CRH to stimulate the release of corticotropin (also known as adrenocorticotropin hormone, ACTH). Corticotropin has a stimulatory action on the adrenal cortex where it induces cortisol production. Cortisol down regulates the release of CRH, AVP and corticotropin.

encodes a receptor for the ACTH hormone, which mediates the production and secretion of glucocorticoids in the adrenal cortex, in particular in the zona fasiculata (Mountjoy et al., 1992, Cone and Mountjoy, 1993). MC_2 belongs to a family of

melanocortin receptors (MCs), which are a subfamily of seven-transmembrane (TM)-domain G-protein-coupled receptors (GPCRs) that are involved in signaling of hormones of POMC origin such as ACTH (Mountjoy et al. 1992, Cone et al. 1993). To date, five MCs have been described, and they have been shown to have a diverse range of functions. MC_1 controls skin pigmentation (Valverde et al., 1995, Corso et al., 2012), MC_3 and MC_4 are major contributors to the regulation of food intake and energy homeostasis (Marsh et al., 1999, Marks et al., 2006, Barb et al., 2010) and MC_5 is highly expressed during embryogenesis but in adults is believed to be involved in exocrine function(Chagnon et al., 1997, Ogawa et al., 2004).

Melanocortin-3 receptor (MC_3) gene is involved in the modulation of energy homeostasis, however how mutations within this gene may affect its function is still not clearly understood. In vivo animal research suggests that both MC_4 and the MC_3 genes are key receptors for melanocortin proteins that are employed in the leptin-melanocortin signaling pathway (Novoselova et al., 2013). However, as certain gene mutations have been identified in people with low body fat levels and are not linked with the dysfunction of these receptors, results remain inconclusive (Shukla et al., 2012, Zegers et al., 2013).

MC_4s are found predominantly in PVN of the hypothalamus and nucleus accumbens. Mutations and/or dysfunction of MC_4 is closely linked with obesity in children (Radosavljevic et al., 2011). Furthermore it has been shown that polymorphism near MC_4 gene is also associated with hyperphagia and more frequent snacking habits (Stutzmann et al., 2009). A study demonstrated that children without MC_4 variant do not achieve the same benefits from lifestyle intervention when compared to normal children confirming that MC_4 has a profound effect on satiety and eating behavior (Zlatohlavek et al., 2013). Further research is needed to unfold the effects of anti-inflammatory mediators on immune cells to elucidate the molecular target (or targets) of a given mediator, thereby leading to advanced drug discovery for the treatment of chronic inflammatory conditions. One such mediator involved in glucocorticoids's function is the anti-inflammatory protein, annexin A1 (AnxA1) (Hutchinson et al., 2011).

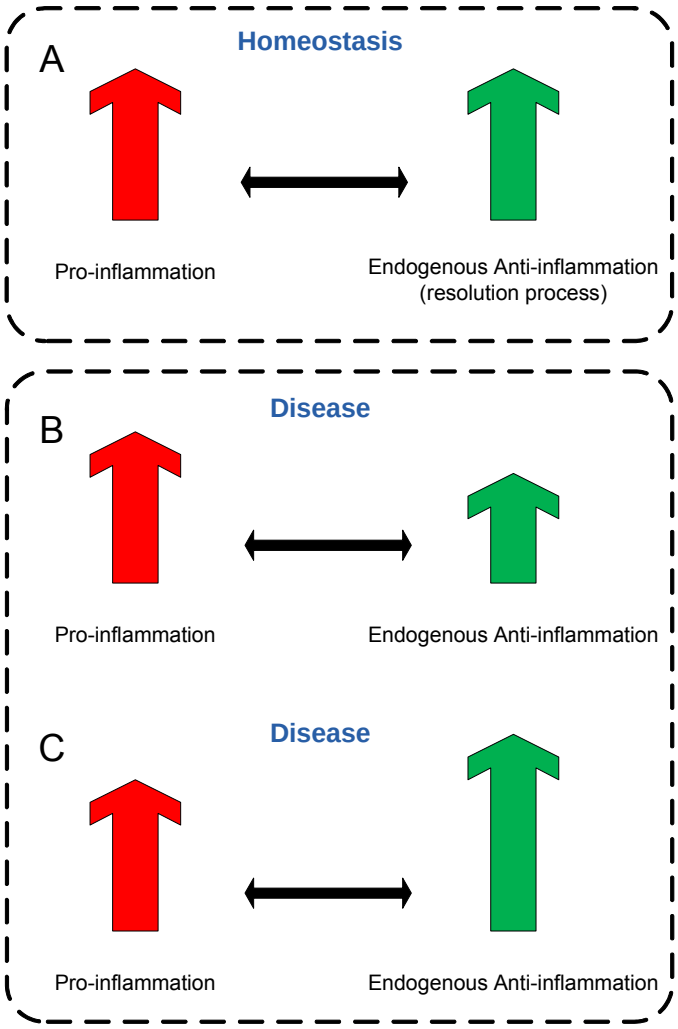
1.1.5.4 AnxA1 as a mediator of resolution

The key biochemical function of annexins is the Ca^{2+} dependent binding or 'annexing' to phospholipid membranes. The main characteristic of this protein is its core domain with four 70 amino acid motifs. The unique roles of annexins depend on the N terminus (comprising of 49 residues), which is thought to be the regulatory region(Lim and Pervaiz, 2007).

AnxA1, discovered in 1970's, a 37-kDa and Ca2+ - and phospholipid-binding protein, previously named macrocortin, renocortin, liponodulin and lipocortin-1, is an endogenous glucocorticoid regulated protein (Goulding et al., 1990, Renshaw et al., 2010). The human recombinant AnxA1 and its biologically active N-terminal peptides modulate a number of systemic anti-inflammatory processes (D'Acquisto et al., 2008b, Pupjalis et al., 2011, Dalli et al., 2011) including reducing eicosanoid synthesis (see Figure 19: Schematic illustration of the arachidonic acid pathway. (Sudlow et al., 1996), antipyretic effects (Davidson et al., 1991), antiendotoxic action (Permana et al., 2006) and reduced neutrophil emigration from the systemic circulation mimicking some of the effects of glucocorticoids . Eicosainoid production via the actions of glucocorticoids is linked with two mechanisms. Acutely, glucocorticoids prevent the phosphorylation/activation of cytosolic phospholipase A2 (cPLA2) through an AnxA1 dependant mechanism (Croxtall et al., 1996) whilst more chronic exposure down-regulates Cox-2 mRNA through an AnxA1 independent mechanism as shown in Figure 19 (Masferrer et al., 1992). AnxA1 reduces cPLA2 and limits COX-2 abundance (D'Acquisto et al., 2008b).
Expression of AnxA1 can be induced by glucocorticoids in various cell types and AnxA1 knock-out mice do not respond to glucocorticoids suggesting that AnxA1 plays a key role as an endogenous homeostatic anti-inflammatory protein that is recruited to aid in the resolution of inflammation (Vago et al., 2012). Studies suggest that AnxA1 is involved in the apoptotic cell 'eat me' signal initiating phagocytosis (McArthur et al., 2010). This action is exerted via an endogenous 'eat me' signal induced by phosphatidyl-serine (PS) molecule that is found on the outer leaflet of

plasma membranes of apoptotic cells. AnxA1 may act as a ligand for PS and therefore, mediate the engulfment of apoptotic cells.

Figure 18: Schematic illustration of the required balance between pro- and anti-inflammatory mediators and pathways.

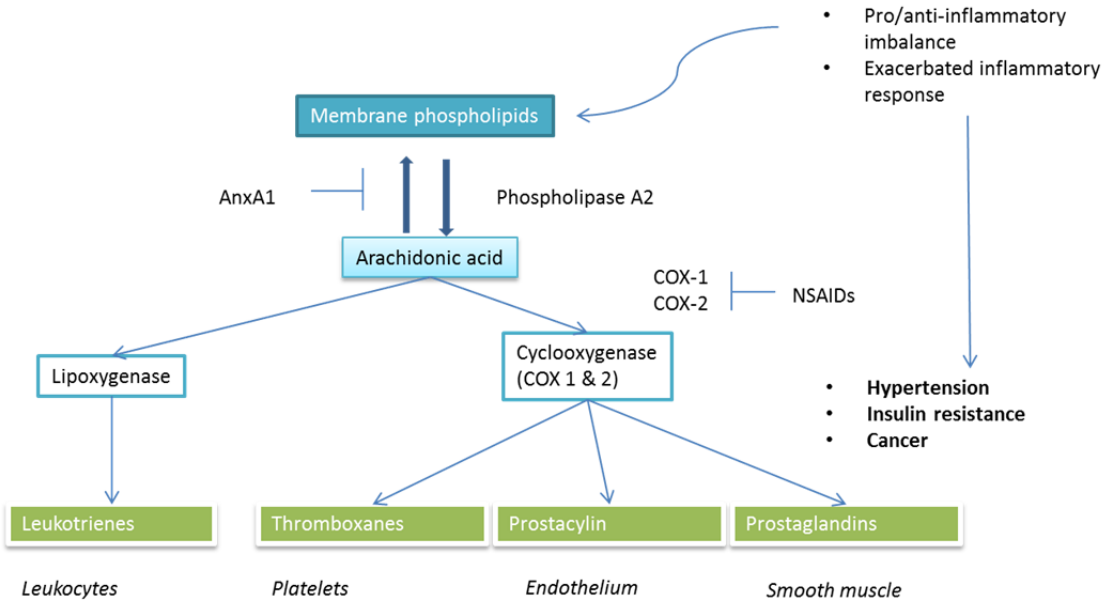


Source: This model was proposed by (Serhan et al., 2007) and was based on several studies. A: Homeostasis between pro-inflammatory molecules and mediators of anti-inflammation is necessary for efficient progression of inflammatory and resolution pathways. This is to ensure killing/removal of the inflammogen takes place during inflammation whilst renewal of normal cell physiology is rebuilt. (B and C) An imbalance on either side of the inflammatory response may lead to disease, i.e. pro-longed pro-inflammation without functioning resolution. On the contrary, lack of pro-inflammatory response where anti-inflammation surpasses may cause immunosuppression and inability to combat infections as identified in chronic inflammatory diseases (arthritis, vasculitis, and atherosclerosis).

The anti-inflammatory protein is highly expressed in cells of the haematopoietic lineage, in particular the polymorphonuclear leukocytes (PMN). The localization of

AnxA1 within the cell varies according to the cell type e.g. in neutrophils AnxA1 is predominantly found in gelatinase granules (Perretti et al., 2000) whilst within the

Figure 19: Schematic illustration of the arachidonic acid pathway.



Source: (Adapted from various literature). AnxA1 exerts its anti-inflammatory action via inhibition of phospholipase A2 along the arachidonic acid pathway and therefore reduces the expression of leukotrienes, thromboxanes, prostacyclins and prostaglandins. An imbalance in the pro/anti-inflammatory response may result in an exacerbated chronic inflammation that is linked hypertension, insulin resistance, and cancer.

macrophage it is mainly located within the cytoplasm (Peers et al., 1993, Bianchi et al., 2003).

Secretion mechanisms of AnxA1 from the cells are not entirely clear, however some evidence suggest that the protein is extruded from the granules on activation leading to abundant levels of AnxA1 on the cell surface (Vong et al., 2007, Perretti et al., 2000). On release from the cell, AnxA1 binds to its receptor regulating the effects of exogenous AnxA1 (Lim and Pervaiz, 2007, Perretti et al., 1996). The two receptors that have been identified to be employed in AnxA1's regulation are formyl peptide receptor 1 (FPR1) and FPR 2 whose acronym is FPR2/ALX since it also conveys the inhibitory signals induced by lipoxin A4 and resolvin D1 (Serhan and Savill, 2005). Both receptors bind to the N terminus of AnxA1 modulating the actions of exogenous AnxA1.

Growing evidence supports AnxA1's role in the resolution of inflammation (Dalli et al., 2013). AnxA1 acts in the resolution of inflammation via macrophage phagocytic clearance of apoptotic neutrophils (Maderna et al., 2005, Dalli et al., 2011, Perretti and Dalli, 2009). It is a process that prevents the exposure of tissues at the inflammatory site to the noxious and potentially immunogenic contents of lytic cells (Babbin et al., 2008, Dalli et al., 2011, Perretti and D'Acquisto, 2009). AnxA1 may also modulate inflammation by interacting directly with NF-κB in an intra-cellular manner (Zhang et al., 2010c, Wang et al., 2011). Hypoxia in the adipose tissue of the obese induces insulin resistance through activation of IκB kinase (IκK)/NF-κB signalling pathway leading to inhibition of perixisome proliferator-activated receptor (PPAR) γ and subsequent transcription of pro-inflammatory markers. The anti-inflammatory effects of AnxA1 may be mediated via down-regulation of NF-κB and up-regulation of PPARγ leading to pro-resolution and decreased levels of pro-inflammatory markers. A number of studies have suggested that AnxA1 deficiency may contribute to the aetiology of inflammatory diseases (Bensalem et al., 2005, Dalli et al., 2010).

AnxA1 has a wide range of functions, including the suppression of inflammation and modulation of the release and signalling of insulin (Perretti and Gavins, 2003). AnxA1 is also regulated by pro inflammatory cytokines, such as IL-6, (Solito et al., 1998) and lipopolysaccharide (LPS), suggesting that AnxA1 may be a key homeostatic mediator, involved in balancing pro inflammation with pro resolution. AnxA1 is expressed in murine and bovine adipocytes (Raynal et al., 1993, Zhao et al., 2010); however, its role in adipogenesis and lipid metabolism in humans warrants further investigation.

A study by Warne *et al*., 2006 demonstrated that AnxA1 gene deletion reduced epididymal fat mass in ANXA1-null mice suggesting that AnxA1 is necessary for the maintenance of adipocyte numbers. The expression of AnxA1 with increased adiposity has not been investigated to date. However, given the balance that exists between pro and anti-inflammatory molecules, it seems plausible that AnxA1 may be altered in individuals with increasing adiposity.

1.1.5.5 Other anti-inflammatory mediators

Galectins belong to the lectins family. There are around 15 different types of lectins. Galectins are predominantly intracellular molecules but can also be found on the surface of cells and extracellularly. They involved in various cellular processes including fat formation and atherosclerosis, cell development and death (Liu and Rabinovich, 2010). Most of the galectins have anti-inflammatory properties with the exception of galectin-3, which may have also pro-inflammatory function depending on its target cells. Studies based on models of inflammatory and autoimmune conditions suggest that galectin-3 is required for a normal functioning of the immune system (Jiang et al., 2009, Volarevic et al., 2012) and may be implicated in malignancy (Radosavljevic et al., 2011).

Since this PhD thesis has been carried out, a number of other mediators have been discovered that may provide a molecular link between chronic systemic inflammation and the aetiology of metabolic syndrome diseases. These, for example include otopetrin 1 (Otop1), an anti-inflammatory mediator that is over-expressed in WAT of obese mice and is regulated by TNFα (Wang et al., 2013). Tumor necrosis factor weak inducer of apoptosis (TWEAK), a cytokine of the tumor necrosis (TNF) superfamily is another potential player in the regulation of chronic inflammation found in obesity. As well as a protective role in inflammation (anti-inflammatory) TWEAK can control many cellular activities including cell growth and cell death and has been linked with cardiovascular disease (Maymo-Masip et al., 2013, Vendrell and Chacon, 2013).

1.1.5.6 Anti-inflammation and calorie restriction mimetics

Whilst the obesity epidemic is on the rise, simultaneously research into the effects of calorie restriction and calorie restriction mimetics is also abundant. Considerable mortality risk can be altered via dietary manipulation, and calorie restriction has been shown to not only lead to weight loss but also improve insulin sensitivity, glucose metabolism, and blood lipid profile. Whilst long term-weight loss significantly improves disease outcomes, calorie restriction diets have been implicated in longevity and prolonging lifespan (Zuin et al., 2010, Lin et al., 2002). In

the light of the current obesity pandemic, it was of interest to investigate a well-established calorie restriction mimetic, resveratrol and its potential effects on improving the metabolic syndrome associated biomarkers in humans.

1.1.5.7 Resveratrol

Resveratrol (3,5,4'-trihydroxystilbene) was first identified from the roots of the white hellebore (*Veratrum grandiflorum*) in the year 1940 (Takaoka, 1940). Later in 1963, resveratrol was also isolated from the roots of a Japanese knotweed, *Polygonum cuspidatum*, a plant used in the traditional medicine (Nonomura et al., 1963) and nowadays supplemental resveratrol is commonly isolated from the *Polygonum cuspidatum* plant. Resveratrol is also found in the skin and seeds of grapes, and therefore, red wine. Red wine and grape juice consumption is significantly inversely correlated with the incidence of coronary heart disease in the French population, and now known as the 'French paradox' (Richard, 1987, St Leger et al., 1979, Renaud and de Lorgeril, 1992). A study on the metabolic effects of resveratrol showed its function as an anti-inflammatory agent inhibiting the arachidonic acid pathway via the 5-lipoxygenase and cyclooxygenase (COX) route in leukocytes (Kimura et al., 1985). However, it was not until 1992 that resveratrol became recognizable exogenous agent due to its implication in the cardioprotective effects (Siemann, 1992). Research during the last two decades presented numerous evidence that resveratrol can prevent or delay the progression of multiple disease states, including cancers, cardiovascular diseases, and ischeamic injuries as well as improve stress resistance and mortality risk (Jang et al., 1997, Bradamante et al., 2004, Wang et al., 2002, Sinha et al., 2002). *In vitro* investigations into the beneficial effects of resveratrol showed universally successful results on multiple direct systems. The exact mechanism of resveratrol function is not fully elucidated to date, however a number of its molecular targets have been identified (Figure 20). COX is a key enzyme in the arachidonic acid - prostaglandin (PG) metabolic pathway. It has two isoforms, COX-1 and COX-2. Both isoforms play a critical role in LPS induced inflammation (Smith, 2008, Simmons et al., 2004). *In vivo* models of cancer demonstrated anti-inflammatory and cancer-protective roles of resveratrol via modulation of PG production down the COX-1 pathway (Jang et al., 1997, Szewczuk

et al., 2004). Resveratrol was shown to act via the inhibition of COX-1 and/or COX-2 enzymes, and the subsequent decrease in PG synthesis (Szewczuk et al., 2004, Subbaramaiah et al., 1998).

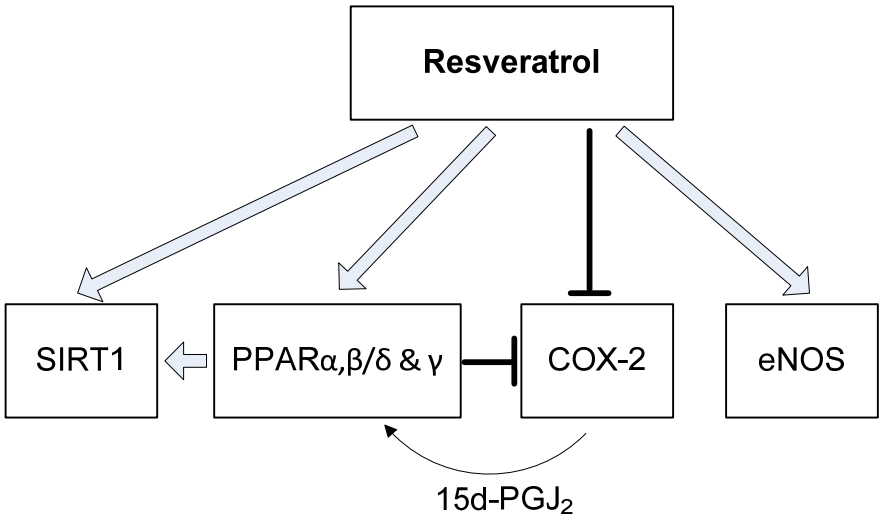
PPARs (α, β, δ and γ) are the nuclear receptor family of ligand-dependent transcription factors involved in a range of metabolic processes including fat and glucose metabolism, cellular development and inflammation (Mangelsdorf et al., 1995, Michalik et al., 2006, Sonoda et al., 2008). It has been postulated and demonstrated that resveratrol also activates all of the PPAR isoforms in endothelial cells (Inoue et al., 2003, Tsukamoto et al., 2010).

Resveratrol also was shown to induce nitric oxide (NO) levels and enhance NO bioavailability *in vivo* (Hattori et al., 2002, Zou et al., 2003, Miatello et al., 2005, Rush et al., 2007, Zhang et al., 2010a). NO has a key function in modulating cardiovascular homeostasis and is produced by endothelial NO synthase (eNOS) (Dudzinski et al., 2006, Dudzinski and Michel, 2007). Dysfunction of NO is implicated in atherosclerosis as normally it relaxes vascular smooth muscle cells, thereby controlling the blood flow, and it has thrombogenic and atherogenic protective properties through its involvement in vasodilatation.

Studies have shown that resveratrol exerts similar lifespan prolonging effects to caloric restriction in lower organisms via activation of sirtuin proteins (Howitz et al., 2003, Wood et al., 2004). To date seven human sirtuins, SIRT1-SIRT7, have been discovered (Frye, 2000). The proteins are distributed in the nucleus, cytoplasm and the mitochondria (Tanno et al., 2007, North and Verdin, 2007, Michishita et al., 2005). SIRT1 protein has been most widely investigated. Sirtuin proteins have a function in inflammation, differentiation, DNA silencing, DNA repair, mitochondrial metabolism, stress resistance and longevity (Horio et al., 2011). Multiple mechanisms have been suggested to be involved in the life-prolonging actions of sirtuins. SIRT1 protein inhibits the transcriptional function of p53 (the tumour suppressor molecule involved in cell death) and therefore, apoptosis (Luo et al., 2001, Vaziri et al., 2001). SIRT7 knock-out cells result in an increased rate of

apoptosis suggesting that the protein deacetylates and inhibits p53 (Vakhrusheva et al., 2008). Sirtuin proteins are also involved in suppression or inactivation of other molecular targets known to cause apoptosis including poly (ADP-ribose) polymerase-1 (PARP)(Rajamohan et al., 2009), heat-shock factor (HSF)-1(Westerheide et al., 2009) and forkhead box O (FOXO)(van der Horst et al., 2004, Kobayashi et al., 2005).

Figure 20: Possible mechanisms of resveratrol's action.



Source: (Adapted from Nakata et al., 2012). The possible molecular targets through which resveratrol exerts its beneficial effects are: induction of eNOS, activation of PPAR α, β, δ and γ, and activation of sirtuin proteins. It has been shown that resveratrol blocks COX-2. The PGD2 metabolite 15-deoxy-Δ12,14 PGJ2 (15d-PGJ2) is a potent natural ligand of PPAR γ.

Moreover, sirtuins have a key function in inhibition of inflammation. SIRT1 deactivates NF-κB, the nuclear factor that modulates inflammation. SIRT1 knock-out mice develop an autoimmune-like disease. SIRT1 also inhibits the activator protein (AP)-1 and reduces COX-2 gene expression in macrophages (Zhang et al., 2010b). Inactivity of SIRT1 in macrophages triggers inflammatory processes stimulated by LPS. Resveratrol is known to activate SIRT1 and inhibit the NF-κB activity induced by LPS (Yoshizaki et al., 2010).

Extensive evidence shows that resveratrol has multiple molecular targets and it mimics the changes to gene expression found in calorie restricted mice (Pearson et al., 2008). In higher organisms, calorie restriction and resveratrol bring about

protective results against a similar type of illnesses (Baur and Sinclair, 2006). It is of interest, therefore to investigate further if resveratrol can improve the metabolic profile of humans.

1.2 Aims of this PhD research

Study 1

The *in vivo* section of this research was set out to conduct a characterization study of adipokines (adiponectin and leptin) and inflammatory markers (CRP, TNF-α, IL-6) secreted into the systemic inflammation; and to investigate the association between these metabolic mediators according to adiposity level, BMI and the distribution of subcutaneous fat (gynoid versus android). An anti-inflammatory plasma protein AnxA1 was also determined and correlated with the metabolic and anthropometric parameters.

Study 2

Alongside, the *in vivo* research study, *in vitro* work was designed to complement this project on relative tissue hypoxia and its effect on inflammatory markers and adipokines. Benefits of *in vitro* work include e.g. examination of cell type specific responses to a range of treatment in isolation where these types of studies cannot be carried out *in vivo* even with an intervention study. The expression of ANXA1 gene in preadipocytes and adipocytes was measured and the role of AnxA1 in adipogenesis was investigated. Alongside adipokines (adiponectin and leptin), the inflammatory markers (CRP, TNFα, IL-10 and IL-6) were also assessed.

Study 3

The third part of the project was a double-blind placebo controlled cross-over study to investigate the impact of oral supplementation with resveratrol, a bioactive anti-inflammatory/oxidant compound found in red wine and the skins and seeds of grapes, on metabolic parameters including fasting blood glucose and cholesterol; blood pressure and inflammatory status. The study was carried out in 15 overweight or obese males.

2 Materials and methods

Study 1: Attenuation of plasma AnxA1 in human obesity

2.1 Research design and methods

2.1.2 Participants and experimental protocol

Informed consent was obtained from 118 healthy male subjects recruited through the advertisement in a local newspaper (i.e. Metro, The Evening Standard). Participants were asked to attend a health screening at the Cavendish Campus of University of Westminster. Participants were given a participants' information sheet and a written informed consent was obtained from all subjects. Participants filled in a medical questionnaire. The exclusion criteria included the use of over the counter non-steroidal anti-inflammatory drugs (NSAIDS), taking prescription medicine or being diagnosed with any disease. Only healthy volunteers were recruited for the study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the University of Westminster (**08/09/22)**.

2.1.3 Anthropometric measurements

2.1.3.1 Height and weight

Weight, height, and anthropometric measurements were taken whilst participants were wearing minimal clothing. BMI was derived from weight (kg) divided by the square of height (m^2). Standing height of the subjects was measured using a stadiometer (Holtain Ltd., Crymych, Dyfed, UK) fixed to the wall and recorded to the nearest 0.1 cm. Body weight measurements were taken as part of the BODPOD procedure, with the integrated scales being calibrated on a daily basis. Normal BMI 18.5 - 25 kg/m^2, Overweight 25.1 - 30 kg/m^2, Obese I 30.1 - 35 kg/m^2, Obese II > 35.1 kg/m^2 were classified according to the World Health Organization (WHO) 1998 clinical guidelines (Bethesda, 1998). Whilst BMI is a proxy for adiposity across populations, some researchers regard BMI a relatively crude index as it does not account for body composition (Garn et al., 1986, Nevill et al., 2006). Indeed, various

studies identified that BMI on its own misdiagnosed healthy adults as overweight and obese after verification of their fat mass (Hortobagyi et al., 1994). For instance, a slender-framed female with significant excess fat may appear as a false negative, and a muscular male as a false positive. Waist was determined by taking a measurement half way between the iliac crest and the lower rib. Hips were measured at the widest part including hip bones and widest part of buttocks to calculate waist to hip ratio (W: H) to the nearest 0.01 cm. A W:H ratio > 0.95 was considered as high (WHO, 2004).

2.1.3.2 Bioelectrical impedance analysis (BIA)

Body fat was measured with BIA, using Tanita Body Composition Analyzer, TBF-300 (Tokyo, Japan). Measurements were obtained whilst the subject was standing with bare feet on the scales. Height, age, and gender were input into the computer of the Tanita scale before obtaining a reading. The technique uses a high-frequency, low-amplitude alternating electric current (50 kHz at 500 to 800 µA) which is sent into the body to measure the voltage drop caused by resistance. The amount of resistance measured (R) is inversely proportional to the volume of electrolytic fluid in bodily tissues. The result is also dependent on the body stature (i.e. ratio of length [L] to cross-sectional area [A]). The following equation is used to calculate the conductive volume $V = \rho L^2/R$ where ρ is an estimate of the specific resistivity of the conductive material (Baumgartner R., 1996). This formula can simply be applied to cylindric conductors with uniform cross-sectional area and homogenous composition (e.g. a wire). As the human body resembles more a series of roughly cylindric conductors with highly structured composition, more evolved equations have been derived to account for these additional parameters.

2.1.3.3 Skinfold analysis

Skinfold analysis was performed by lifting a fold of skin and subcutaneous fat away from the underlying muscle and bone and the skinfolds were recorded using a skinfold caliper (Harpenden, Burgess Hill, UK) at four sites: tricep, bicep, subscapular and supraspinale as shown in Figure 21, Figure 22, Figure 23, and Figure 24. Skinfolds were measured three times at each site to the nearest 0.1 mm. The tester picked up the skinfold at the four sites making sure that only a layer of skin and the underlying

fat tissue is pinched, but not the muscle tissue. The calipers were then used to measure the fold, 1 cm below and at right angles to the pinch, and a reading in millimetres (mm) taken two seconds later. If values did not agree within 0.2 mm, additional determinations were made, and a mean of the two closest values was calculated. All skinfold measurements were taken on the right side of the body by the author of this PhD (trained Level One Anthropometrist, International Society for the Advancement of Kinanthropometry) for consistency.

Various investigators have determined equations for calculating the total body fat level using 4, 3, or 7-point skinfold measures. The most employed equation is that used by Durnin and Womersley, 1974 that includes 4 types of skinfolds. The sum of bicep, tricep, subscupular and supraspinale skinfolds is fed into body density equation:

$$Body\ density = C - (M) \times \text{LOG10 of all 4 skinfolds}$$

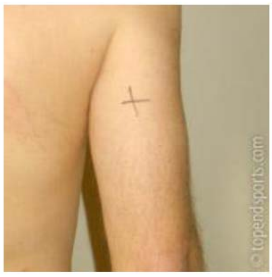
with C and M being constants according to gender and age (Durnin and Womersley, 1974). The body density value is then used in the Siri equation to estimate percentage body fat, (Siri, 1956):

$$per\ cent\ body\ fat\ = (495\ \div body\ density) \div 450$$

2.1.3.4 Skinfold landmarks and pinch

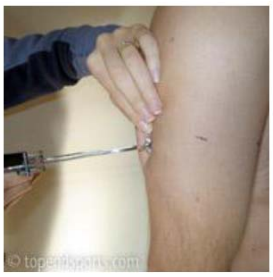
Figure 21: Triceps skinfold landmark and pinch illustration.

Triceps Skinfold



Landmark

- At the level of the mid-point between the acromiale (lateral edge of the acromial process, e.g. bony tip of shoulder) and the radiale (proximal and lateral border of the radius bone, approxiately the elbow joint), on the mid-line of the posterior (back) surface of the arm (over the triceps muscle).



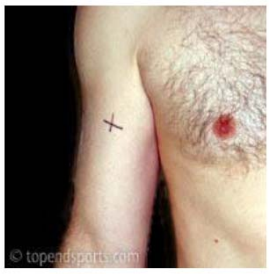
Pinch

- The arm should be relaxed with the palm of the hand facing forwards.
- A vertical pinch, parallel to the long axis of the arm, is made at the landmark.

Source: topendsports.com The figures show skinfold landmark and provide guidelines on accurate measurement of tricep skinfold.

Figure 22: Biceps skinfold landmark and pinch illustration.

Biceps Skinfold



Landmark

- At the level of the mid-point between the acromiale (lateral edge of the acromial process, e.g. bony tip of shoulder) and the radiale (proximal and lateral border of the radius bone, approximately the elbow joint), on the mid-line of the anterior (front) surface of the arm (over the biceps muscle).

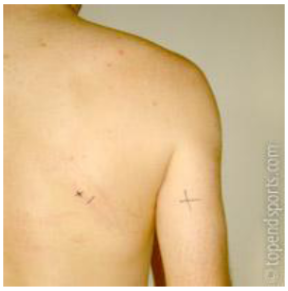
Pinch

- The arm should be relaxed with the palm of the hand facing forwards.
- A vertical pinch, parallel to the long axis of the arm, is made at the landmark.

Source: topendsports.com. The figures show skinfold landmark and provide guidelines on accurate measurement of bicep skinfold.

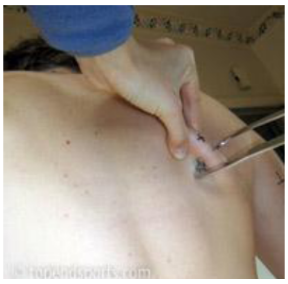
Figure 23: Subscapular skinfold landmark and pinch illustration.

Subscapular Skinfold



Landmark

- The lower angle of the scapula (bottom point of shoulder blade)
- If there is difficulty finding this landmark, get the subject to reach behind their back with their right arm, while feeling for the movement of the scapula



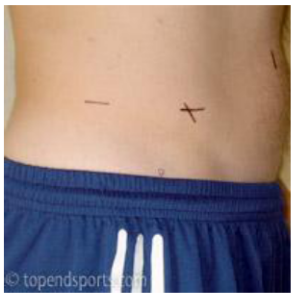
Pinch

- The pinch is made following the natural fold of the skin, approxiately on a line running laterally (away from the body) and downwards (at about 45 degrees).

Source: topendsports.com. The figures show skinfold landmark and provide guidelines on accurate measurement of subscapular skinfold.

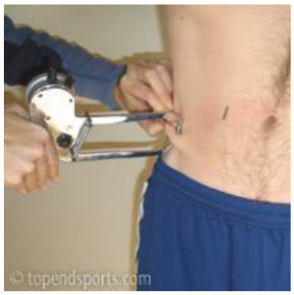
Figure 24: Supraspinale skinfold landmark and pinch illustration.

Supraspinale Skinfold



Landmark

- The intersection of a line joining the spinale (front part of iliac crest) and the anterior (front) part of the axilla (armpit), and a horizontal line at the level of the iliac crest.



Pinch

- The pinch is directed medially (towards the centerline) and downward, following the natural fold of the skin (at an approximate angle of 45 degrees).

Source: topendsports.com. The figures show skinfold landmark and provide guidelines on accurate measurement of supraspinale skinfold.

2.1.3.5 Air displacement plethysmography

Air displacement plethysmography (ADP) was used to assess total body fat level using daily-calibrated BODPOD (Life Measurement, Inc., Concord, California, USA)(McCrory et al., 1998). Daily calibration of the test cylinder volume was carried out before the start of testing following the standard procedure. Manufacturer criteria for a successful calibration was a mean difference (N= 5) of $\pm$ 100 ml in cylinder volume and SD <75 ml (Inc., 2005). The within and between day coefficients of variation (CV) in volume measurements of the cylinder were 0.10 % and 0.13 %, respectively (data not shown). The BODPOD device also was calibrated using a three-step standard procedure every time before a participant entered the BODPOD cylinder (Fields et al., 2002). The within subject CV for ADP % fat based on repeated measures in our laboratory was 3.5 % , which is within the range of within subject CV (2.3 - 4.5 %) (Fields et al., 2002) for % fat. ADP is a safe, non-invasive method used commonly in research and its validity was compared with the dual-energy x-ray absorptiometry (DEXA. The data showed a strong correlation and no statistically significant bias (Wilson et al., 2012) or produced a positive bias of 1.7 % (Wagner et al., 2000). Other studies also confirmed that the BODPOD gives comparable results to the DEXA technique i.e. it overestimated body fat % in women by 0.98 % and in men by 1.46 % (Shafer et al., 2008) or in 10 -18 year old children (Lockner et al., 2000) the BODPOD underestimated total body fat level by 1.9 %.

Subjects' weight was determined to the nearest 0.01 kg on the weighing scale integral to the BODPOD system. Following a standard calibration of the BODPOD, they were asked to enter the chamber for body volume measurement, wearing tight-fitting clothing (i.e. a pair of Speedos® or tight fitting underwear), with a swim cap to minimize the volume trapped in hair. Subjects were asked to remove all jewellery. Three repeated test procedures, each lasting approximately 40 seconds, were conducted. The full test required 3 – 4 minutes. The BODPOD system software uses Arcamedius's principle as in underwater weighing, but instead of using the water displacement it uses air to measure actual body volume, after correction for lung

volume, along with body weight in air to calculate body density (by dividing body mass (kg) by body volume (l)). From body volume the fraction of body fat (% BF) was calculated using the Siri equation based on the assumed densities of 0.9 kg/l for fat and 1.1 kg/l for fat-free mass (Siri, 1956).

2.1.3.6 Estimation of the agreement between methods of estimating body fat level. (BODPOD, skinfold caliper and BIA).

This analysis was set out to determine if total body fat estimation via BODPOD, skinfold caliper and BIA can be used interchangeably.

2.1.3.6.1 Method

All data was tested for normal distribution using Kolmogoroff-Smirnov analysis. Agreement between three measurement techniques for body composition was assessed using bias and 95 % limits of agreement (bias ± 1.96 SD), according to the method of Bland and Altman (Bland and Altman, 2003). Heteroscedasticity was determined by plotting a scatter diagram of the absolute difference against the mean values and computing Pearson's correlation coefficient. Statistical significance was assumed at $p < 0.05$. Data were analysed using Statistical Package for the Social Sciences (SPSS, version 19 for Windows, Chicago, IL, USA).

2.1.3.6.2 Outcome

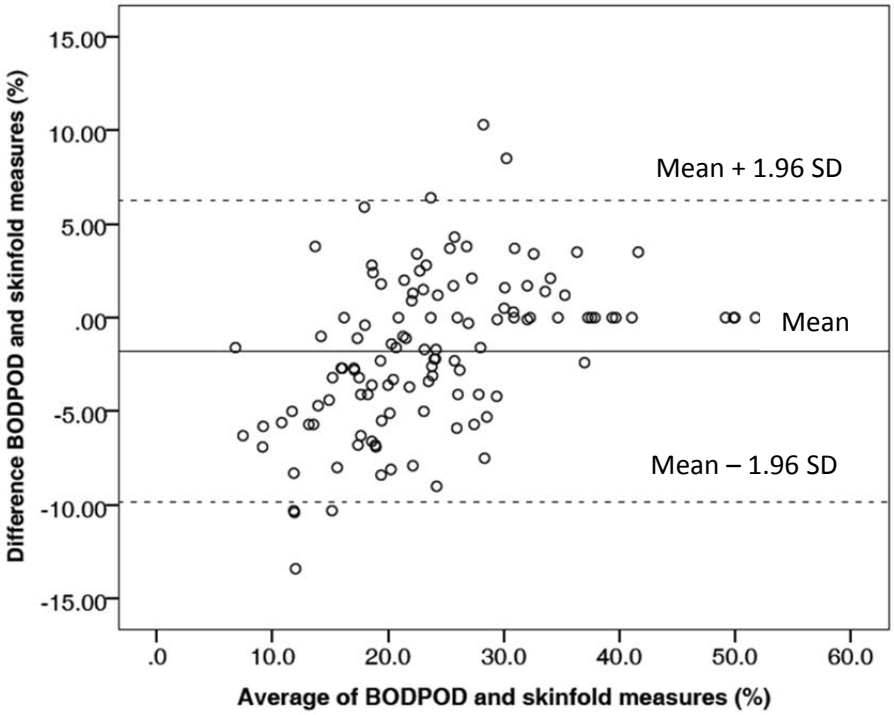
The three techniques of calculating body fat level showed significant variations in the values obtained with BODPOD and skinfold measures showing significantly higher body fat values when compared to BIA measurements ($P < 0.001$). The BODPOD body fat measures were also statistically significantly different from skinfold measures showing significantly lower body fat values when compared to skinfold analysis ($P < 0.001$) (see Table 2).

Table 2: Comparison of percentage body fat measurements.

Subjects	BODPOD	Skinfold measures	BIA
N = 118	23.1 ± 10.2 **# γ**	24.9 ± 8.3 **γ**	20.0 ± 6.6

Mean ± SD, BIA bioelectrical analysis, SD standard deviation, # significantly different from skinfold measures ($p < 0.001$), γ significantly different from BIA ($P < 0.001$).

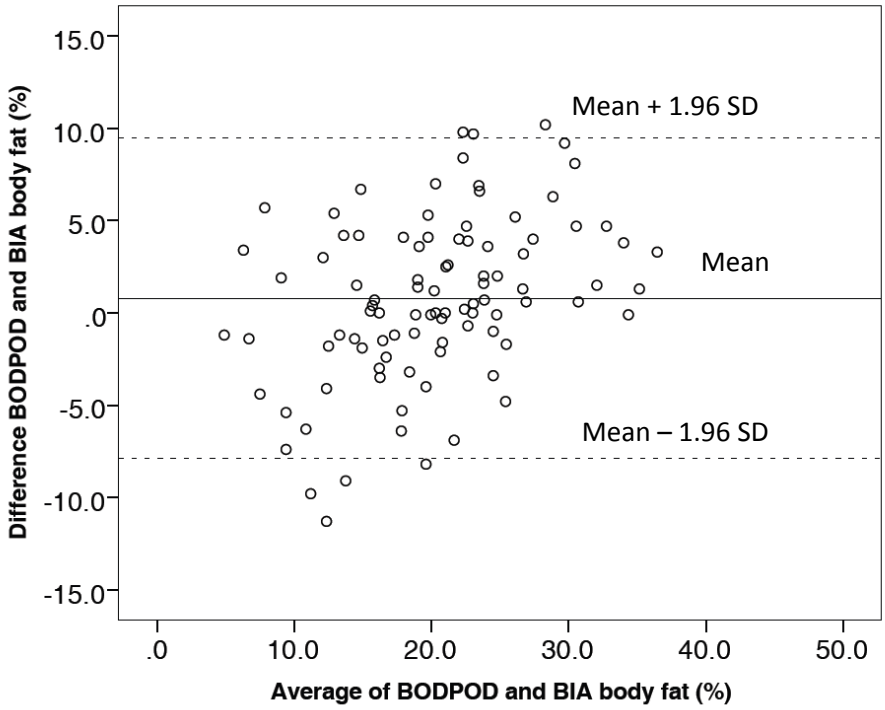
Figure 25: A Bland and Altman plot of difference against mean for the BODPOD body fat and skinfold measures.



Agreement between BODPOD and skinfold measurements, showing bias (-1.8) and limits of agreement (-9.9 %, +6.3 %).

The plot of difference against mean for BODPOD body fat and skinfold measures showed a significant bias (-1.8) and a statistically significant trend ($R = 0.46$, $P < 0.01$) (see Figure 25). The limits of agreement of the difference between the two techniques indicate that 95 % of the results for percentage body fat determined by the skinfold measures were between 9.9 % lower and 6.3 % higher than results from the BODPOD measurement.

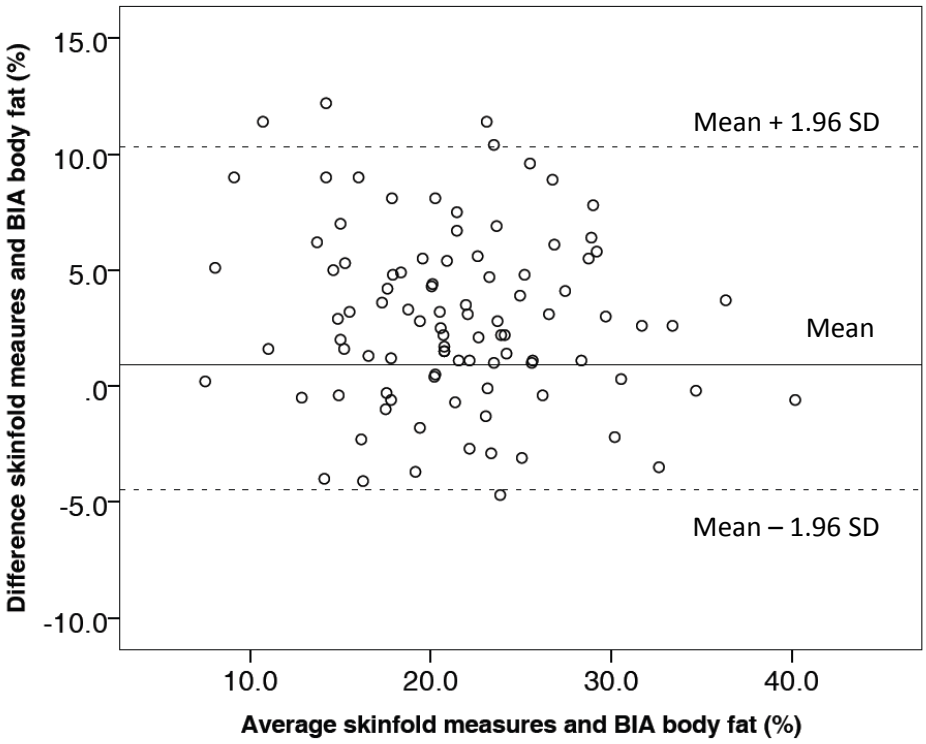
Figure 26: A Bland and Altman plot of difference against mean for the BODPOD and BIA body fat.



Agreement between BODPOD and BIA body fat, showing bias (0.80) and limits of agreement (-7.9 %, +9.5 %).

The plot of difference against mean for BODPOD body fat and BIA showed a significant bias (0.80) and a statistically significant trend ($R = 0.39$, $P < 0.01$) (see Figure 26). The limits of agreement of the difference between the two techniques indicate that 95 % of the results for percentage body fat determined by the skinfold measures were between 7.9 % lower and 9.5 % higher than results from the BODPOD measurement.

Figure 27: A Bland and Altman plot of difference against mean for the skinfold measures and BIA body fat.



Agreement between skinfold measures and BIA body fat, showing bias (2.9) and limits of agreement (-4.5 %, +10.3 %).

The plot of difference against mean for skinfold measures and BIA showed a significant bias (2.9) and a statistically significant negative trend (R = -0.24, P = 0.02) (see Figure 27). The limits of agreement of the difference between the two techniques indicate that 95 % of the results for percentage body fat determined by the skinfold measures were between 4.5 % lower and 10.3 % higher than results from the BIA measurement.

In this study, the precision of the threee techniques was not assessed. However, other studies found body fat assessment via BODPOD to have reliability with values of CV at 1.7 % (McCrory et al., 1995), 3.4 % (Vescovi et al., 2001), and 2.5 % (Miyatake et al., 1999). Comparison of body fat assessment values estimated by means of BIA with results from the BODPOD and skinfold measurement, yeilded significantly lower readings by 3.1 % and 4.9 %, respectively. Similar results were found in an evaluation study of percentage body fat with BIA in collegiate wrestlers,

where the analysis showed an under-estimation of 1.5 % and 1.9 % when compared to BODPOD and skinfold meaurement (Dixon et al., 2005). On the contrary, others (Vasudev et al., 2004) have shown a good agreement between body fat measurements derived from BIA and skinfold thickness in 162 South Indian urban men and women when compared to the gold-standard dual-energy X-ray absorptiometry (DEXA). Similar statistical values were obtained in an assessment of agreement between BODPOD and BIA in healthy subjects over 60 years of age (Aleman-Mateo et al., 2004). A study carried out in 155 college aged Japanese females also investigated the agreement between BIA and skinfold measurement and their analysis arrived at values which were 3.0 % higher than results received by skinfold assessment (Kitano et al., 2001). Most recent study demonstrated that BMI, skinfold and DEXA methods produced comparable strenghts of correlations with metabolic parameteres suggesting that these methods can be used interchangeably (Hariri et al., 2013).

The existent literature, thus presented indicates an inconsistency in the agreement between BIA with BODPOD and skinfold analysis, which may be due to the use of different BIA devices and the inter-individual variability in taking skinfold measurements. Nevertheless, the investigated model of BIA, as used in this study, was statistically significantly differenet from BODPOD ($P < 0.001$) (see Table 2) and therefore, indicating that it does not provide valid data on body fat mass.

The difference between the BODPOD and skinfold measurement was also significantly different ($P < 0.001$) (see Table 2), with the skinfold measures values being consistenly higher than the BODPOD readings. The current study was a cross-sectional study, with individuals' body fat level ranging from ultra lean to risky. Some studies have reported a significant difference between BODPOD and skinfold measures in specific populations. For example, the BODPOD yielded average values that were 2.4 % and 1.9 % higher than results from skinfold measurements in female college athletes and a lean subset (Vescovi et al., 2002). Conversely, a study on collegiate wrestlers (Utter et al., 2003) found BODPOD body fat results to be statistically significantly lower than skinfold measurements, whilst a similar

investigation on a related population showed no difference between the means of BODPOD and skinfold body fat determinations (Dixon et al., 2005). In respect to these findings, there is no general agreement regarding the discrepancies between BODPOD and skinfold total body fat assessment. Skinfold measures are associated with a number of mesurement errors, mainly due to high inter-individual variability between assessors including finding the right site to perform the measurements and taking accurate readings needs a considerable amount of practice and involves some degree of personal judgement. Regrettably, the BODPOD's accuracy could not be validated against DEXA in this project. Others investigated the comparison of BODPOD against DEXA and found that for example in collegiate football players (Collins et al., 1999) and in 10 -18 year old children (Lockner et al., 2000) the BODPOD underestimated total body fat level by 1.9 % and 2.9 % , respectively. Similar findings were derived between the BODPOD and DEXA in white college-aged female athletes and non-athletes (Maddalozzo et al., 2002, Ballard et al., 2004) whilst an overestimation of 1.7 % by the BODPOD was found in a heterogenous sample of black men (Wagner et al., 2000).

Current evidence denotes that the BODPOD's accuracy is less valid in lean individuals as compared to participants with average or high body fatness (Vescovi et al., 2001). However, the BODPOD was shown to be appropriate for assessing body fat level in obese populations with adiposity level up to 40 % (McCrory et al., 1995, Petroni et al., 2003, Demerath et al., 2002) and has a precision of 2 % (Clark et al., 2002). To date accuracy of BODPOD in individuals with extremely high adiposity level (above 40 %) has only been evaluated in one study (Das et al., 2003).

Overall, BODPOD body fat level assessment yields reliable data in studies recruiting subjects within and above the average range of percentage body fat (Ritz et al., 2007), which lies within the inclusion criteria for this study and justifies the choice of BODPOD values for the analysis in the current research programme.

2.1.4 Metabolic measurements

2.1.4.1 Measurement of biomarkers

2.1.4.1.1 Arterial blood pressure

The arterial blood pressure was measured while in a seated position using an automatic manometer (Omron, Mannheim, Germany). Both systolic and diastolic blood pressures were assessed after a 15 minute rest. The blood pressures were taken three times and an average was calculated from the values. Hypertension is described as systolic BP > 130 mmHg and diastolic BP > 80 mmHg (WHO, 2004).

2.1.4.1.2 Blood glucose, total blood cholesterol.

Blood glucose and total cholesterol were measured via a needle prick sample following a minimum 12 hours fast. A HemoCue® Ltd (Derbyshire, UK) blood monitor and Accutrend® Roche (West Sussex, UK) cholesterol monitor were used, respectively. The normal range for the fasting blood glucose and cholesterol are 3.5 – 5.6 mmol/l and < 5.0 mmol/l, respectively (Tirosh et al., 2005, Rossouw et al., 1985).

2.1.4.1.3 Quantification of plasma glycated low density lipoprotein (LDL), plasma annexin A1, plasma insulin and adipocytokines.

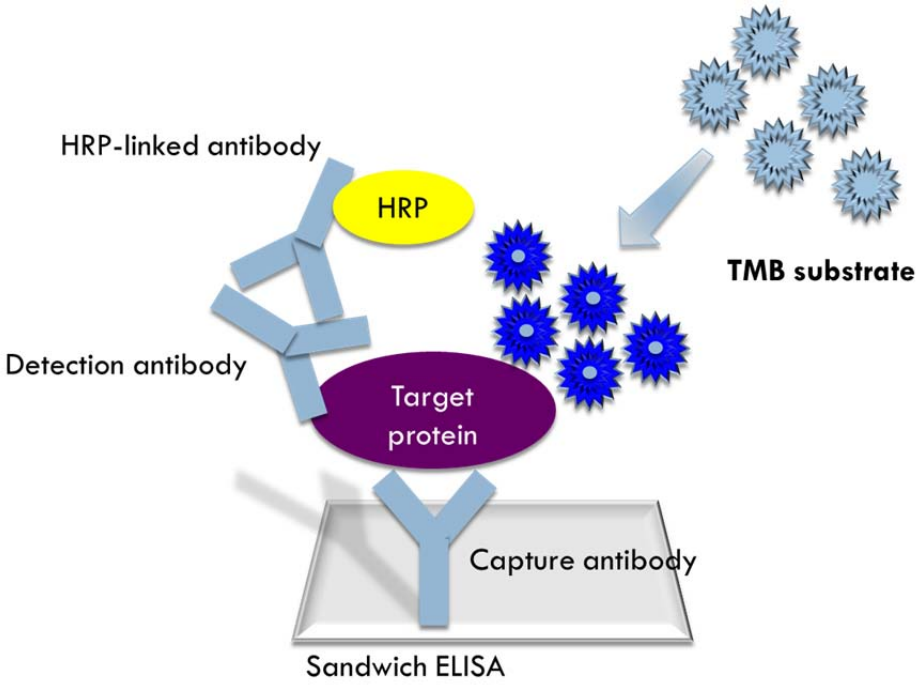
Approximately 20 milliliters of blood was taken by venepuncture from each volunteer following morphometric measurements and blood pressure assessment. All blood samples were placed on ice immediately and centrifuged after 15 minutes, then aliquoted and stored at minus 80 °C. Glycacor Enzyme Linked Immunosorbent Assay (ELISA) (Philadelphia, PA) was used for the quantitation of plasma glycated LDL. Specific ELISAs from R&D Systems (Abingdon, UK) were used to measure fasting plasma adipocytokines. We measured fasting plasma insulin, cortisol, adiponectin, leptin, CRP, IL-6, IL-10 and TNF α. Insulin resistance (IR) was calculated using the homeostatic model of assessment (HOMA)-IR. HOMA-IR indices were calculated as follows: HOMA = fasting insulin (μIU/ml) x fasting glucose (mmol/L)/22.5; HOMA β cell = (20*Fasting insulin (μIU/ml))/(Fasting glucose (mmol/l) – 3.5); and FIRI (fasting insulin resistance index) = (Fasting glucose (mmol/l)*Fasting Insulin (μIU/ml)) /25 (Uwaifo et al., 2002). According to this method, high HOMA-IR indices indicate low insulin sensitivity. Insulin sensitivity was also assessed using the quantitative insulin

sensitivity check index (QUICKI). The QUICKI index is used to analyse the data based on logarithms and the reciprocal of the level of fasting glucose and insulin. HOMA index is a similar proxy however, it does not include the log-transformation within its formula and therefore, QUICKI provides a more accurate calculation for a wider range of insulin measurement function (Borai et al., 2011). This has been also confirmed by others (Straczkowski et al., 2004).

2.1.4.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA technique uses the principle of the antigen-antibody interaction and allows efficient quantitation of antibody or antigen concentration in various samples including blood, plasma, serum, or supernatant. Various types of ELISAs are commercially available which employ the basic principle of immunological interaction between single or multiple antigen and antibody in a sample.

Figure 28: Schematic diagram of sandwich ELISA.



A sandwich ELISA measures the amount of antigen between two layers of antibodies such as detection and capture antibody as shown in the figure above. Proteins are first captured by the capture antibody and the target antibody is detected by the detection antibody. The detection (secondary antibody) is linked with an enzyme such as HRP, which under an introduction of a substrate leads to colour change. The amount of colour developed is proportional to the amount of antibody/antigen present in the sample.

The most commonly employed sandwich ELISA measures the amount of antigen between two layers of antibodies (i.e. capture and detection antibody) (see Figure 28). The substance to be quantified must contain at least two antigen-binding sites to enable antibody attachment. Two antibodies are generally employed in a sandwich assay, including monoclonal or polyclonal antibodies that can be used as the capture and detection antibodies. Monoclonal antibodies enable a highly sensitive detection of target antigen due to its monovalent affinity i.e. binding to the same epitope. A polyclonal antibody is generally used as the capture antibody to bind as much of the antigen as possible. Sandwich ELISA enables a quick, efficient, and sensitive measurement of proteins without the need to prior purification of the samples.

In the technique, the antigen is first adsorbed on a solid support, i.e. a 96-well ELISA plate. The primary antibody is incubated with the antigen, washing off the excess using an appropriate buffer. The primary antibody is then incubated with an enzyme-labelled secondary antibody raised against it conjugated to an enzyme. The secondary antibody may be labelled with an enzyme such as alkaline phosphatase or horseradish peroxidase (HRP). In the case of labelling with HRP/AP, developing is carried out following incubation in the presence of a substrate that generates a colour change. The amount of colour production is quantified via a spectrophotometric plate reader at a target wavelength and is proportional to the concentration of the antibody/antigen present in the sample.

2.1.4.3 Human glycated LDL ELISA assay

2.1.4.3.1 Principle of the assay

This assay was obtained from Glycacor, Exocell (Philadelphia, USA). A monoclonal mouse antibody, ES12, is used in this competitive ELISA, which is capable of recognising a single epitope on glycated apolipoprotein B (apoB) in the LDL molecule. The glycated LDL has a half-life of 2.5 – 5 days whilst glycohaemoglobin is stable for 100 days and glycoalbumin for 17 days. Quantification of glycated LDL reflects the average blood glucose concentration during the preceding week, whilst glycated albumin is used to estimate average glucose concentration over the preceding 2-3

weeks. The glycation of apoB, the main protein molecule of LDL, is a post-secretory process that has been implicated in atherosclerosis in diabetes mellitus (Johnson and Baker, 1986). The mean glycated LDL in 100 assays of samples from healthy subjects was found to be 0.63 to 1.79 mg/dl with the mean 1.12 ± 0.29 SD (mg/dl). Briefly, the glycated LDL from standards and samples is added to a 96-well plate already containing glycated LDL in a solid phase. Then ES12 mouse monoclonal antibody is added to each well that binds to the immobilized glycated LDL or the soluble glycated LDL from samples and standards. The plate is incubated over night to ensure complete competitive binding. The antibody conjugated with the glycated LDL in the solid phase is detected using HRP-conjugated goat anti-mouse antibody. Due to the competitive nature of this assay, the colour development with tetramethylbenzidine (TMB) is inversely proportional to the concentration of glycated LDL.

2.1.4.4 Human annexin A1 ELISA assay

2.1.4.4.1 Principle of the assay

Human AnxA1 protein levels were determined in the human plasma using an in-house ELISA which has previously been described (Goulding et al., 1990, Yazid et al., 2009, McArthur et al., 2010). Briefly, human recombinant AnxA1 protein was used to produce a standard curve of between 1-2000 ng/ml. Samples and standards were assayed in duplicate on a 96 well high binding ELISA plate (Costar, USA). Capture antibody [clone 1b (D'Acquisto et al., 2008b)] was diluted to a final concentration of 10 μg/ml in bicarbonate buffer, pH 9.6 and was incubated overnight at 4 °C. Blocking was achieved using 1 % solution of BSA for 1 hour at 37 °C. Anti-rabbit AnxA1 polyclonal antibody (Zymed, Invitrogen, UK) was used for detection (final concentration 1 μg/ml in 1 % tween-20/PBS buffer pH 7.4). Detection was achieved using an alkaline phosphatase conjugated goat anti-rabbit secondary antibody (1:1000, Sigma, UK), which was then incubated with phosphatase substrate (Sigma, UK). The samples were measured on a plate reader at dual wavelength of 405 nm and off-set at 540 nm. Conditioned media from primary human neutrophils was used as a positive control in this assay. Briefly, neutrophils were isolated and incubated at a concentration of 1 x10^6/ml as previously described (Renshaw et al., 2010) with

dexamethasone (10^{-9}M) for 1 hour at 37 °C in a shaking water bath and then centrifuged at 4000 g for 5 minutes and supernatant immediately frozen at minus 80 °C in aliquots.

2.1.4.4.2 Typical data

A standard curve was generated for each set of samples assayed. The typical data is shown in Table 3 and a typical standard curve is shown in Figure 29. Mean CV was 7.09 % for AnxA1 intra-assay precision (see Table 4).

Table 3: A standard curve is generated based on AnxA1 concentration (pg/ml) and optical density (O.D.) read at 405 nm (λ correction 540) as measured during the ELISA assay.

pg/ml	O.D.	Average	Corrected
2000	3.16 2.92	3.04	3.04
1000	2.81 2.70	2.75	2.75
500	2.67 2.53	2.60	2.60
250	2.39 2.21	2.30	2.30
125	2.06 1.93	2.00	1.99
62.5	1.57 1.43	1.50	1.50
31.25	1.15 0.98	1.07	1.07
15.63	0.73 0.79	0.76	0.76
7.81	0.45 0.55	0.50	0.50
3.91	0.18 0.21	0.20	0.20
0.00	0.0016 0.0013	0.00	-

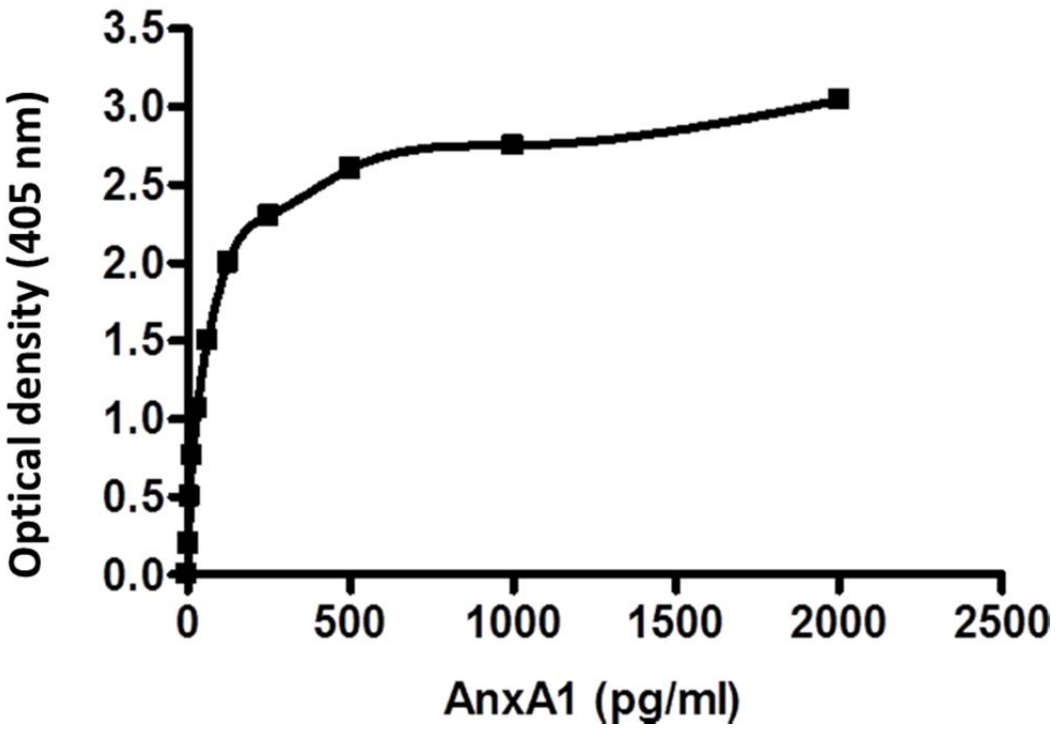
A standard curve is generated based on AnxA1 concentration (pg/ml) and optical density (O.D.) read at 405 nm (λ correction 540) as measured during the ELISA assay.

Table 4: Intra assay precision analysis based on O.D. duplicates for annexin A1 concentration derived during ELISA.

	Intra-assay precision										
[AnxA1]	**2000**	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	**15.63**	**7.81**	**3.91**	
n	2	2	2	2	2	2	2	2	2	2	
mean	3.04	2.75	2.60	2.30	2.00	1.50	1.07	0.76	0.50	0.20	
SD	0.17	0.07	0.10	0.12	0.09	0.10	0.12	0.04	0.08	0.02	
CV (%)	5.51	2.72	3.78	5.30	4.60	6.49	10.92	5.81	15.09	10.67	
Mean CV (%)											**7.09**

Coefficient of variation calculated from O.D. duplicates determined during an AnxA1 ELISA. Mean CV was 7.09 % for AnxA1 intra-assay precision.

Figure 29: Typical standard curve for AnxA1 concentration and optical density.



Source: A four-parameter logistic (4-PL) curve-fit for determination of AnxA1 plasma samples was generated using computer software GraphPad Prism 4 (San Diego, California). Diluted samples were multiplied by the appropriate dilution factor.

2.1.4.5 Adiponectin ELISA

2.1.4.5.1 Principle of the assay

The R&D DuoSet® ELISA Development System (DY1065, Abingdon, UK) uses the quantitative sandwich enzyme immunoassay principle. A 96-well microtitre plate is

pre-coated with a monoclonal capture antibody specific for the adiponectin hormone. The standards, positive and negative controls along with appropriate samples are added into the wells and any adiponectin present is captured by the immobilized antibody. A washing step enables removal of any unbound substances. After the washing a monoclonal detection antibody specific for adiponectin is added. This is followed by a subsequent washing step to eliminate any unbound antibody reagents. Streptavidin-HRP enzyme is then aliquoted into each well, followed by addition of a substrate solution. The amount of colour produced is proportional to the amount of adiponectin bound in the initial step. Hydrogen peroxide and tetramethylbenzidine is used to stop the colour development and the amount of adiponectin is measured via a spectrophotometer at a target wavelength.

Table 5: Typical data for adiponectin concentration (pg/ml) and optical density (O.D.).

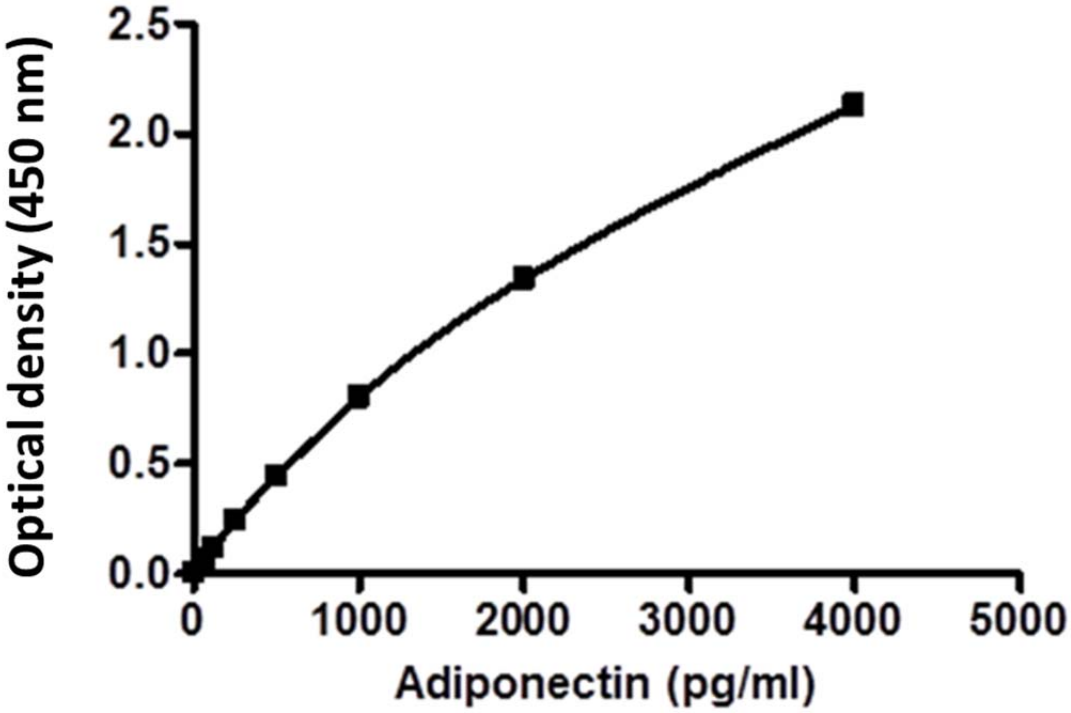
pg/ml	O.D.	Average	Corrected
4000	2.21 2.08	2.15	2.13
2000	1.39 1.32	1.36	1.34
1000	0.78 0.83	0.81	0.80
500	0.46 0.43	0.45	0.44
250	0.26 0.24	0.25	0.24
125	0.14 0.13	0.13	0.12
62.5	0.07 0.07	0.07	0.06
0	0.01 0.01	0.01	0

A standard curve is generated based on adiponectin concentration (pg/ml) and optical density (O.D.) read at 450 nm (λ correction 540) as measured during the ELISA assay.

i. Typical data

A standard curve was generated for each set of samples assayed. The typical data is shown in Table 5 and a typical standard curve is shown in Figure 30. Mean CV was 3.99 % for adiponectin intra-assay precision (see Table 6).

Figure 30: Typical standard curve for adiponectin concentration and optical density.



Source: A four-parameter logistic (4-PL) curve-fit for determination of adiponectin plasma samples was generated using computer software GraphPad Prism 4 (San Diego, California). Diluted samples were multiplied by the appropriate dilution factor.

Table 6: Intra assay precision analysis based on O.D. duplicates for adiponectin concentration derived during ELISA.

	Intra-assay precision							
[Adiponectin]	**4000**	**2000**	**1000**	**500**	**250**	**125**	**62.5**	**0**
n	2	2	2	2	2	2	2	2
mean	2.15	1.36	0.81	0.45	0.25	0.13	0.07	0.01
SD	0.09	0.05	0.04	0.02	0.01	0.00	0.00	0.00
CV (%)	4.24	3.65	4.57	4.15	5.40	1.84	4.66	3.38
Mean CV (%)								**3.99**

Coefficient of variation calculated from O.D. duplicates determined during an adiponectin ELISA. Mean CV was 3.99 % for adiponectin intra-assay precision.

2.1.4.6 Leptin, IL-6, IL-10, IL-17 and TNFα ELISA

2.1.4.6.1 Principle of the assay

The R&D DuoSet® ELISAs Development System (Abingdon, UK) was used to quantify the above adipocytokines. The principle of the assays was as described for R&D Systems adiponectin ELISA.

2.1.4.6.2 Typical data

Typical data for the above assays was as shown in the adiponectin ELISA.

2.1.4.7 Cortisol ELISA

2.1.4.7.1 Principle of the assay

The R&D Systems Cortisol Immunoassay (KGE008, R&D Systems, Abingdon, UK) was used for determination of plasma cortisol.

Table 7: Typical data for cortisol concentration (ng/ml) and optical density (O.D.).

ng/ml	O.D. 450 nm	Average	Corrected
NSB	0.010 0.007	0.00915	-
0	1.305 1.302	1.3039	1.29475
0.156	1.178 1.200	1.189	1.17985
0.312	0.998 1.035	1.0165	1.00735
0.625	0.765 0.799	0.78185	0.7727
1.25	0.488 0.662	0.5752	0.56605
2.5	0.418 0.449	0.4335	0.42435
5	0.222 0.287	0.2546	0.24545
10	0.141 0.151	0.1462	0.13705

A standard curve is generated based on cortisol concentration (ng/ml) and optical density (O.D.) read at 450 nm (λ correction 540) as measured during the ELISA assay.

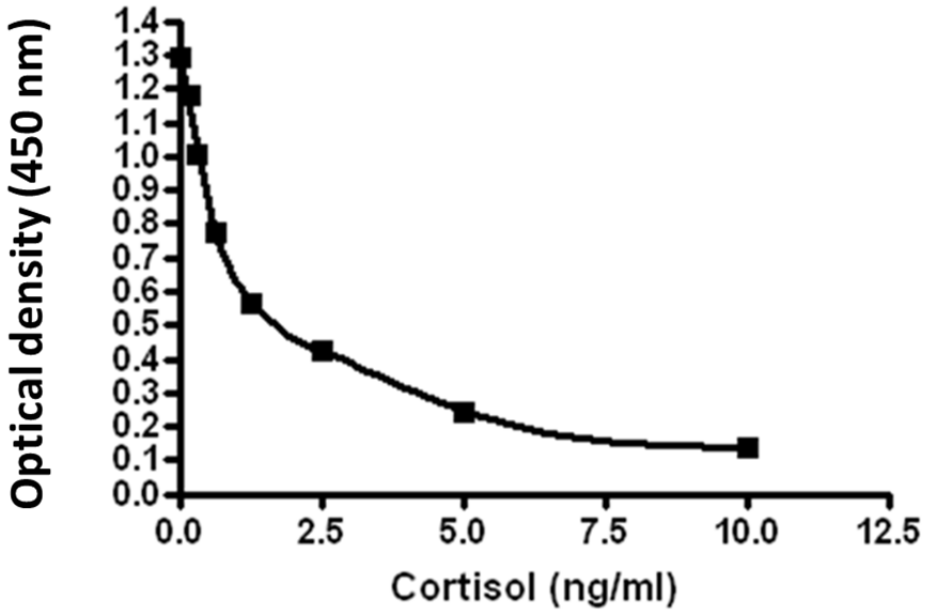
Competitive binding was the main principle of the cortisol ELISA assay used in this study. Briefly, the amount of cortisol found in a study sample competes with the HRP-labelled cortisol for binding sites on the antibody. The next step involves an incubation period during which the monoclonal antibody binds to the goat anti-mouse antibody precoated on the microplate. After the incubation any excess unbound sample and conjugate is washed off, and the enzyme activity is measured

via the colour development produced after adding substrate solution. The microplate is read at 450 nm using a spectrophotometer.

2.1.4.7.2 Typical data

A standard curve is generated for each set of samples assayed. All samples were assayed in duplicates. The typical data is shown in Table 7 and a typical standard curve is shown in Figure 31. Intra-assay coefficient of variation for cortisol ELISA is 1.68 % (see Table 8).

Figure 31: Typical standard curve for cortisol concentration and optical density.



Source: A four-parameter logistic (4-PL) curve-fit for determination of cortisol plasma samples was generated using computer software GraphPad Prism 4 (San Diego, California). Diluted samples were multiplied by the appropriate dilution factor.

Table 8: Intra assay precision analysis based on O.D. duplicates for cortisol concentration derived during ELISA.

	Intra-assay precision								
[Cortisol]	**0**	**0.156**	**0.312**	**0.625**	**1.25**	**2.5**	**5**	**10**	
N	2	2	2	2	2	2	2	2	
Mean O.D.	1.991	1.991	1.991	1.991	1.991	1.991	1.991	1.991	
SD	0.003	0.016	0.026	0.024	0.123	0.022	0.046	0.007	
CV (%)	0.13	0.78	1.31	1.21	6.17	1.12	2.33	0.36	
Mean CV (%)									**1.68**

Coefficient of variation calculated from O.D. duplicates determined during a cortisol ELISA. Mean CV was 1.68 % for cortisol intra-assay precision.

2.1.4.8 CRP ELISA

2.1.4.8.1 Principle of the assay

The R&D Systems Quantikine Human CRP Immunoassay (DCRP00, R&D Systems, Abingdon, UK) is a 4.5 hour solid-phase ELISA designed to measure human CRP in plasma.

Table 9: Typical data for CRP concentration (ng/ml) and optical density (O.D.).

ng/ml	O.D. 450 nm	Average	Corrected
50	2.676 2.746	2.711	2.701
25	1.262 1.496	1.37855	1.36855
12.5	0.781 0.797	0.78925	0.77925
6.25	0.476 0.453	0.46455	0.45455
3.12	0.241 0.291	0.2662	0.2562
1.56	0.158 0.147	0.1527	0.1427
0.78	0.063 0.067	0.0654	0.0554
0	0.009 0.010	0.01	-

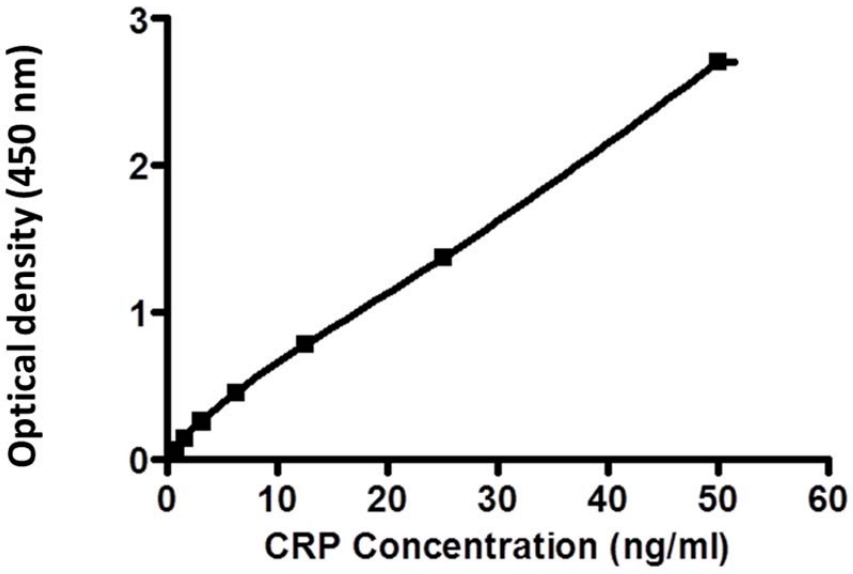
A standard curve is generated based on CRP concentration (ng/ml) and optical density (O.D.) read at 450 nm (λ correction 540) as measured during the ELISA assay.

CRP ELISA assay kit used in this study employed the principle of the sandwich ELISA as described in Figure 28.

2.1.4.8.2 Typical data

A standard curve is generated for each set of samples assayed. All samples were assayed in duplicates. The typical data is shown in Table 9 and a typical standard curve is shown in Figure 32. Intra-assay coefficient of variation for CRP ELISA is 6.03 % (see Table 10).

Figure 32: Typical standard curve for CRP concentration and optical density.



Source: A four parameter logistic (4-PL) curve-fit for determination of CRP plasma samples was generated using computer software GraphPad Prism 4 (San Diego, California). Diluted samples were multiplied by the appropriate dilution factor.

Table 10: Intra assay precision analysis based on O.D. duplicates for CRP concentration derived during ELISA.

	Intra-assay precision								
[CRP]	50	25	12.5	6.25	3.12	1.56	0.78	0	
N	2	2	2	2	2	2	2	2	
Mean O.D.	2.71	1.38	0.79	0.47	0.27	0.15	0.07	0.01	
SD	0.05	0.17	0.01	0.01	0.04	0.00	0.00	0.00	
CV (%)	1.83	12.01	1.49	3.50	13.36	4.96	4.00	7.07	
Mean CV (%)									**6.03**

Coefficient of variation calculated from O.D. duplicates determined during a CRP ELISA. Mean CV was 6.03 % for CRP intra-assay precision.

2.1.4.9 Insulin ELISA

2.1.4.9.1 Principle of the assay

The DRG Insulin Enzyme Immunoassay Kit (Cat No., 2935, DRG Instruments GmbH, Marburg, Germany) was used for detection of plasma insulin from the human samples. The DRG Insulin ELISA Kit is a solid phase ELISA that utilises the sandwich principle as described in Figure 28.

2.1.4.9.2 Typical data

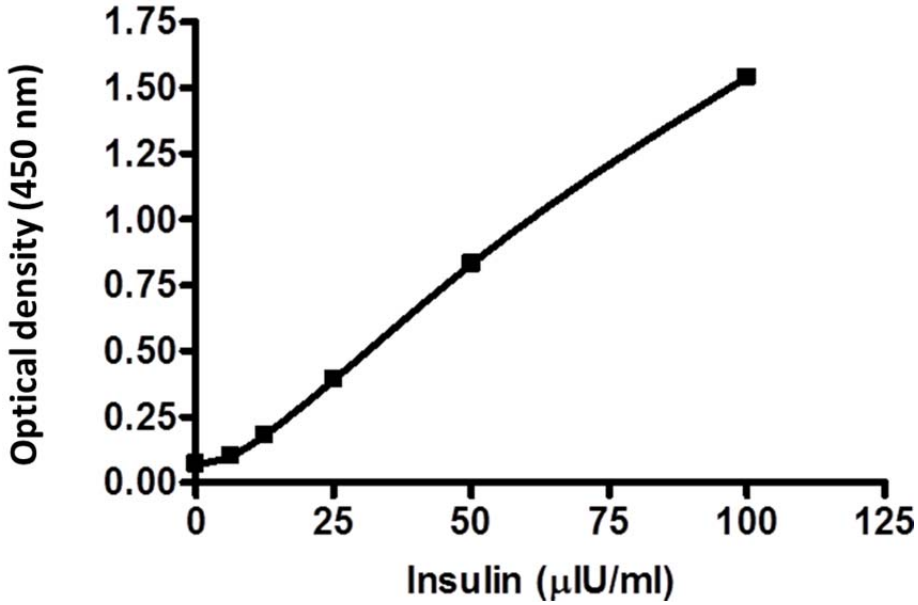
Table 11: Typical data for insulin concentration (µIU/ml) and optical density (O.D.).

µIU/ml	O.D. 450 nm	Average	Corrected
0	0.073 0.064	0.07	0
6.25	0.105 0.104	0.10	0.04
12.5	0.170 0.182	0.18	0.11
25	0.379 0.393	0.39	0.32
50	0.842 0.816	0.83	0.76
100	1.45 1.63	1.54	1.47

A standard curve is generated based on insulin concentration (µIU/ml) and optical density (O.D.) read at 450 nm (λ correction 540) as measured during the ELISA assay.

A standard curve is generated for each set of samples assayed. All samples were assayed in duplicates. The typical data is shown in Table 11 and a typical standard curve is shown in Figure 33. Intra-assay coefficient of variation for insulin ELISA was 1.81 % (see Table 12).

Figure 33: Typical standard curve for insulin concentration and optical density.



Source: A four-parameter logistic (4-PL) curve-fit for determination of Insulin plasma samples was generated using computer software GraphPad Prism 4 (San Diego, California).

Table 12: Intra assay precision analysis based on O.D. duplicates for insulin concentration derived during ELISA.

	Intra-assay precision						
[Insulin]	**0**	**6.25**	**12.5**	**25**	**50**	**100**	
n	2	2	2	2	2	2	
mean	1.93	1.93	1.93	1.93	1.93	1.50	
SD	0.00	0.00	0.00	0.01	0.02	0.13	
CV (%)	0.31	0.03	0.47	0.52	0.96	8.56	
Mean CV (%)							**1.81**

Coefficient of variation calculated from O.D. duplicates determined during an insulin ELISA. Mean CV was 1.81 % for insulin intra-assay precision.

2.1.5 Statistical analyses

All data was checked for normal distribution and non-normally distributed data was corrected using the natural logarithm of the original values. We assessed associations among variables with the use of Pearson's correlation coefficient. Data was checked for normal distribution and accepted if skewness and kurtosis were between minus 2 and 2. Linear regression analyses were carried out to test for significance. Two regression models were fitted to determine the relations between body composition and the biomarkers measured. The models included a simple

linear relation between an independent variable and dependent variable and the log of the dependent variable and the independent variable. Categorical and continuous variables were tested using ANOVA followed by Tukey's *post hoc* test. All data are expressed as the mean $\pm$ standard error of mean (SEM). 2-tailed statistical significance ($p < 0.05$) was applied to all tests. All analyses were conducted using Prism (GraphPad Software Inc., San Diego, CA) for the calculation of the ELISA results and ANOVA followed by Tukey's *post hoc* test and for the remainder of the data using Statistical Package for Social Sciences (IBM SPSS Statistics, Version 19, NY).

2.1.6 Sample size calculation

Investigations into a casual-effect relationships or a difference between two groups generally are tested with statistical analysis of the effect, confidence intervals, and P value. The confidence interval reflects the estimated range of values for the unknown/tested population parameter. P value signifies the likelihood of the observed effect/treatment being random. Along the confidence interval and P value, the power of a study is routinely included in the statistical analysis. The power of a study relates to the chance of estimating a difference between variables tested. The higher the power of a study is, the better the chance of detecting an effect. The power of a study depends largely on sample size e.g. studies with a small sample size may not detect a difference/effect of a treatment especially if the true difference is small (Whitley and Ball, 2002). A simple calculation was extrapolated to estimate a sample size by deriving the standardized difference of the variable being tested (Altman, 1982).

$$Standardized\ difference\ =\ target\ difference/standard\ deviation$$

To calculate our sample size we used blood pressure results taken from a study looking at 263 patients with severe sepsis or septic shock (Whitley and Ball, 2002). Their mean blood pressure was 95 and 81 mmHg, respectively. The SD was estimated to be 18 mmHg, therefore:

$$Standardized\ difference\ =\ (95-81)/18\ =\ 0.78$$

Taking into account the standardized difference of 0.78, P value at 0.05 and assuming the power of the study at 80 % , using a sample size nomogram (Whitley and Ball, 2002) we calculated that the necessary sample size to detect a difference is 52 (minimum 26 participants in each category of the study) participants.

Study 2: AnxA1 peptide reduces inflammation in an in vitro model of obesity

2.2 Research design and methods

2.2.2 In vitro models of hypoxia

In this study, we adopted an *in vitro* cell culture approach, where a hypoxic environment was designed to mimic the conditions within adipose tissue as closely as possible. The cells were incubated in a hypoxic incubator (2 % oxygen) to artificially create as closely as possible the reduced oxygen conditions experienced by adipocyte cells in human subjects with increased central subcutaneous adiposity. A study by Ye *et al.,* 2007, showed that PO_2 in the epididymal fat pad mass of *ob/ob* obese mice (15.2 mmHg) was 70 % lower than in the lean mice (47.9 mmHg) demonstrating that obesity leads to tissue hypoxia. The 1 % oxygen (7.6 mmHg) is close to the adipose tissue oxygen level (2 %, 15.2 mmHg) and was used *in vitro* to mimic the hypoxic environment. This allowed us to directly correlate reduced oxygen conditions with a precise cytokine profile of primary human adipocytes.

Adipocyte cells were initially cultured using standard techniques in an atmosphere of 95 % O_2/5 % CO_2 to allow cells to proliferate normally. We have carried out a number of experiments including:

- We measured the expression of AnxA1, FPR1, FPR2/ALX, adiponectin, leptin, CRP, and IL-6 in preadipocytes and mature adipocytes under normoxic conditions.
- We measured the effect of 4, 8, and 24-hour hypoxia treatment (1% O_2) on mature adipocytes and the expression of AnxA1, FPR1, FPR2/ALX, adiponectin, leptin, CRP, and IL-6.
- We also measured the effect of the N-terminal peptide of AnxA1 (peptide Ac2-26) on the expression of inflammatory markers (CRP and IL-6) and adipose tissue specific hormones (adiponectin and leptin) after 4, 8, and 24 hour hypoxia treatment (1 % O_2).

These experiments allowed us to determine the degree to which oxygen reduction can influence inflammatory cytokine and AnxA1 release by this cell type and the effect of AnxA1 on inflammatory markers during hypoxic conditions.

2.2.3 Materials

A kind donation of all required SGBS preadipocyte cells has been made from Dr Peter King, St Bartholomew's Hospital, Queen Mary University of London. This cell line was established by Wabitsch, et al., 2001 and since then it has been widely used to study adipocytes physiology and behaviour. The cells are easy to proliferate and provide an almost limitless source of a human preadipocyte cell line with high magnitude of differentiation into mature adipocytes (Wabitsch et al., 2001) therefore, providing a useful and efficient model for human fat cell biology research. The culturing conditions require standard media (DMEM and F12 media) and reagents (T3, rosiglitazone and insulin) that improve cell proliferation and differentiation, which are readily available and cost-effective. SGBS cells can be passaged up to 30 times in appropriate conditions (Wabitsch et al., 2001). Ac 2-26 was obtained from Pepceuticals (Enderby, UK). Rosiglitazone was purchased from Cayman (Ann Arbor, MI, USA). 3, 3', 5-triiodothyronine was obtained from IRMM (Geel, Belgium). QuantiTect® Primers and SYBR® Green-based real time RT-PCR assay were obtained from Qiagen (Mainz, Germany). Unless, stated otherwise, all reagents or chemicals used were from Sigma-Aldrich (Dorset, UK).

2.2.4 Cell Culture

Cell culture is used for growing prokaryotic or eukaryotic cells that have been isolated from an organism in artificially created conditions necessary for their growth. It involves either the culture of cells collected directly (primary cells), or the use of an immortalized cell line (such as SGBS cells used in this study). Cell culture enables the study of cell biology in an isolated environment with the use of variety of treatments that are not easily employed in an organism e.g. a human. This includes providing the cells with optimum conditions of temperature, gas mixtures (CO_2/0_2 ratio) and a nutrient environment that reflects the extracellular environment *in vivo*. This is achieved by maintaining the cells in growth medium in a cell-incubator set at

pre-determined temperature and gas supply. The growth medium is adjusted to a specific pH and contains specific nutrients, and growth factors.

SGBS cells used in this study were maintained in DMEM/F12 medium in a 1:1 ratio containing 4.5 g/l glucose. The media was further supplemented with 10 % foetal bovine serum (FBS) to provide the cells with essential components such as growth factors. FBS was heat treated at 56 °C for 30 minutes prior to use to inactivate heat labile cell growth inhibitors. Phenol red was included as a pH indicator. A change in the colour of the medium from orange/red to yellow indicated that the cells were being cultured at too high a density. Glutamine (2 mM), HEPES (10 nM) were added to media. 100 U/ml penicillin/streptomycin antibiotics were added to prevent bacterial contamination. SGBS cells (passages 10 to 25) were seeded into negatively charged 25 cm^2 (T25) or 75 cm^2 (T75) tissue culture flasks containing the media and were cultured as monolayers in an incubator at 37 °C (95 % O_2, 5 % CO_2) changing the growth media every 72 hours. All cell culture protocols were carried out in a sterile environment in a tissue culture cabinet wiped clean with Mycoplasma-off ® solution (Minerva Biolabs, Berlin, Germany) prior to experiments.

2.2.5 Cell subculture

Cells are subcultured to propagate the cell population from an ongoing culture or to set up fresh cultures for experimental studies. Confluent cells were dislodged from the bottom of a flask in a process called trypsinisation, by adding a low volume and concentration of trypsin/EDTA (0.1 %/0.2 %) solution to the cells. The enzymes that are contained within trypsin dislodge the cells from the anchorage sites binding them to the surface of the cultured vessel.

2.2.5.1.1 Methodology

Once 75 % confluence was attained, SGBS cells were trypsinised to promote further propagation or use in experiments. Culture medium was aspirated from T25 or T75 flasks containing the SGBS cells and appropriate amount of trypsin and serum free medium was added to the flasks (3 ml and 9 ml respectively). Cells were placed in an incubator at 37 °C (95 % O_2/5 % CO_2) for 5 minutes and then removed and flasks tapped firmly at the sides to aid disassociation. After confirming visually under an

inverted microscope that disassociation was complete, 4 ml or 12 ml DMEM/F12 medium (T25 and T75, respectively) was added into the flasks to inhibit the action of trypsin. The cell suspension was then transferred to a centrifugation tube and cells pelted by centrifugation at 1000 rpm for 3 minutes. The supernatant was aspirated and the pellet resuspended in 10 ml of fresh DMEM/F12 medium by gently pipetting up and down. A proportion of this cell suspension (based on a cell count) was seeded into fresh culture vessels (T25/T75 flasks) containing the appropriate volume of growth medium for propagation. For experimental incubations, the cell suspension was seeded into the culture vessels (6-well plates) containing the appropriate quantity of experimental medium. All culture flasks with treated cells were kept in an incubator at 37 °C (95 % O_2/5 % CO_2).

2.2.6 Cryopreservation and thawing of cells

Cryopreservation allows storage of stocks of cells to prevent the need to have all cell lines in culture at the same time. Adequate cryopreservation is fundamental when working with cells of limited life span. Cryopreservation minimizes the risk of microbial contamination and cross contamination with other cell lines; it prevents the allelic drift and maintains the morphological features as well as allows carrying out experiments using cells at a consistent passage number.

Extensive research has been carried out to develop successful cryopreservation and resuscitation of a broad range of cell lines of different cell class. The precise methodologies of adequate cryopreservation and resuscitation differ for cell types of various origins. However, the main principles involve a slow cooling at a rate of -1 °C to -3 °C per minute and rapid thawing in a 37 °C for 3 to 5 minutes. Here is a general summary of key points that need to be considered for successful cryopreservation of most cell lines:

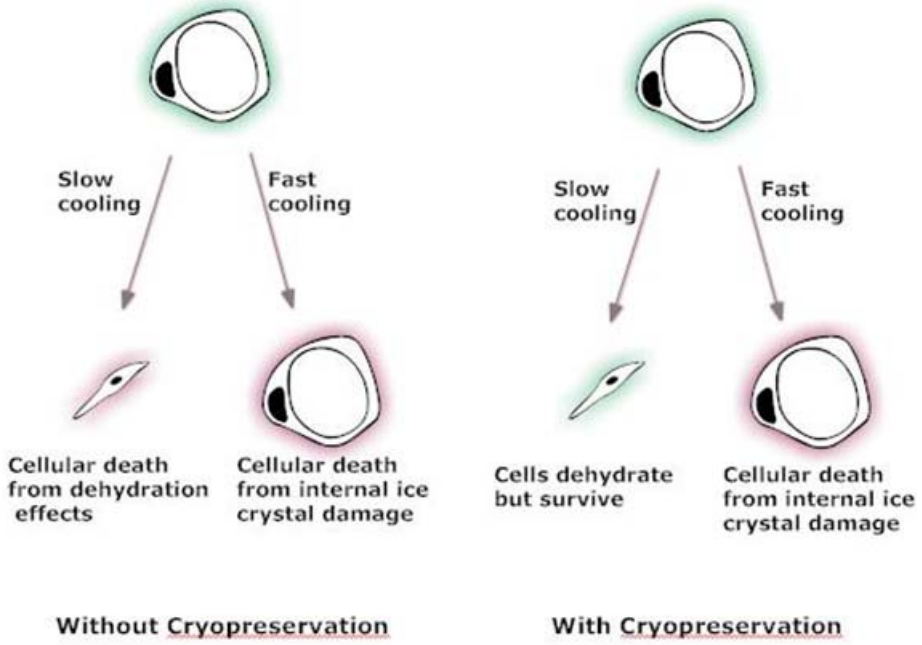
- Cells must be free of microbial, viral and fungal infection with a viability of > 90 %
- Cells should be below their maximum cell density and the culture medium should be changed 24 hours before cryopreservation
- A high concentration of serum is recommended i.e. at 90 %

- 10 % of dimethyl sulphoxide (DMSO) is recommended to protect the cells from damage due to ice crystals formation during freezing.

Cells are subject to storage at cryogenic temperatures (-196 °C) as these conditions prevent biochemical processes that result in cell death, so enabling cells to be stored for an indefinite period of time and remain viable when thawed following a standard procedure. Under cryogenic storage, cells do not undergo any detectable alterations, and therefore, cellular ageing and evolution are minimized.

Cryopreservation involves first combining the cells with a cryopreservant (growth medium containing 10 % dimethylsulphoxide (DMSO)) and then transferring the cells into a cryogenic storage vessel. The cryoprotectant prevents damage to the cells (including ice crystal formation) during the process of slow freezing. Although its precise mechanism is not completely understood, DMSO provides excellent cryoprotection and is one of the most widely employed cryoprotectants. However, DMSO is a powerful polar solution capable of having toxic effect on cell cultures and therefore, it is vital; to use it at an appropriate concentration (5 % to 15 % v/v) diluted in FBS prior to addition to cell suspension. The resulting inoculums is then added in aliquots to screw-cap sterile cryovials, the cryovials are then labeled and placed at -80 C overnight before being transferred into liquid nitrogen (-196 °C) for a long-term storage. This is to allow gradual freezing of the cells and prevent the rapid dehydration of the cells that might occur due to sudden exposure to cryogenic temperature (see Figure 34). Reconstitution of cryogenically stored cells requires the removal of the cryoprotective agent as quickly and gently as possible and replacement with the culture medium. First, the cryogenic vials are removed from their storage and placed in a warm water bath at 37 °C with gentle agitation. Rapid thawing facilitates rehydration of the cells without the appearance of damaging ice crystals within the cells. Following their recovery, the cells are transferred into a tissue culture flask containing growth medium and placed in an incubator.
SGBS cells were reconstituted from cryogenic storage by placing the frozen cryovial containing the cells in a water bath at 37 °C and gently agitating the cryovial to thaw the cells rapidly.

Figure 34: Effect of the rate of freezing on cell survival.



Source: (Image adapted from www.corning.com) The rate of freezing is critical for cell survival since cooling too rapidly results in cell death. Optimum freezing occurs at a cooling rate that is slow enough to prevent intracellular ice crystal formation while being rapid enough to prevent cell dehydration.

The vial was then taken into a sterile tissue culture hood and the contents were transferred immediately to a 15 ml centrifuge tube into which 10 ml of fresh growth medium was added. The cell suspension was centrifuged at 1000 rpm for 5 minutes after which the cell pellet was resuspended in a further 10 ml of fresh DMEM/F12 (1:1) growth medium and transferred into a T25/T75 tissue culture flask at optimal density. The seeding density was calculated based on a prior estimation of cell count.

2.2.7 Cell counting using a haemocytometer

Estimation of cell number is essential during cell culture experiments in order to quantify the concentration of cells. Cell count is generally carried out using a light microscope and a haemocytometer, a thick glass microscope slide with a rectangular indentation that creates a chamber of defined dimensions (area and depth). Grids of perpendicular lines are etched in each chamber, with each grid consisting of nine primary squares, each measuring 1 mm^2 (see Figure 35).

2.2.7.1.1 Methodology

Cell count was performed by retrieving cells from culture vessels by trypsinisation as described previously. Following the centrifugation step, the pellet was resuspended in 10 ml DMEM/F12 (1:1) media. A 10 µl aliquot of the cell suspension was withdrawn and diluted with 90 µl PBS. A coverslip was placed upon the haemocytometer chamber, and the diluted cell suspension was carefully introduced into the chamber using a plastic Pasteur pipette. The number of cells in four 1 mm square areas (see Figure 35) was counted using a light microscope at 100 X magnification, and cell number per ml cell suspension determined by applying the formula:

$$Cell\ number\ per\ ml\ of\ cell\ suspension = mean\ cell\ number\ per\ 0.1\ mm^3 \times dilution\ factor \times 10^4$$

2.2.8 Differentiation of pre-adipocytes into mature adipocytes – SGBS cell culture

2.2.8.1.1 Media

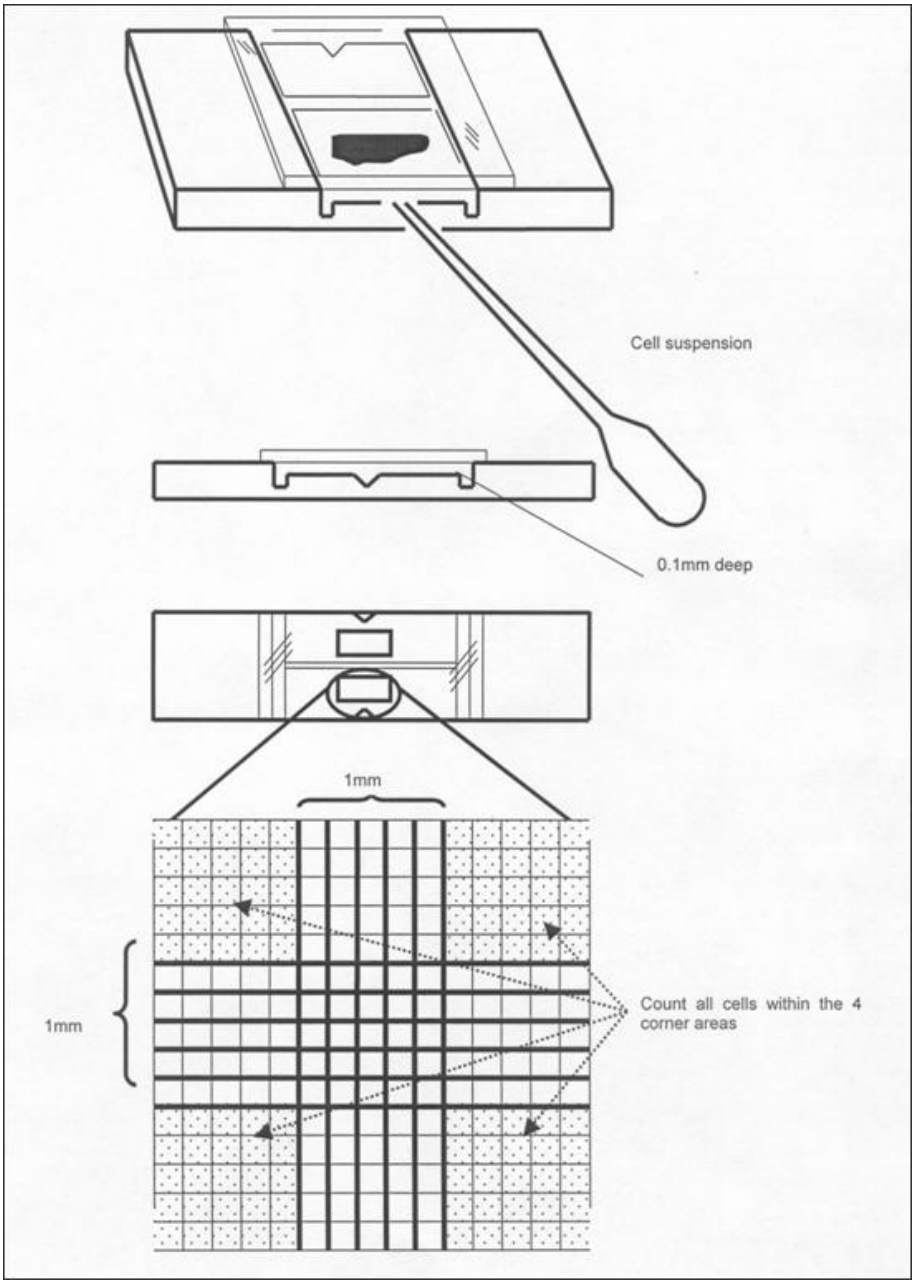
Serum free basal medium (OF) was prepared by adding to DMEM/Nutrient Mix F12 (Gibco BRL, #31330-038, 500ml); 0,008g biotin (SIGMA, B-4639, 1g), 0,004g pantothenate (SIGMA, #P-5155, 100g) and 10 ml penicillin / streptomycin (GIBCO BRL, #15140-114, 100ml) and it was stored at 4 °C.

Serum-containing medium was prepared using the OF basal medium and adding 10 % foetal bovine serum (FCS) (Gibco BRL, #10270-106, 500ml). It was important that the serum was not heat-inactivated, was steriled through filtartion at 0.2 µm and stored in 50 ml aliquots at –20 °C.

Adipogenic medium was prepared using the OF medium and adding 0,01mg/ml human transferrin (T8158, Sigma), $2x10^{-8}$M insulin (I9278, Sigma), 10^{-7}M cortisol (H0888, Sigma) 0,2 nM triiodothyronine (T_3) (IRMM, 469).

Quickdifferentiation medium was prepared using the Adipogenic medium and adding 25nM dexamethasone (SIGMA, #D-1756), 500µM IBMX (SIGMA, I-5879), 2µM rosiglitazone (BRL 49653, Cayman).

Figure 35: Schematic representation of a haemocytometer.



Source: (Image available online **www.who.int**.) A glass microscope slide has a rectangular indentation creating a chamber. The counting chamber consists of 1 x 1 mm (1 mm2) squares that are further divided. The device enables the accurate counting of cells in a specific volume of fluid.

2.2.8.1.2 Protocols

Defrosting cells

Cell were defrosted quickly in 37°C water bath. Freezing medium was diluted immediately after thawing in OF+10 % FCS (~20ml). Cells were centrifuged for 3.5

minutes at 1500 RPM. The supernatant was removed and the pellet was re-suspended in OF+10 % FCS. Cells were seeded in appropriate flasks at the desired density.

Freezing cells

The cells were frozen in medium containing 90 % FCS and 10 % DMSO. Firstly, the cells were trypsinized, then counted and washed in OF medium, and pelleted by centrifugation. The supernatant was discarded and freezing medium was added in dependence of cell number at 1-2 mio.cells/ml/vial. Cells were immedietly transferred for 100 minutes above evaporating N_2 or in a freezing machine where cells got frozen down in a controlled process.

Filtration

Cells were trypsinized when confluent (not too dense, never overgrown) e.g. feeding media was removed, cells were washed with PBS, PBS was removed and 1 times trypsin/EDTA (SIGMA, #T-4174), (volume that covers cells) was added. The cells were incubated ~5min at 37° C. When the cells were detached, they were suspended in OF +10 % FCS. Cells were counted and seeded in optimal density.

Differentiation of pre-adipocytes into adipocytes

Cells were grown to near confluence (~80 %) and washed three times with PBS. Quickdifferentiation medium was added and cells were incubated for 4 days.
The media was removed after 4 days and the cells were maintained in the adipogenic medium for further 10 days. Medium was changed every three days.

2.2.9 Oil red O quantification

2.2.9.1.1 Principle

Oil red O quantification utilises the principle of physical properties of dyes, that have higher solubility in a lipid based solvent when compared to a vehicular solvent. Oil red O, the Sudan red and the Sudan black dyes belong to the polyazo group of dyes and can all be used for fat lipid quantification using the method described below.

2.2.9.1.2 Methodology

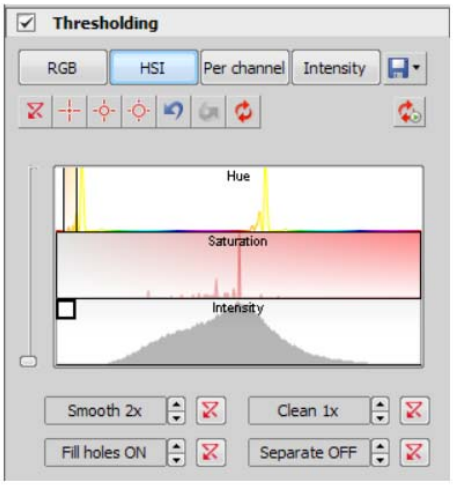
After 14 days of adipogenic differentiation, the lipid droplets inside the adipocytes were stained with Oil red O (O 0625, Sigma). Cells were carefully washed with DPBS and fixed with fixative solution (4 % formaldehyde, 75 mM sodium phosphate buffer pH 7, 1.5 % methanol) for 30 minutes at room temperature. Cells were then washed 3 times with DPBS and stained with Oil Red O solution for 2 to 3 hours in the dark. Oil Red O dye was prepared by making up a 1 mg/ml stock solution in 60 % triethyl phosphate. After allowing the crystals to dissolve for 1 hour, a working solution of the dye of 60:40 in distilled water was prepared and filtered through Whatmann filter paper. After staining the cells, the dye solution was removed and washed twice with DPBS to remove unbound dye. Stained cells were covered with DPBS and either stored at 4 °C or quantified for adipogenesis.

2.2.9.1.3 Quantification of differentiation

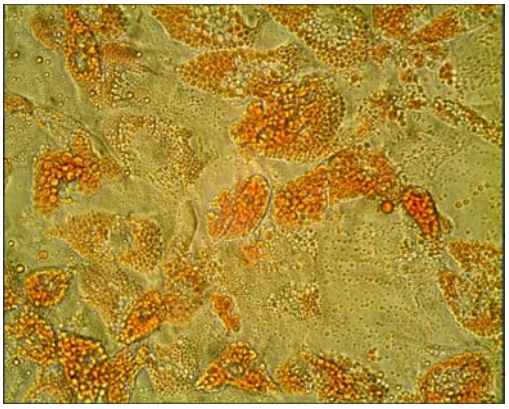
Adipogenic differentiation for quantification was carried out in 6-well formats unless otherwise stated. Cells containing oil Red O stained lipid droplets indicative of adipocytes formation were counted using NIS-Elements, software version 3.0 (Nikon UK Limited, Surrey). The amount of oil red O dye was proportional to the amount and size of adipocytes. NIS-Elements automated counting and analysis software allows automated detection and counting of objects of interest. Furthermore, it can measure the intensity of coloured dye via its intensity thresholding tool i.e. using simple binary layers (the simplest way of measuring and defining areas of interest). In this study quantification of oil red O was measured using thresholding based on hue, saturation and intensity (HIS). The range for intensity and saturation was set to full and hue adjusted to fine control the colour as the main identifier of the areas of interest (as shown in Figure 36). The density of oil red O contained in adipocytes formed was expressed as an average of 6 wells.

Figure 36: Quantification of oil red O using thresholding based on hue, saturation and intensity.

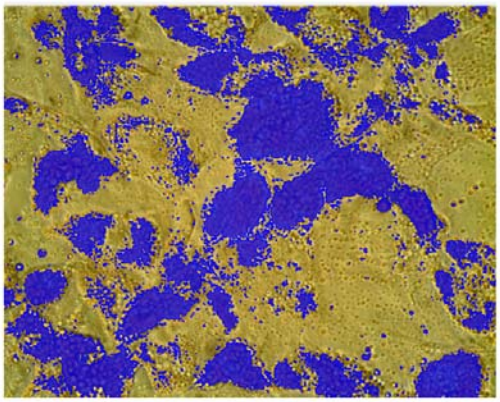
A



B



C



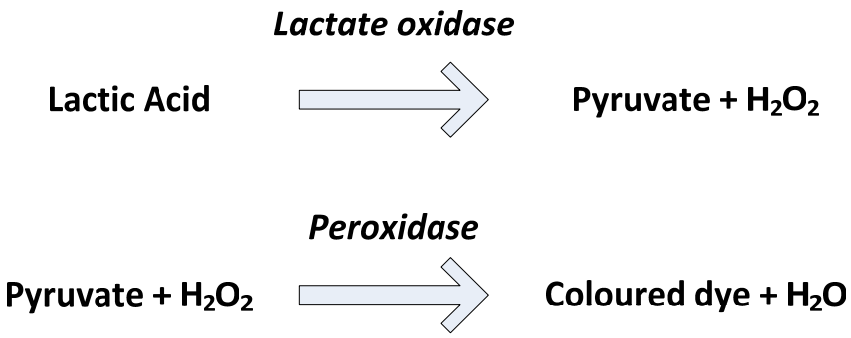
A: Hue and saturation are set to full, whilst intensity is adjusted to control the colour as the main identifier of oil red O as shown in **B** SGBS adipocytes stained with oil red O (x40 magnification) and **C** SGBS adipocytes identified for density measured using HIS thresholding (x40 magnification) using NIS-Elements software (Nikon UK Limited, Surrey)

2.2.10 Lactate Assay

2.2.10.1.1 Principle

During anaerobic respiration, glucose is metabolized to pyruvate, which in turn is converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDC). During this metabolic process, lactate is generated as a by-product by the action of the enzyme lactate dehydrogenase. PDC activity is considered to be constant and therefore, measurement of lactate production can be used as a marker of anaerobic glycolysis.

Figure 37: Principle of the lactate assay.



Lactic acid which is released from the cells undergoes oxidation via lactate oxidase enzyme to form pyruvate and hydrogen peroxide (H_2O_2). Peroxidase then catalyses the condensation of chromogen precursors in presence of the formed hydrogen peroxide to produce a coloured dye. The amount of coloured dye that can be measured is proportional to the amount of lactic acid produced.

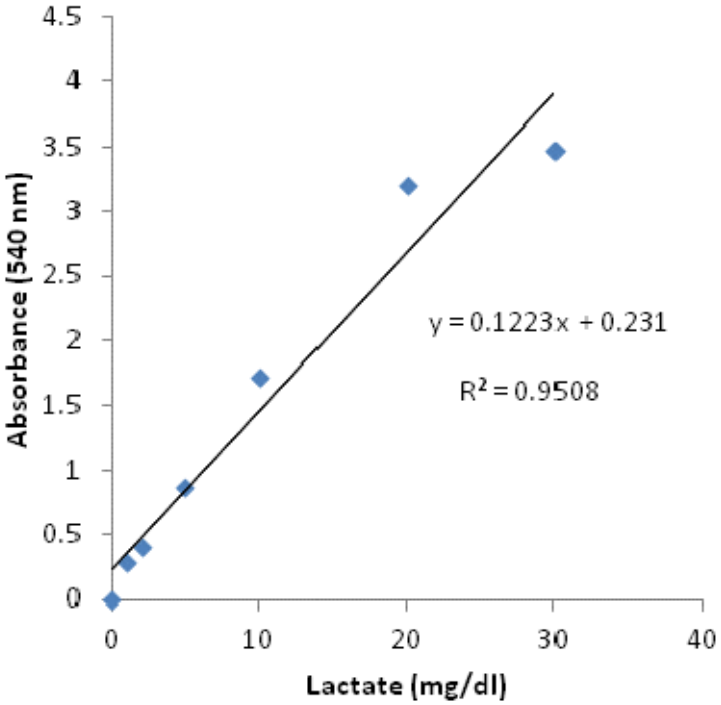
Lactate is produced in proliferating cells and during anaerobic conditions such as e.g. exercise. *In vivo* blood lactate arises predominantly from muscle cells and erythrocytes and is metabolized by the liver. Abnormally high concentrations of lactate have been associated with pathological conditions including diabetes, cancer, and lactate acidosis. The normal blood concentration levels of lactate in humans are around 1 to 2 mmol/l and reflect both production and metabolism. In this study, lactate production was estimated to measure glucose metabolism in the SGBS adipocytes in hypoxia. A commercial lactate assay kit was used, that uses the principle of lactate conversion to pyruvate and hydrogen peroxide by the enzyme lactate oxidase. Peroxidase then catalyses the condensation of chromogen precursors in presence of the formed hydrogen peroxide to produce a coloured dye having an absorption maximum of 540 nm. The lactate concentration in the sample is determined by an enzymatic assay, which is directly proportional to the increase in absorbance at 540 nm. The reaction is illustrated in Figure 37).

2.2.10.1.2 Methodology

Media was collected from adipocytes incubations and the concentration of lactate was measured. Serial dilutions of lactate standard were prepared within a concentration range of 0 to 30 nM from a lactate standard solution and aliquoted

into a 96 well plate. Adipocytes incubation media samples (30 µl per well) were added to plate wells. Equal volumes (270 µl per well) of lactate reagent were added to each well of the plate. The mixture was left to incubate for 10 minutes at room temperature. Absorbance was then read spectrophotometrically at 540 nm in a microplate reader (Wallac Victor 2, Perkin-Elmer; MA, USA). A typical standard curve for the lactate assay used to calculate lactate concentration is shown in Figure 38.

Figure 38: A typical lactate calibration curve.



Absorbance as read spectrophotometrically at 540 nm in a microplate reader (Wallac Victor 2, Perkin-Elmer; MA, USA). Typical lactate concentration (mg/dl).

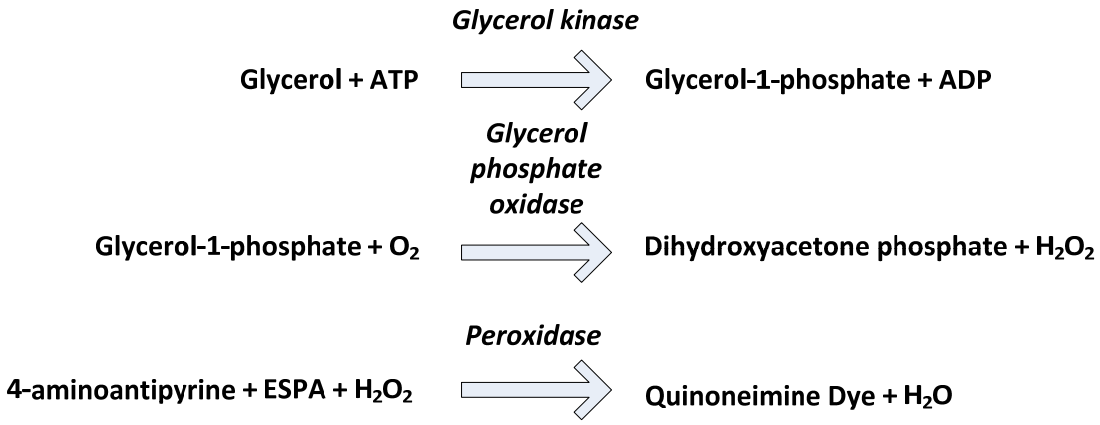
2.2.11 Glycerol Assay

2.2.11.1.1 Principle

The Free Glycerol Determination Kit was used to measure free, endogenous glycerol using coupled enzyme reactions. The rate of lipolysis can be measured using the principle of TAGs metabolism. TAGs are esters of three FAs and a glycerol molecule. Therefore, a measurement of free glycerol produced during lipolysis can be used to quantify net TAG breakdown. Adenosine-5′-triphosphate (ATP) phosphorylates glycerol which is released in the media and forms glycerol-1-phosphate (G-1-P) and adenosine-5′-diphosphate (ADP). Glycerol kinase (GK) is the enzyme that catalyses

this reaction. Then a different enzyme called glycerol phosphate oxidase (GPO) oxidises G-1-P to form dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2).

Figure 39: Glycerol assay enzymatic reactions.



Adenosine-5′-triphosphate (ATP) phosphorylates glycerol which is released in the media and forms glycerol-1-phosphate (G-1-P) and adenosine-5′-diphosphate (ADP). Glycerol kinase (GK) is the enzyme that catalyses this reaction. Then a different enzyme called glycerol phosphate oxidase (GPO) oxidises G-1-P to form dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Peroxidase enzyme enables the coupling of hydrogen peroxide (H_2O_2) with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to yield a quinoneimine dye. The absorbance levels as measured at 540 nm are proportional to the amount of the free glycerol present in the tested samples.

Peroxidase enzyme enables the coupling of hydrogen peroxide (H2O2) with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to yield a quinoneimine dye. The absorbance levels as measured at 540 nm are proportional to the amount of the free glycerol present in the tested samples. The enzymatic reactions involved in this assay are illustrated in Figure 39.

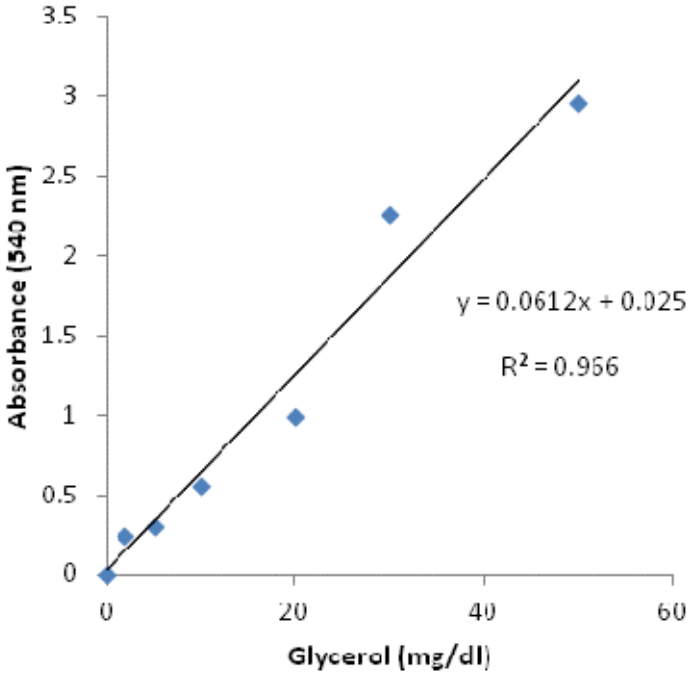
2.2.11.1.2 Methodology

The free glycerol reagent provided in the commercial assay kit was prepared by reconstitution in 40 ml of pure deionised water. After reconstitution, the solution contained following concentrations of reagents:

- ATP 0.75 mM
- Magnesium salt 3.75 mM
- 4-Aminoantipyrine 0.188 mM
- *N*-Ethyl-*N*-(3-sulfopropyl) 2.11 mM

- *m*-anisidine, sodium salt
- Glycerol kinase (microbial) 1,250 units/L
- Glycerol phosphate oxidase 2,500 units/L (microbial)
- Peroxidase (horseradish) 2,500 units/L
- Buffer pH 7.0 ± 0.1
- Sodium azide, 0.05 %

Figure 40: Glycerol assay standard curve.



Absorbance was recorded at 540 nm in a microplate reader (Wallac Victor 2, Perkin-Elmer, MA, USA). A typical standard curve for the glycerol assay used to calculate glycerol concentrations (mg/dl).

A series of glycerol standards in the concentration range 0 to 50 nM were prepared from a glycerol standard solution (0.26 mg/ml) and 50 µl were aliquoted in duplicates into the wells of a sterile 96 well plate. Adipocytes medium of equal amount (50 µl) collected previously were then added into the plate wells. 200 µl of glycerol reagent was added into each well. The reaction plate was left to incubate at room temperature for 15 minutes and absorbance was recorded at 540 nm in a microplate reader (Wallac Victor 2, Perkin-Elmer, MA, USA). A typical standard curve for the glycerol assay used to calculate glycerol concentrations is shown in Figure 40.

2.2.12 Hypoxia treatments

At day 15 after induction of differentiation, fully differentiated SGBS cells were exposed to hypoxia. To create a hypoxic environment (1% O_2), cells were placed in a MIC-101 modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA), flushed with a mixture of 1 % O_2, 5 % CO_2 and 94 % N_2, sealed and incubated at 37 °C. Adipocytes were cultured in the hypoxic environment for 4, 8, and 24 hours and the control group was cultured under normoxic conditions (21% O_2). Cells were harvested after treatment for different time periods.

2.2.13 Protein quantification

2.2.13.1.1 Preparation of cell lysate

To determine protein content, protein needs to be collected first from the cells under investigation. Protein samples can be collected from cells by causing the cells to lyse. Lysis buffer enables efficient cell lysis and protein solubilisation while avoiding protein degradation.

The radioimmunoprecipitation assay (RIPA) is widely used lysis buffer that enables the extraction of protein from cells. Prior to use, the buffer is supplemented with protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, aprotinin, pepstatin) and phosphatase inhibitors (Na_3VO_4 and NaF) to prevent protein degradation. RIPA buffer composition is shown below:

- 1 x RIPA Buffer
- 150 mM NaCl
- 1 % Na deoxycholate
- 1% NP-40
- 0.1% SDS
- 25 mM Tris-HCl pH 7.6

2.2.13.1.2 Methodology

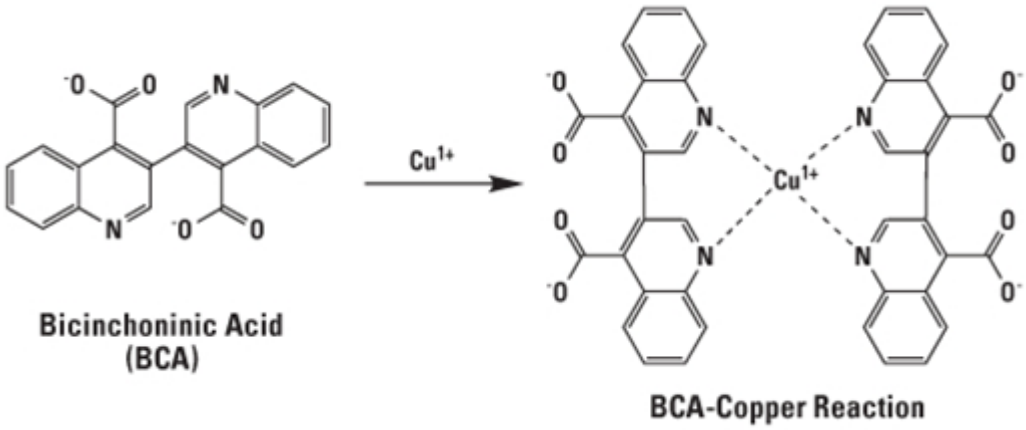
SGBS cells were plated out into 6-well plates as described previously (Section 2.3.4). After aspirating the growth media, test media (2 ml per well) was added to each well and the plates were incubated ay 37 °C (95% O_2/5% CO_2) for 72 hours. Protein extraction was then carried out as follows: supernatant was aspirated from each well

and the adherent cells washed twice with ice-cold PBS (2 ml). 500 µl ice cold 1 x RIPA buffer (Pierce, Illinois, USA) was then added to each well and they were placed in an ice bath for 5 minutes. Adherent cells were then scraped from the wells using sterile plastic cell scrapers and the cell suspension transferred into 1.5 ml eppendorf tubes. The tubes were placed in ice for 10 minutes to allow complete cell lysis after which they were centrifuged at 14000 g for 10 minutes at 4 °C. The pellet was discarded and the supernatant transferred into fresh tubes. An aliquot (100 µl) from each sample was withdrawn to be used for determining protein concentration using bicinchoninic acid (BCA) assay kit. All cell lysate sample tubes were stored at -20 °C.

2.2.14 Bicinchoninic acid assay (BCA)

Quantification of protein is necessary for standardization of data. The basis for most protein quantification assays is the biuret reaction, in which, in presence of an alkaline environment containing sodium potassium tartrate, a coloured chelate complex forms between peptides and cupric ions. The Pierce BCA kit (Pierce, Illinois, USA) is a sensitive and selective colourimetric assay, which combines the reduction of Cu^{2+} to Cu^{1+} by peptides and enables the selective colourimetric identification of the cuprous cation (Cu^{1+}) by BCA. In the first step, copper chelates with proteins forming a blue coloured complex in an alkaline media. This is followed by a colourimetric detection reagents reacting with the cuprous cation (Cu^{1+}) resulting in formation of a purple-coloured reaction product because of the reaction between BCA and cuprous ion. The coloured reaction product thus formed exhibits linear absorbance with increasing protein concentrations and can be measured between the wavelengths 550 nm to 570 nm (see Figure 41).

Figure 41: Principle of BCA assay.



Source: (Image available online www. piercenet.com) The Pierce BCA kit (Pierce, Illinois, USA) is a sensitive and selective colourimetric assay, which combines the reduction of Cu^{2+} to Cu^{1+} by peptides and enables the selective colourimetric identification of the cuprous cation (Cu^{1+}) by BCA. The figure shows the chelation reaction between BCA and copper.

2.2.14.1.1 Methodology

A series of protein (bovine albumin, albumin, BSA) reference standards ranging from 20 – 2000 µg/ml were prepared by diluting an appropriate amount of 2 mg/ml stock albumin solution in DI H_2O (deionized water). Cell lysate samples collected previously (Section 2.2.12) were prepared for protein assay by centrifugating at 13000 rpm for 1 minute to pellet the debris. In a 96-well assay plate, 25 µl of blank (DI H_2O), standards and samples were added in duplicate and 200 µl of BCA working reagent then added to each well. The plate was shaken thoroughly on a plate shaker, covered and incubated at 37 °C for 30 minutes and then allowed to cool to room temperature. Absorbance was measured at 562 nm in a plate reader. A standard curve was prepared by plotting the absorbance of the standards against their concentration, and protein concentrations of the samples determined from this.

2.2.15 Gene expression quantification

Studying the expression of a particular gene and gaining insight into its regulation provides an insight into the control of biological (e.g. metabolic) processes. Alterations in the expression of individual genes or groups of genes reflect key cellular decisions relating to survival, growth, and differentiation. Regulation of gene

expression is orchestrated, in part, by transcription factors that either repress or activate the expression of genes. mRNA analysis requires isolation and purification of RNA from cells followed by quantification and analysis. A number of techniques have been developed for RNA analysis, including agarose gel electrophoresis (northern blotting) and subsequently more specialized and sensitive techniques such as real-time polymerase chain (RT-PCR), quantitative RT-PCR (qRT-PCR) and microarrays.

2.2.16 Total ribonucleic acid (RNA) isolation

Isolating intact RNA is necessary to determine the expression of a particular gene in a target tissue and is therefore the first step in RNA analysis. Since presence of even trace amounts of protein or DNA contaminants can affect the accuracy of gene profiling, it is imperative that the isolated RNA is of high purity. This is further complicated by the presence of ubiquitous enzymes termed RNases that degrade RNA during extraction. Therefore, to achieve a high yield of RNA of suitable purity it is vital to ensure that during tissue handling as well as RNA isolation all reagents and apparatus are RNase free.

RNA isolation involves complete cell lysis or tissue disruption either mechanically by vortexing or sonication or use of a strong denaturant (such as guanidine isothiocyanate (GITC), chloroform or phenol). RNA is extracted from cells by first detaching and then collecting cells by centrifugation. Following this, cells are lysed by suspending the cells in a denaturant solution followed by vortexing.

During this project TRIzol® Reagent (Gibco, 15596-026) was used to isolate total RNA from SGBS cell samples. TRIzol® is a mono-phasic solution of phenol and guanidine isothiocyanate that enables a simple method of cell lysis, maintenance of RNA but disruption and dissolution of other cell components.

2.2.16.1.1 Methodology

Aproximately 5 x 10^6 cells were routinely used for total RNA isolation. Cell were lysed in TRIzol® reagent, centrifuged and transferred into labelled eppendorf tubes. Chloroform (C 2432, Sigma) was added in 1:5 ratio following centrifugation to separate the solution into an aqueous phase and an organic phase (e.g. 200 µl of

chloroform per 1 ml of TRIzol®). The solution was centrifuged at 10,000xg for 15 minutes at 2 to 8 °C. The aqueous phase which exclusively contains RNA was carefully transferred into newly labelled tubes. The organic phase contains the DNA and proteins which can be recovered by sequential precipitation. Isopropyl alcohol (I 9516, Sigma) was then added in 1:1 ratio and samples were incubated for 1 hour at minus 80 °C. After the incubation samples were allowed to defrost for 10 minutes then centrifuged for 20 minutes at 12,000 g at 2 to 8 °C. The aqueous solution was removed and the pellet washed twice in 75 % ethanol (E7023, Sigma) diluted in nuclease free water (pellet was mixed by vortexing, then centrifuged at 7,500 g for 5 minutes at 2 to 8 °C). The waste was carefully removed and RNA allowed drying for 10 minutes, then RNA was resuspended in 10 µl of nuclease free water. RNA concentration was quantified using the Nanodrop system. All reagents were molecular biology grade.

2.2.17 RNA quantification

Prior to analyzing mRNA expression, it is necessary to estimate its concentration and purity. Nucleic acids absorb UV light maximally at 260 nm; therefore by determining the sample absorbance spectrophotometrically at this wavelength it is possible to quantify RNA concentration. If the RNA sample is diluted in water, absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (1 OD 260 Unit = 40 µg/ml of RNA). RNA quantification in a sample can thus be calculated using the formula:

$$A260\ x\ dilution\ x\ 40\ =\ [RNA]\ \mu g/ml$$

Pure RNA exhibits an A260/A280 ratio within the range of 1.8 – 2.0 therefore, by determining the ratio of absorbance at 260 nm and 280 nm ($_{A260/280}$) using a spectrophotometer such as proteins. A ratio of less than 1.7 exhibited by the RNA sample confirms that is contaminated and of low purity.

The Nanodrop ND-1000 (Labtech International; East Sussex, UK) is a UV-Vis spectrophotometer that can be used to determine the concentration and purity of an RNA sample by measuring absorbance at 260 nm. NanoDrop ND-1000 uses surface tension alone to hold the sample in place, eliminating the need for cuvettes.

In addition, the ND-1000 can measure highly concentrated samples without the need for dilution, therefore samples as little as 1 µl can be measured accurately.

2.2.17.1.1 Methodology

The concentration and quality of RNA in total RNA extracts was tested using the NanoDrop ND-1000 spectrophotometer. Briefly, the 'Nucleic Acid' application from the software was selected to measure RNA samples. A blanking cycle was performed first, in which a blank sample (1 µl DNase/RNase free water) was loaded onto the measuring pedestal. The blanking sample was then wiped from the measuring pedestal and 1 µl test sample was pipetted onto the measurement pedestal. The sampling was then closed and a spectral measurement was initiated using the operating software on the PC. On completion of the measurement cycle, the sampling arm was opened and the sample was wiped from the pedestal to prevent sample carryover in successive measurements. Purity of the RNA samples was also determined by analyzing the ratio of absorbance at 260 and 280 (a260/280 subscript).

2.2.18 RT-PCR Primers

QuantiTec® Primer Assay (Qiagen, Mainz, Germany) for use with Qiagen kits for SYBR® Green-based real-time RT-PCR were used during this project for qRT-PCR. The assay includes a mix of lyophilized forward and reverse primers for a specific target. A list of primers used during this project is provided below:

- GAPDH QT 01192646
- AnxA1 QT 00078197
- FPR1 QT 00199745
- FPR2/ALX (FPRL1) QT 00204295
- HIF1 alpha, QT 00083664
- Adiponectin QT 0014091
- Leptin QT 00030261
- IL6 QT 00083720
- CRP QT 00203371

2.2.19 Reverse transcription (first strand cDNA synthesis)

Reverse transcription of first strand synthesis is a process in which a messenger RNA (mRNA) template is copied into its complimentary DNA (cDNA) using a reverse

transcriptase enzyme (RT), a primer, deoxynucleoside triphosphates (dNTPs) and an RNase inhibitor. The cDNA obtained can be subsequently amplified by reverse transcription PCR. The enzyme reverse transcriptase is generally used for first strand cDNA synthesis and requires a double-stranded primer that allows 5' to 3' synthesis of cDNA by annealing to the 3' end of every mRNA. Several types of primers are available for this reaction, including oligo (dT) primers, random (hexamer) primers and gene specific primers, each having their inherent advantages. Oligo primers generate cDNA synthesis by annealing to the poly A sequences at the mRNA 3' end and initiating synthesis from this position. This 3' bias helps in achieving accurate cDNA from RNA. However, reverse transcription of any transcript lacking a polyA tail cannot be carried out. Random hexamer priming does not have the 3' bias as it is not specific for one end of the transcript, however it could result in truncated cDNA synthesis due to annealing of a primer at the 5' end. Gene specific primers target the specific transcript of interest thus leading to significant reduction in complexity and high cDNA yield. These however, are more susceptible to secondary structure within the primer that can result in reduction of reverse transcription efficiency. In a typical reverse transcription reaction, the initial step is to anneal the primer to the mRNA secondary structure by incubating mRNA with the primer and then allowing this to chill quickly on ice. This s followed by adding dNTPs, RNase inhibitor, reverse transcriptase and RT buffer to the reaction mix and carrying out the reaction at 42°C for 1 hour to make the cDNA. Finally, the reaction is heated to 70 °C to inactivate the enzyme.

2.2.20 Quantitative real-time polymerase chain reaction

In the polymerase chain reaction a heat-stable DNA dependent polymerase such as Taq polymerase is used to amplify DNA by *in vitro* enzymatic replication. The technique generates millions of copies of DNA by amplification of a DNA fragment using single stranded DNA as template and DNA oligonucleotides (primers) as initiators for DNA synthesis. The amplification is achieved by repeated cycles of three phases of heating (denaturation), cooling (annealing) and extension generally carried out in a regulatable heating block termed a thermocycler. This can be programmed to heat and cold in the sequence required.

Kinetic or real-time polymerase chain reaction is a modification of the PCR technique that utilizes the general principle of reverse transcription-polymerase chain reaction to simultaneously amplify and quantify target DNA present in as ample in real-time since amplification of DNA can be monitored while it occurs. In qRT-PCR, while the DNA samples are amplified they can be quantified simultaneously by a fluorescent probe or by an intercalator-based method. In either case, a sensitive camera connected to the thermocycler measures the fluorescence in each well of the 72 well plate at frequent intervals.

Fluorescent reporter probe based real time PCR or TaqMan PCR is the more accurate or reliable technique. In this method, DNA samples are amplified via the Taq DNA polymerase and fluorescent markers are used to measure the DNA after each cycle of PCR. The probe is a single stranded oligonucleotide of 20 -26 nucleotides with a fluorigenic reporter at one end and a quencher of fluorescence at the opposite end. The fluorigenic probe binds to the target DNA along with the primers during annealing. Specific PCR products generate a fluorescent signal that is detected and measured by the thermocycler. In each reaction, the threshold cycle (Ct) is determined from the geometric increase in fluorescence corresponding to the exponential increase of the product.

In this project, the gene expression analysis was carried out using two-step TaqMan PCR by an absolute quantification method using an Applied Biosystems 7500 fast RT-PCR system (Applied Biosystems, Cheshire, UK). The Applied Biosystems 7500 system uses TaqMan chemistry and enables rapid analysis of multiple samples in a single 72 well plate. This method enables an absolute quantity of a single nucleic acid target sequence within a sample to be determined. Reverse transcription as well as amplification was carried out simultaneously. Absolute quantification requires the construction of an absolute standard curve for a particular amplicon, and this enabled the precise determination of copy number per cell or total RNA concentration. A stock solution was prepared by pooling cDNA from each sample. Standards were prepared by serial dilution of the cDNA stock solution over a large

range. These were then assayed in duplicate alone and with the positive and negative standards. Ct values for each dilution that were obtained are log values of the initial copy number and the standard curve was therefore, computed by plotting Ct values against logarithm of the initial copy numbers. A housekeeping gene that is expressed at a constant level in most cell types, GAPDH, was used as an endogenous control to normalize the expression pattern of each gene.

2.2.21 Statistical significance

Data were normalized against the housekeeping gene GAPDH using the following primer sets: GAPDH (QT 01192646), AnxA1 (QT00078197), FPR2/ALX (QT 00204295), FPR1 (QT 00199745), adiponectin (QT 00014091), and leptin (QT00030261) and analysed using Relative Expression Software Tool (REST)-2009 (Qiagen, Hilden, Germany). The mathematical model used is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample groups and control groups. Subsequently, the expression ratio results of the investigated transcripts are tested for significance by a Pair Wise Fixed Reallocation Randomisation Test and plotted using SE estimation *via* a complex Taylor algorithm, as previously described (Pfaffl et al., 2002).

Study 3: Is there a free ride? Resveratrol as calorie restriction mimetic.

2.3 Research design and methods

2.3.2 Composition of the food supplement

The nutritional food supplement was obtained as a generous donation from Solgar UK Ltd. (Tring, Herts) in the form of capsules each containing 250 mg/day; from 500 mg of Polygonum cuspidatum; (from root).

2.3.3 Participants and experimental protocol

The present study was a prospective double blind placebo controlled crossover design. Informed consent was sought from volunteers (already part of the previously submitted characterisation study, **App. No. 08/09/22**). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the University of Westminster (**App.No. 09/10/47).** A sample size calculation using IL-6 as a primary outcome parameter indicated a sample size of 15 to achieve a study power of 80 %. Significance for effect of intervention was accepted at $P \leq 0.05$.

2.3.3.1 Inclusion criteria

Inclusion criteria for the study were healthy male, BMI >25, aged 18 -60 yrs, not taking anti-inflammatory drugs during study period or not on any other medication.

2.3.3.2 Procedure

Subjects were randomly assigned to either resveratrol or placebo (brown sugar) groups. Volunteers attended a health screening at the Cavendish Campus of University of Westminster 4 times. Subjects were given a participants information sheet and signed a consent form. The health screening consisted of taking measurements of the blood pressure, height, weight, total % body fat by air displacement plethysmography using BODPOD (Life Measurement, Inc., Concord, California, USA), and waist to hip ratio. The subjects gave two 20ml Vacutainer tubes of venous blood twice, once before the oral glucose tolerance test and again after the test. Venous blood samples were taken by a trained phlebotomist. Blood plasma

was stored in a -80 °C freezer and at a later date analysed for the levels of key adipokines, inflammatory markers and for the metabolic markers (i.e. plasma glucose, cholesterol and insulin).

Subjects were given capsules containing either resveratrol or casein and instructed to take one tablet daily (250mg resveratrol) for two weeks. After two weeks, blood samples were taken again to measure the above parameters. A two week washout period was then observed by all subjects before returning to the study and receiving the crossover supplement (i.e. active if previously placebo and vice versa).

Prior to re-commencing (i.e. after the washout period) blood samples were taken. Subjects were instructed as previously to take one capsule per day for two weeks before returning to have final blood samples taken. Aside from the transient discomfort of venepuncture there are no risks for subjects. A trained phlebotomist obtained all blood samples.

2.3.3.3 Anthropometric measurements

Body weight, height and body composition were measured as described in Chapter 2.2.2 with the exception of skinfold and BIA to determine total body fat level. BODPOD was used to measure total body fat percentage.

2.3.3.4 Metabolic measurements

Blood pressure, fasting blood glucose and cholesterol were measured as described in Chapter 2.2.3.

2.3.3.5 Oral Glucose Tolerance Test

The Oral Glucose Tolerance Test (OGTT) is the gold standard test for the diagnosis of diabetes mellitus, although it is often possible to establish this diagnosis without resorting to a full OGTT. OGTT also allows the diagnosis of impaired fasting glycaemia (IFG), impaired glucose tolerance (IGT) and gestational diabetes. IFG and IGT indicate increased cardiovascular risk. A variation of the OGTT (collecting blood at half-hour intervals for both glucose and growth hormone) is considered the gold standard test for the diagnosis of acromegaly. A prolonged OGTT may also be useful to demonstrate reactive hypoglycaemia.

2.3.3.5.1 Procedure

The volunteers were instructed to follow a normal diet (>150 g carbohydrate daily) for at least 3 days prior to the test, and undertake normal physical activity and were instructed to fast for a minimum of 12 hours prior to testing. Baseline blood glucose was measured and a venous blood sample was taken for the determination of the concentration of the insulin hormone. A glucose solution was prepared by dissolving 75 g anhydrous glucose in 200 ml water. Before commencing OGTT, the volunteer was informed that he needed to remain in the waiting area for 2 hours without eating, drinking or smoking. Volunteers were instructed to drink the glucose solution over a maximum of 15 minutes (ideally within 5 minutes) and the time was noted. The volunteer then remained seated within the waiting area for 2 hours, without eating, drinking or smoking. The test was invalid if these conditions were not imposed. 2 hours after administering the glucose, a second venous blood sample for glucose and insulin was collected and Labelled with the patient's name and the sample date and time (or "2 hour"). Samples were centrifuged and plasma was aliquoted into 2 ml vials and stored in a -80 C freezer for an analysis at a later date.

2.3.3.5.2 Complications

Some individuals may experience faintness, nausea and vomiting after taking the glucose solution. This is often more significant during pregnancy and therefore the risk in this study was very low as we only recruited males. If it was anticipated, instead of the glucose solution, a Lucozade drink was offered as it is better tolerated. Where necessary, volunteers were asked to lie down. If the patient vomited, the test was not completed unless this happened towards the end of the test (after 1 hour).

2.3.3.5.3 Interpretation

Table 13: Guidelines for interpreting OGTT results (mmol/l).

	Fasting glucose (mmol/l)		2-hour glucose (mmol/l)
Normal	< 6.1	*and*	< 7.8
Impaired fasting glycaemia	≥ 6.1 and < 7.0	*and*	< 7.8
Impaired glucose tolerance	< 7.0	*and*	≥ 7.8 and < 11.1
Diabetes mellitus	≥ 7.8	*and/or*	≥ 11.1

Source: (WHO, 2006). A low sugar diet prior to the OGTT may lead to a more normal result than that obtained on a normal diet. Either a high 2-hour or a raised fasting glucose may indicate diabetes Diabetes: In view of the implications of a diagnosis of diabetes upon a volunteer's lifestyle, and the possible employment implications, all such diagnoses need confirmation by a referral to a specialist doctor. Impaired fasting glycaemia and impaired glucose tolerance: These conditions are regarded as risk factors indicating an increased long-term probability of developing diabetes, rather than diagnoses in their own right.

2.3.3.6 Measurements of inflammatory markers

The inflammatory markers, adipokines and AnxA1 were determined using techniques as described in Section 2.1.2.

2.3.4 Statistical analyses

Statistical comparison of the aforementioned blood parameters were assessed of relative effects of resveratrol versus placebo and performed using Microsoft Excel 2007. Summary statistics included means and standard errors of the mean for differences between groups and differences between baseline and 14 days of intervention. Data was checked for normal distribution using the Kolmogrov-Smirnov goodness of fit test. ANOVAs were employed to examine effect of intervention plus intervention-time effects. Paired student's t-test was used to check for statistical significance between placebo and resveratrol treatment. 2-sided statistical significance was assumed at $p \leq 0.05$.

3 Attenuation of plasma AnxA1 in human obesity

Abstract

Context: Obesity related metabolic disorders are characterized by mild chronic inflammation as a result of adipocytokine production from fat tissue leading to dysregulation in the pro/anti-inflammatory systemic balance. AnxA1, is an endogenous glucocorticoid regulated protein, which mediates systemic anti-inflammatory processes. The expression of AnxA1 with increased adiposity has not been investigated to date and it is plausible to hypothesize that AnxA1 may have a role in the pro-resolving phase of inflammation in the adipose tissue.
Objective: Our objective was to examine the association between the anti-inflammatory mediator, AnxA1 and body composition and inflammatory markers in healthy humans.
Design, participants, and interventions: We recruited 118 healthy males (mean ± S.E.M. age 40.46 ± 0.96 years, body mass index (BMI) 27.6 ± 0.7 kg/m^2) for an *in vivo* study measuring both metabolic and anthropometric parameters. Plasma AnxA1 levels were correlated with levels of adiposity and distribution of fat, BMI, waist to hip ratio (WHR), and plasma adipocytokines.
Main outcome measures: Our results show that plasma AnxA1 protein is significantly inversely correlated with BMI ($p < 0.001$), total body fat (BF) % ($P = 0.037$) waist to hip ratio ($P < 0.001$) in healthy male subjects suggesting that as central fat mass increases the concentration of plasma AnxA1 decreases.
Results: Plasma AnxA1 was also significantly correlated with CRP ($P = 0.002$) and leptin ($P = 0.002$). Plasma adiponectin, leptin, and CRP showed significant trends with BMI, %BF and WHR as previously reported.
Conclusions: These data demonstrate that AnxA1 could potentially represent a (fat) depot specific biomarker whose decline with increasing central adiposity may relate to the phenomena of increasing systemic inflammation and associated disease risk.

3.1 Introduction

Adipose tissue is vital for normal function of the human body; however, excess adiposity which is defined as an increased BMI of > 25 kg/m^2 is strongly linked with the cause of type 2 diabetes, cardiovascular disease and certain types of cancer. Certain metabolic, physiological and lifestyle factors such as dyslipidaemia, arterial hypertension, large waist size, BMI >25 kg/m^2 and low activity levels have been identified as increasing risk factors for metabolic syndrome (Otani, 2011, Zalesin et al., 2011). It is surprising that to date the exact underlying pathology of these disease states is still unknown and yet to be discovered. However, there is evidence showing that the metabolic syndrome is characterised by a mild chronic inflammation found in the systemic circulation (Yudkin, 2007, Yudkin, 2003). To date, the exact underlying cause of these disease states is yet to be discovered. It has been shown that obesity induces local inflammation in the adipose tissue (Yudkin, 2007, Yudkin, 2003, Nishimura et al., 2009a). Excessive NF-kappa B activity is believed to be associated with the development of type 2 diabetes and cardiovascular risk as obese individuals express high circulating protein levels such as CRP, TNF-α and IL-6 (Berg and Scherer, 2005, Grimble, 2002, Pickup and Crook, 1998).The adipokines and pro-inflammatory markers are also linked to insulin sensitivity, glucose metabolism, inflammation and atherosclerosis and they may provide a molecular link between excess adiposity and the development of type 2 diabetes and cardiovascular disease (Ye, 2011). It is thought that the signals sent from WAT directly influence insulin resistance and inflammation (Trayhurn, 2005, Wood et al., 2009). As a result, circulating levels of adipokines and pro-inflammatory markers may be valuable as biomarkers to estimate the risk of other disease states associated with obesity. Whilst there is a considerable body of literature regarding adipokines and pro-inflammatory cytokines (Do et al., 2006, Trayhurn and Wood, 2004, Wood et al., 2009) the anti-inflammatory adipocytokines warrant further exploration. It is also of interest to study some of the anti-inflammatory mediators in order to understand more fully the inflammatory-hormonal nexus of the adipose tissue. AnxA1 is an anti-inflammatory mediator and we proposed that it may also have a role in the function of the adipose tissue inflammation. AnxA1, a 37-kDa and Ca2+ - and phospholipid-binding protein, is an endogenous glucocorticoid regulated protein, which mediates

systemic anti-inflammatory processes (D'Acquisto et al., 2008b, Pupjalis et al., 2011, Dalli et al., 2011) including reducing eicosanoid synthesis (Sudlow et al., 1996), antipyretic effects (Davidson et al., 1991), an antiendotoxic action (Wu et al., 1995) and reduced neutrophil emigration from the systemic circulation (Perretti, 1998). AnxA1 plays an important role in the resolution of inflammation via macrophage phagocytic clearance of apoptotic neutrophils (Maderna et al., 2005, Dalli et al., 2011, Perretti and Dalli, 2009). It is a process that prevents the exposure of tissues at the inflammatory site to the noxious and potentially immunogenic contents of lytic cells (Babbin et al., 2008, Dalli et al., 2011, Perretti and D'Acquisto, 2009). The molecular mechanisms by which AnxA1 modulates these cellular responses are not fully understood. However, AnxA1 was associated with NF-kappa B and it was shown to suppress its transcriptional activity by preventing NF-kappa B binding to DNA (Zhang et al., 2010c, Wang et al., 2011) and this may explain some of AnxA1's anti-inflammatory actions. A number of studies have suggested that AnxA1 deficiency may contribute to the aetiology of inflammatory diseases (Bensalem et al., 2005, Dalli et al., 2010). AnxA1 has a wide range of functions, including the suppression of inflammation and modulation of the release and signalling of insulin (Parente and Solito, 2004, Grigorescu et al., 1991). AnxA1 is expressed in mice adipocytes (Raynal et al., 1993), however its function in adipogenesis and lipid metabolism warrants further exploration. A recent study investigated the expression of AnxA1 in patients with significant carotid stenosis. Their data showed that high AnxA1 expression might bring about a stabilising outcome in asymptomatic patients who display less atherosclerotic deposits (Cheuk and Cheng, 2011). Since the aetiology of atherosclerosis surrounds an inflammatory process, AnxA1 may play an essential role in preventing plaque complications or disease progression. The expression of AnxA1 with increased adiposity has not been investigated to date. However, given the evidence showing AnxA1's role in resolving inflammation, it is plausible that AnxA1 may be altered in individuals with increasing adiposity.

3.2 Objectives

Our objective was to examine the association between the anti-inflammatory mediator, AnxA1 and body composition and inflammatory markers in healthy male subjects.

3.3 Results

3.3.2 Participants

Overall, 118 healthy male subjects were recruited for this study. Out of the study group 47 were of normal weight (mean ± SD age 40.87 ± 10.41 years, BMI 22.68 ± 1.91 kg/m^2 , %BF 16.23 ± 6.13 , WHR 0.86 ± 0.05), 45 were overweight (mean ± SD age 41.44 ± 10.29 years, BMI 26.98 ± 150 kg/m^2, %BF 22.23 ± 6.27, WHR 0.92 ± 0.05), 13 were obese class I (mean ± SD age 43.38 ± 11.21 years, BMI 31.91 ± 1.54 kg/m^2, %BF 32.87 ± 5.04, WHR 1.03 ± 0.08), and 13 were obese class II (mean ± SD age 32.62 ± 6.58 years, BMI 43.72 ± 6.94 kg/m^2, %BF 41.21 ± 7.10 % , WHR 1.04 ± 0.08) (see Table 14).

Table 14: Frequencies of participants by BMI (kg/m^2), %BF & WHR categories and age range (years).

	N = 118	Age group (years)				Total
		<30	**30.1 - 40**	**40.1 - 50**	**>50.1**	
BMI	Normal	9	12	16	10	**47**
	Overweight	5	16	15	9	**45**
	Obese I	1	3	6	3	**13**
	Obese II	6	5	2	0	**13**
Total		**21**	**36**	**39**	**22**	**118**
%BF	Lean	6	5	3	2	**16**
	Moderately lean	6	12	16	4	**38**
	Excess fat	2	11	12	11	**36**
	Risky	7	8	8	5	**28**
Total		**21**	**36**	**39**	**22**	**118**
WHR	High	6	14	17	16	53
	Normal	15	22	22	6	65
Total		**21**	**36**	**39**	**22**	**118**

Total number of participants was N=118. The number of obese class I and II subjects was relatively low (N == 13) when compared to subjects with normal BMI (N = 47) and overweight (N = 45). The distribution of subjects across %BF was lean (N = 16), moderately lean (N = 38), excess fat (N = 36), and risky (N = 28).

The participants were healthy but at a slightly high (N = 36) and high risk (N =28) of developing metabolic syndrome due to having excess and risky level of total body fat, respectively (see Table 14 and Table 16).

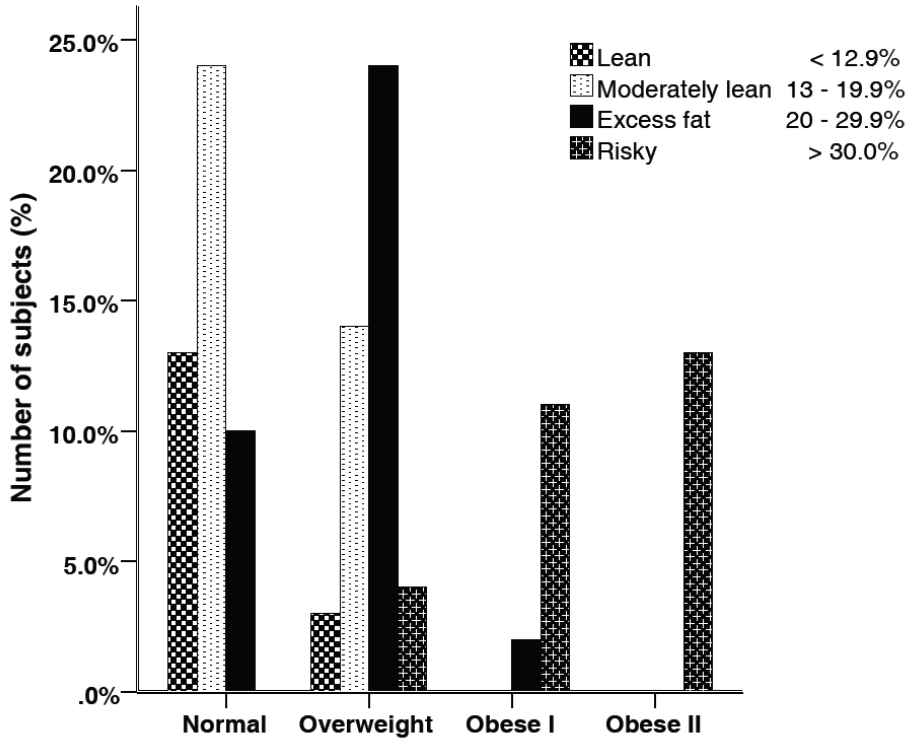
Figure 42 indicates that a preponderance of the study cohort falls into the category of overweight and obese with excess fat when not accounted for age. It is interesting to note therefore, that BMI indices, as described by the Quetelet values indicated in Table 14, do not reflect 'overweight' as a majority fraction of the total number of subjects, as well as body fat percentage as determined by the BODPOD does. It is also important to note that majority of obese II subjects were below 40 years of age and that the study was lacking obese I and obese II subjects in the above 40 and 50 age group categories (see Table 14). It also should be noted that to detect age related significant difference ($P < 0.05$, power of study = 80 %) according to our sample size calculation, we would need a minimum 26 participants in each arm. Table 14 indicates that the age group < 30 and >50 does not meet this requirement with N values equal to 21 and 22, respectively. The same accounts for BMI category in the obese I and obese II class (N=13 for both) and for lean %BF category, where N= 16.

3.3.3 Metabolic parameters

Overall, mean age for the study cohort was found to be 40.46 ± 10.39 years and Table 15 indicates that the participants overall were at a slightly high all-cause mortality risk due to being overweight with mean BMI 27.66 ± 6.95 kg/m^2 and having excess body fat with mean level of 23.10 ± 10.18 %. The blood parameters showed the mean blood glucose level to be within the normal range and a normal concentration of total blood cholesterol except the obese subjects had marginally high fasting blood glucose concentration (mean = 5.74 ± 3.86 mmol/L). However, the mean glycated LDL (1.76 mg/dl) was comparable with the mean concentration (1.66 ± 0.66 mg/dl) found in 70 separate plasma samples from patients with diabetes mellitus (Dolhofer and Wieland, 1980, Guthrow et al., 1979).

Blood pressure as indicated by systolic and diastolic measures is unremarkable, with no age group displaying clinical hypertension. Homeostatic model assessment for insulin resistance (HOMA) IR , HOMA $_{\beta\text{-cell,}}$ quantitative insulin sensitivity check index (QUICKI) and fasting insulin sensitivity index (FISI) were derived from fasting blood glucose and insulin measurements and were all found to be within the normal range showing a normal glucose and insulin function when compared with values found in other studies (Kim et al., 2011, Lee et al., 2011b) (see Table 16).

Figure 42: Distribution of BMI (kg/m2) and total body fat % of all participants.



Morphometric data showing all-cause mortality risks according to BMI classification (normal BMI, normal/low risk; overweight, moderate risk; obese I, high risk; obese II, very high risk). BMI classifications are as follows: normal BMI, 18.5–25 kg/m^2; overweight, 25.1–30 kg/m^2; obese class I, 30.1–35 kg/m^2; obese class II, >35.1 kg/m^2. Within the BMI range, the distribution of subjects (n, %) according to body fat levels is shown.

We did not find any significant differences across BMI groups and any of the indices of glucose metabolism, however there was a significant correlation between blood glucose concentration of the normal and obese I BMI (P = 0.044, Table 16) displaying the obese I subjects borderline glucose intolerant.

Table 16 shows the means and distribution of the level of adipocytokines. Table 18 shows the means ± SD for the hormones and inflammatory mediators measured according to BMI distribution. We noticed that plasma adiponectin level decreased whilst BMI increased except in the obese II category where we see a mean of 3.75 ± 2.56 µl/ml whilst the mean for the overweight and obese I subjects are 3.20 ± 1.78 and 2.93 ± 1.13 µl/ml, respectively.

Table 15: Means of metabolic parameters of the study cohort.

N = 118	Min	Max	Mean	Std. Deviation	S.E.M.
Age (years)	19.00	61.00	40.46	10.39	0.96
BMI (kg/m^2)	16.80	56.40	27.66	6.95	0.64
%BF	4.30	51.80	23.10	10.18	0.94
WHR	0.78	1.14	0.92	0.08	0.01
Glucose (mmol/L)	3.30	18.50	4.76	1.38	0.13
Cholesterol (mmol/L)	3.08	7.19	4.83	0.82	0.08
Glycated LDL (mg/ml)	0.34	3.44	1.76	0.55	0.05
Systolic BP (mmHg)	100.00	178.00	124.14	14.17	1.30
Diastolic BP (mmHg)	56.00	116.00	79.52	9.63	0.89
HOMAIR	0.00	0.80	0.11	0.12	0.01
HOMA$_{\beta\text{-cell}}$	-0.69	1.9	0.20	0.28	0.03
QUICKI	0.27	0.72	0.38	0.08	0.01
FIRI	0.06	12.91	1.85	1.94	0.18

Overall subjects (N = 118) were healthy with most metabolic parameters within the normal ranges, with the exception of BMI (mean = 27.66 kg/m^2) and %BF (mean = 23.1 %) which were in the overweight and excess fat category, respectively.

We have found plasma leptin to be positively correlated with BMI ($p > 0.001$, $R = 0.807$), %BF ($p > 0.001$, $R = 0.848$) and WHR ($p < 0.001$, $R = 0.706$) (see Figure 43, 44, and 45) as previously reported (Wauters et al., 2000). Plasma leptin was also significantly correlated with both systolic ($p = 0.021$, $R = 0.225$) and diastolic BP ($p < 0.001$, $R = 0.386$), (see Table 16).

Plasma adiponectin level was inversely correlated with BF % ($p = 0.037$, $R = -0.192$) and WHR ($p = 0.009$, $R = -0.239$) but not with BMI ($p = 0.128$, $R = -0.141$) (see Table 16, Figure 46 and Figure 47). Table 18 also shows other significant correlations found

Table 16: Anthropometric and metabolic characteristics of participants according to BMI range.

Variable	Normal (N =47)	Overweight (N = 45)	Obese I (N = 13)	Obese II (N = 13)
Age (years)	40.87 ± 1.5	41.44 ± 1.5	43.38 ± 3.1	32.62 ± 1.8 * §
BMI (kg/m^2)	22.6 ± 0.28	26.98 ± 0.22 ***	31.91 ± 0.42 *** §§§	43.72 ± 1.93 *** §§§ ###
%BF	16.23 ± 0.67	22.23 ± 0.69 ***	32.87 ± 1.27 *** §§§	41.21 ± 2.08 *** §§§ ###
WHR	0.86 ± 0.007	0.92 ± 0.008 ***	1.03 ± 0.02 *	1.04 ± 0.02 *** §§§
Glucose (mmol/L)	4.61 ± 0.07	4.64 ± 0.08	5.74 ± 1.06 *	4.72 ± 0.19
Cholesterol (mmol/L)	4.66 ± 0.08	4.99 ± 0.13	4.90 ± 0.26	4.85 ± 0.32
Glycated LDL (mg/dl)	1.77 ± 0.54	1.76 ± 0.55	1.66 ± 0.67	1.87 ± 0.41
Systolic BP (mmHg)	121.49 ± 2.00	124.82 ± 2.0	127.70 ± 5.5	127.85 ± 3.1
Diastolic BP (mmHg)	77.62 ± 1.3	78.44 ± 1.4	83.92 ± 2.3 *	85.69 ± 3.1 *
HOMAIR	0.12 ± 0.02	0.09 ± 0.01	0.18 ± 0.06	0.11 ± 0.04
HOMA$_{\beta\text{-cell}}$	0.22 ± 0.05	0.18 ± 0.03	0.21 ± 0.06	0.46 ± 0.27
QUICKI	0.37 ± 0.01	0.40 ± 0.01	0.37 ± 0.02	0.39 ± 0.02
FISI	2.02 ± 0.24	1.41 ± 0.18	2.84 ± 0.96	1.81 ± 0.73
Insulin (µIU/ml)	10.90 ± 1.4	7.48 ± 0.92	11.25 ± 2.5	9.59 ± 3.7

Anthropometric and metabolic characteristics of participants (N = 118). Categorical and continuous variables were tested using ANOVA followed by Tukey's post hoc test; 2-tailed statistical significance was applied to all tests. *P < 0.05, ***P < 0.001 vs.normal; §P < 0.05, §§§P < 0.001 vs. overweight; ###P < 0.001 vs. obese I.

between the variables measured. Plasma cortisol was positively correlated with BMI ($p = 0.022$, $R = 0.211$, see Figure 60), diastolic BP ($p = 0.029$, $R = 0.201$) and plasma leptin concentration ($p = 0.026$, $R = 0.217$, see Table 16 and Table 18). Plasma CRP concentration was significantly associated with BMI ($p < 0.001$, $R = 0.442$), %BF ($p < 0.001$, $R = 0.358$) and WHR ($p < 0.001$, $R = 0.382$) as shown in Figure 49, Figure 50, and Figure 51.

Table 17: Descriptives of adipokine and inflammatory mediators.

Variable	Min	Max	Mean	Std. Deviation	S.E.M.
Insulin (µIU/ml)	0.28	49.65	9.49	8.84	0.81
Adiponectin (µl/ml)	0.33	12.86	3.75	2.41	0.22
Leptin (pg/ml)	11.70	1391.30	386.72	343.88	33.56
Cortisol (ng/ml)	6.63	188.66	59.65	41.13	3.79
CRP (pg/ml)	0.06	9.57	1.45	1.57	0.14
IL-6 (pg/ml)	0.00	332.98	16.04	47.41	4.36
TNFα (pg/ml)	0.59	906.98	46.76	115.89	10.67
IL-10 (pg/ml)	1.34	1230.87	30.15	116.20	10.70
Annexin A1 (pg/ml)	4.35	312.34	91.41	64.12	5.90
IL-17 (pg/ml)	0.14	360.75	24.02	49.56	4.56

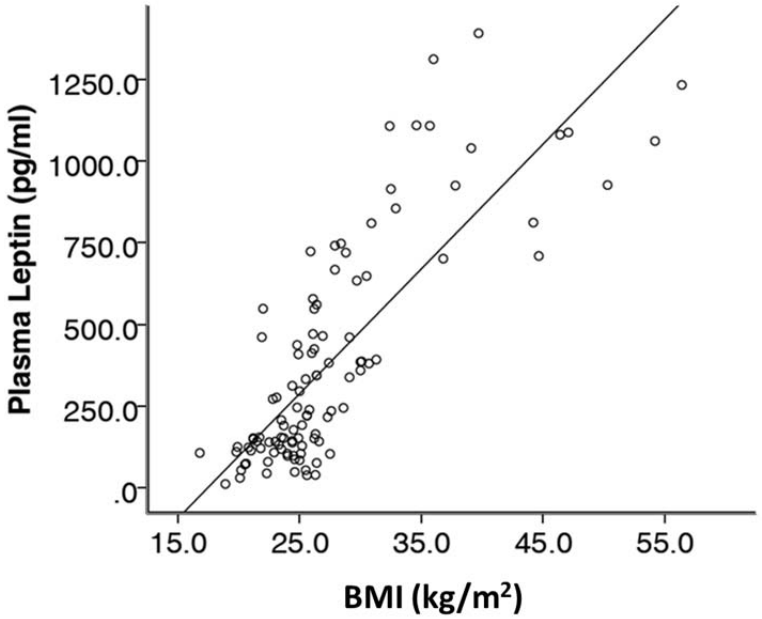
The min, max, mean, SD and SEM values of hormones and cytokines measured in the fasting blood of the subjects (N= 118).

Table 18: Fasting blood hormone, adipokine and inflammatory markers profile for the participants according to the BMI range.

Variable	Normal (N =47)	Overweight (N = 45)	Obese I (N = 13)	Obese II (N = 13)
Annexin A1 (pg/ml)	118.29 ± 10.5	88.84 ± 7.2	57.30 ± 13.5 **	37.19 ± 12.6 *** §
Cortisol (ng/ml)	52.19 ± 5.1	57.30 ± 6.8	76.35 ± 12.1	78.04 ± 10.3
Adiponectin (µl/ml)	4.49 ± 0.43	3.20 ± 0.26 *	2.93 ± 0.31	3.75 ± 0.71
Leptin (pg/ml)	164.42 ± 17.3	347.73 ± 34.9 ***	733.83 ± 98.9 *** §§§	1029.86 ± 59.1 *** §§§ ##
CRP (pg/ml)	0.81 ± 0.12	1.37 ± 0.24	2.76 ± 0.40 ** §§§	2.75 ± 0.53 *** §§§
IL-6 (pg/ml)	15.17 ± 7.4	19.28 ± 8.5	17.44 ± 7.2	28.38 ± 14.4
TNFα (pg/ml)	30.60 ± 10.4	52.51 ± 21.9	89.66 ± 43.0	42.36 ± 20.2
IL-10 (pg/ml)	17.93 ± 3.5	24.36 ± 6.7	108.48 ± 93.6	16.03 ± 5.1

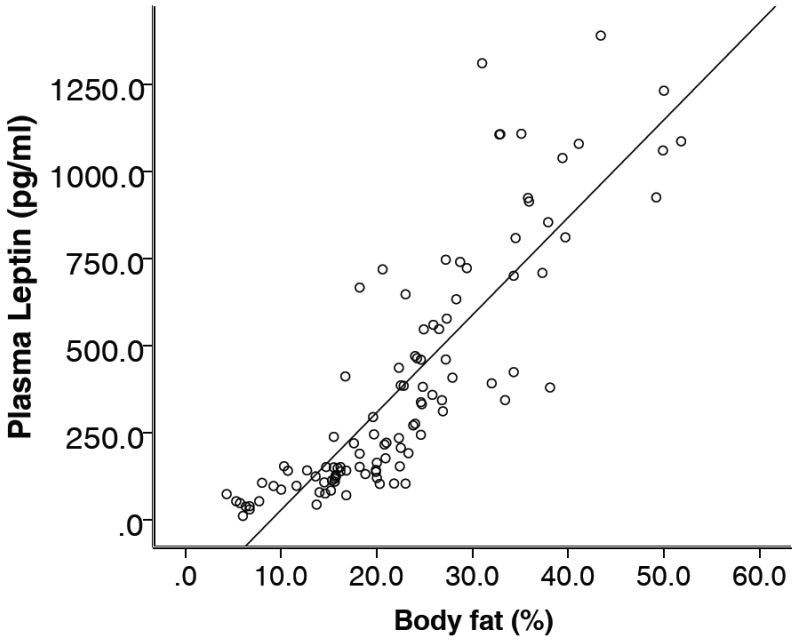
Fasting blood hormone, adipokine, and inflammatory marker profiles for the participants (N = 118). Values are expressed as means ± SEM. Categorical and continuous variables were tested using ANOVA followed by Tukey's post hoc test; 2-tailed statistical significance was applied to all tests. *P < 0.05, **P < 0.01, ***P < 0.001 vs. normal; §P < 0.05, §§§P < 0.001 vs. overweight; ##P < 0.01 vs. obese I.

Figure 43: Correlation between plasma leptin (pg/ml) and BMI (kg/m^2).



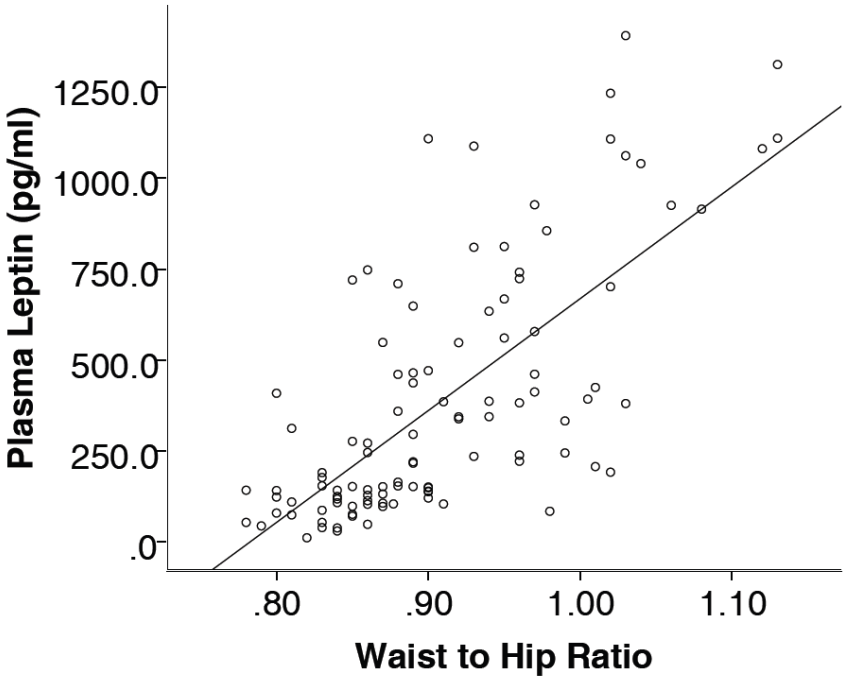
The figure shows a significant correlation (R = 0.807**, P < 0.0001) between plasma leptin concentration plotted against BMI (kg/m^2) (N = 118) where * - P < 0.05, ** - P < 0.01, *** - P < 0.001 (N = 118).

Figure 44: Correlation between plasma leptin (pg/ml) and body fat (%).



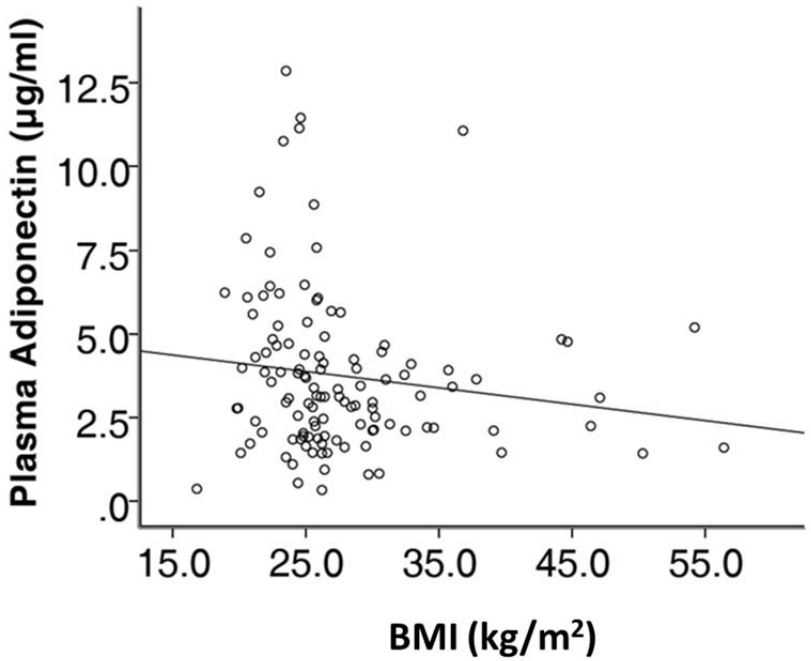
The figure shows a significant correlation (R = 0.848**, P <0.0001) between the plasma leptin concentration plotted against body fat % (N = 118).

Figure 45: Correlation between plasma leptin (pg/ml) and WHR.



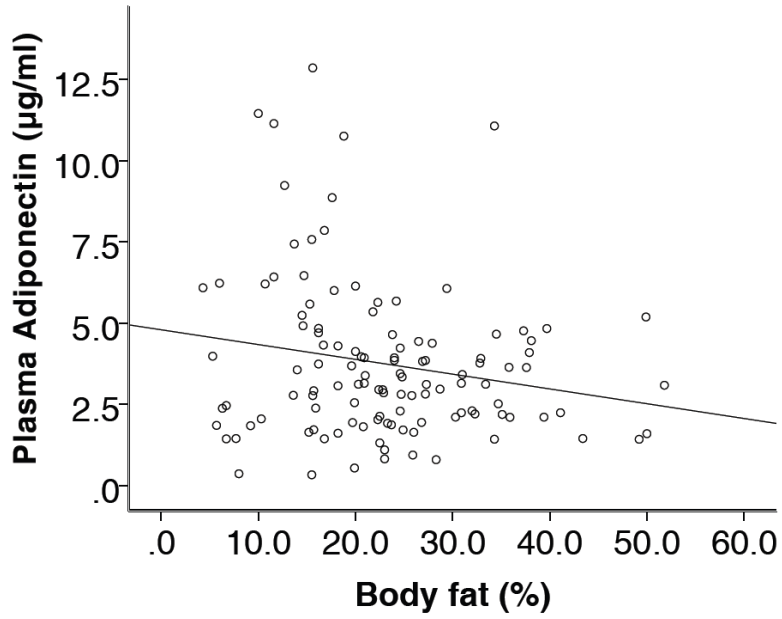
The figure shows a significant correlation (R = 0.706**, P < 0.0001) between plasma leptin concentration plotted against WHR (N = 118).

Figure 46: Correlation between plasma adiponectin and BMI (kg/m^2).



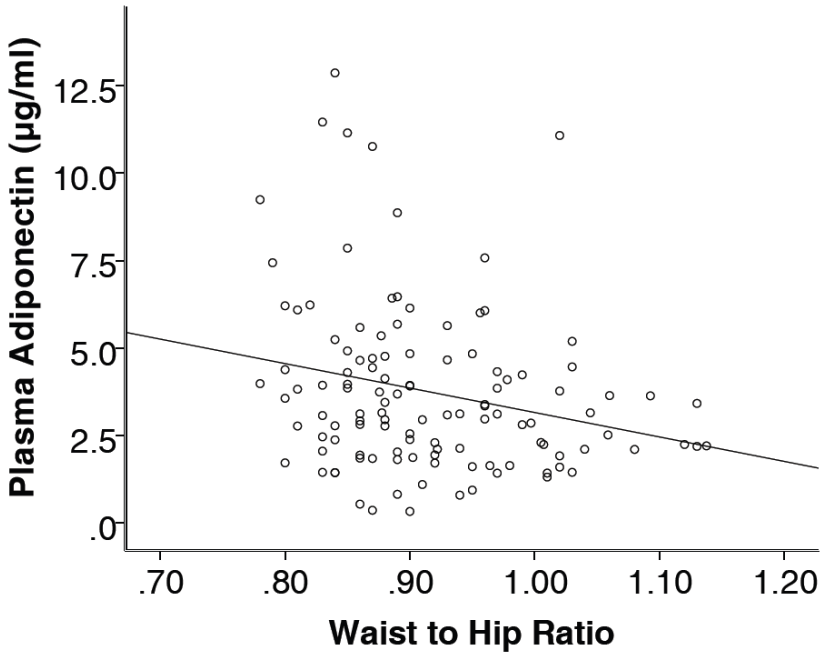
The figure shows no significant correlation (R = -0.141, P = 0.128) between the plasma adiponectin concentration plotted against BMI (kg/m^2) (N = 118).

Figure 47: Correlation between plasma adiponectin (µg/ml) and body fat (%).



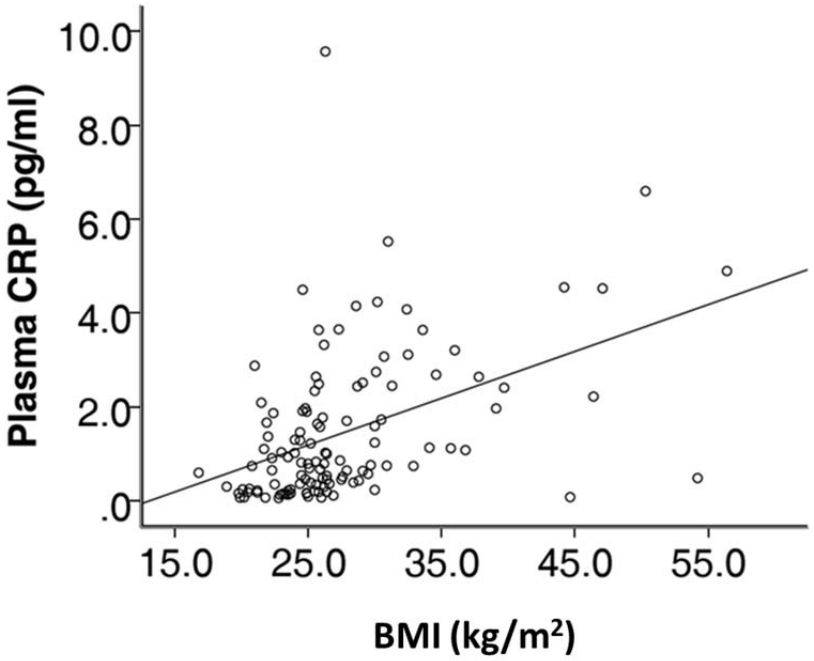
The figure shows the significant correlation (R = -0.192*, P = 0.037) between plasma adiponectin concentration plotted against body fat % (N = 118).

Figure 48: Correlation between plasma adiponectin (µg/ml) and WHR.



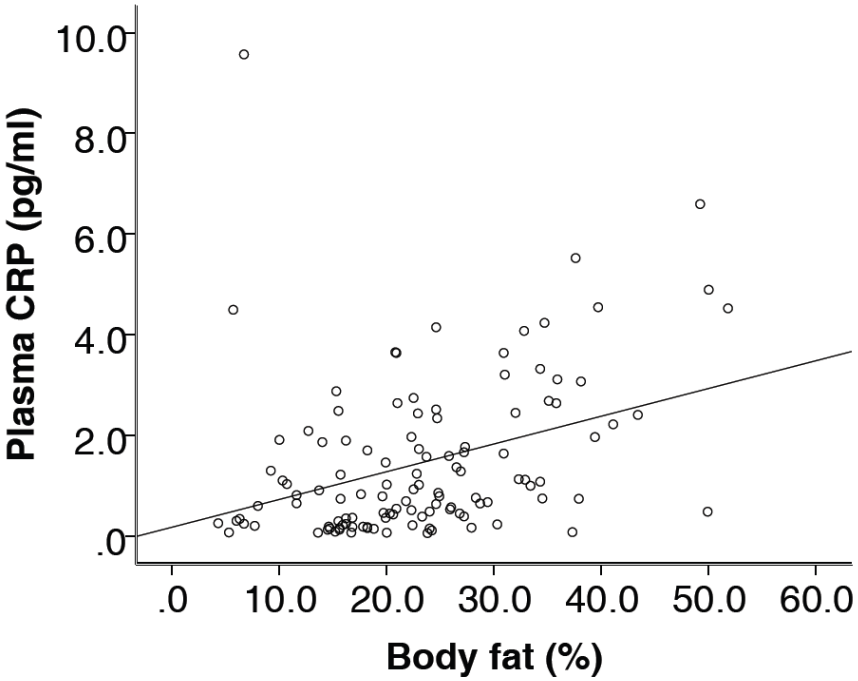
The figure shows the significant correlation (R = -0.239**, P = 0.009) between the plasma adiponectin concentration plotted against WHR (N = 118).

Figure 49: Correlation between plasma CRP (pg/ml) and BMI (kg/m^2).



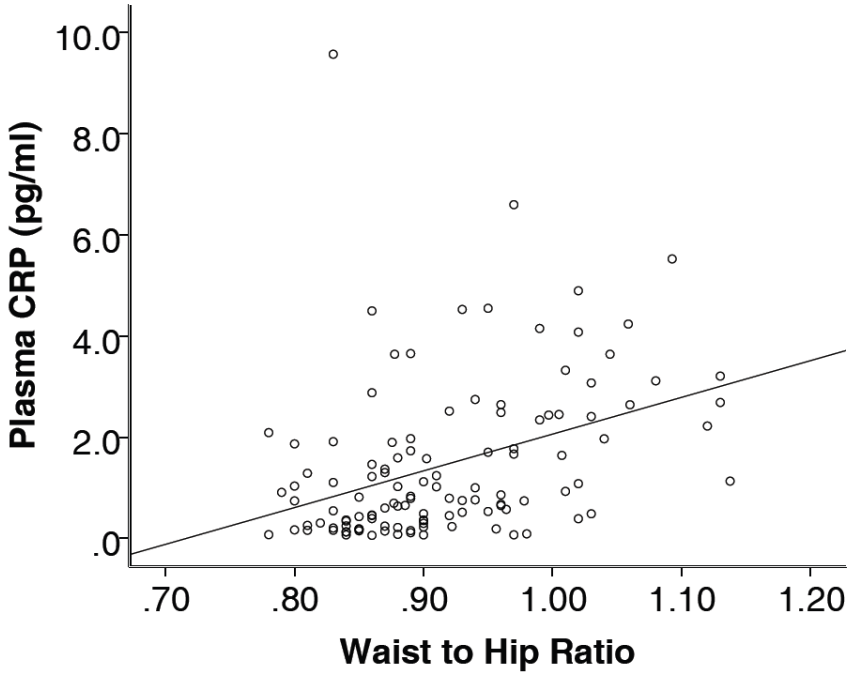
The figure shows the significant correlation (R = 0.442**, P < 0.0001) between the plasma CRP concentration plotted against BMI (kg/m^2) (N = 118).

Figure 50: Correlation between plasma CRP (pg/ml) and body fat %.



The figure shows the significant correlation (R = 0.358**, P < 0.0001) between plasma CRP concentration plotted against body fat % (N =118).

Figure 51: Correlation between plasma CRP (pg/ml) and WHR.

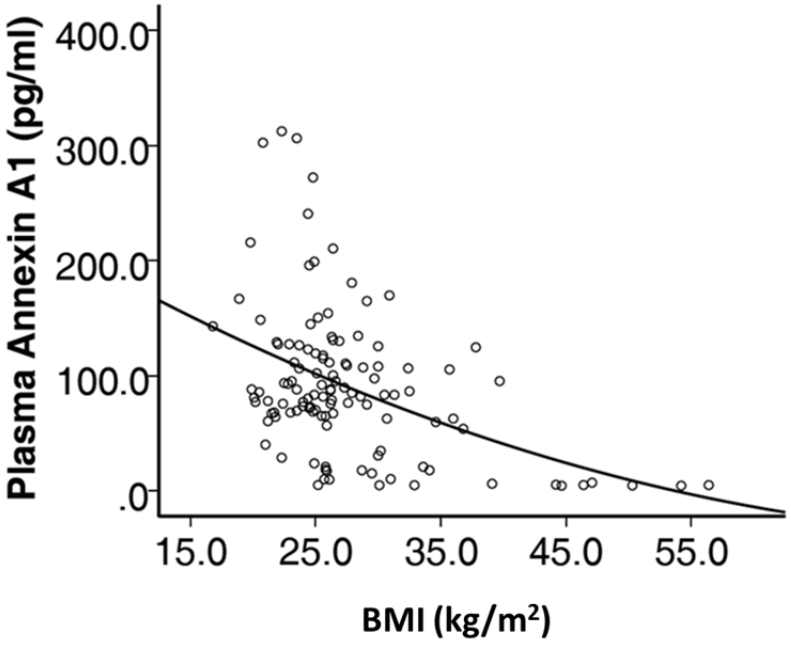


The figure shows the significant correlation (R = 0.382**, P < 0.0001) between the plasma CRP concentration plotted against WHR (N = 118).

3.3.4 Annexin A1

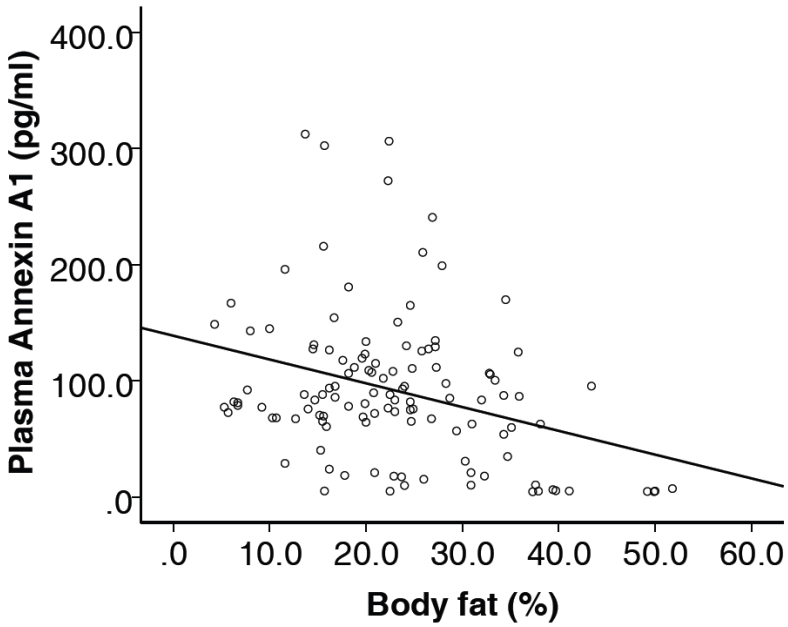
Our results show that plasma AnxA1 protein is significantly inversely correlated with BMI (p <0.001, R = -0.424), BF % (p = 0.037, R = -0.192) and WHR (P = 0.009, R = -0.239) as shown in Figure 52, 53 and 54 suggesting that as (central) fat mass increases the concentration of plasma AnxA1 decreases. Figure 55, Figure 56, and Figure 57, represent the level of plasma AnxA1 according to the BMI, %BF and WHR range. It was interesting to note that plasma AnxA1 was similar in the normal and overweight at (mean± S.E.M) 118.29 ± 10.50 and 88.85 ± 7.20 pg/ml whilst it decreased 2 and 4-fold in the obese I (57.30 ± 13.50 pg/ml) and obese II (37.20 ± 12.60 pg/ml), respectively. A similar trend was found across the BF % range (Figure 56) and WHR (see Figure 57). While age and blood glucose or any of the HOMA of insulin resistance appears to have no correlation with AnxA1 concentrations, our data do display trends towards inverse relations between AnxA1 and total blood cholesterol (p = 0.06, R = -0.0424) as shown in Figure 61. A further analysis of the blood lipids i.e. a correlation between glycated LDL and AnxA1, indeed did show a significant positive association (P = 0.017, R=0.22) as demonstrated in Figure 62.

Figure 52: Correlation between plasma annexin A1 (pg/ml) and BMI (kg/m^2).



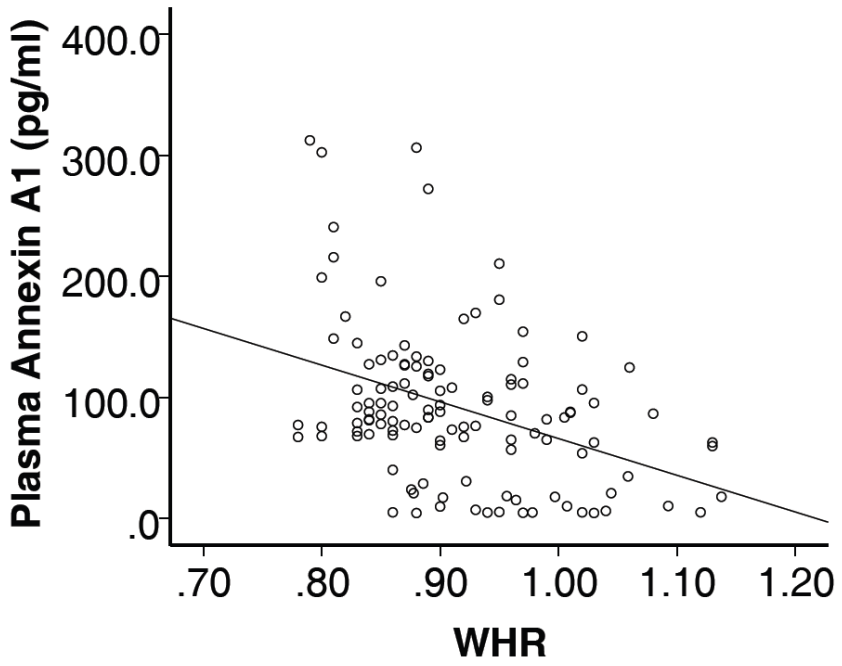
The figure shows a significant correlation (R = -0.424**, P < 0.001) between the plasma annexin A1 concentration (pg/ml) plotted against the BMI (kg/m^2) (N = 118).

Figure 53: Correlation between plasma annexin A1 (pg/ml) and total body fat % determined via air displacement plethysmography.



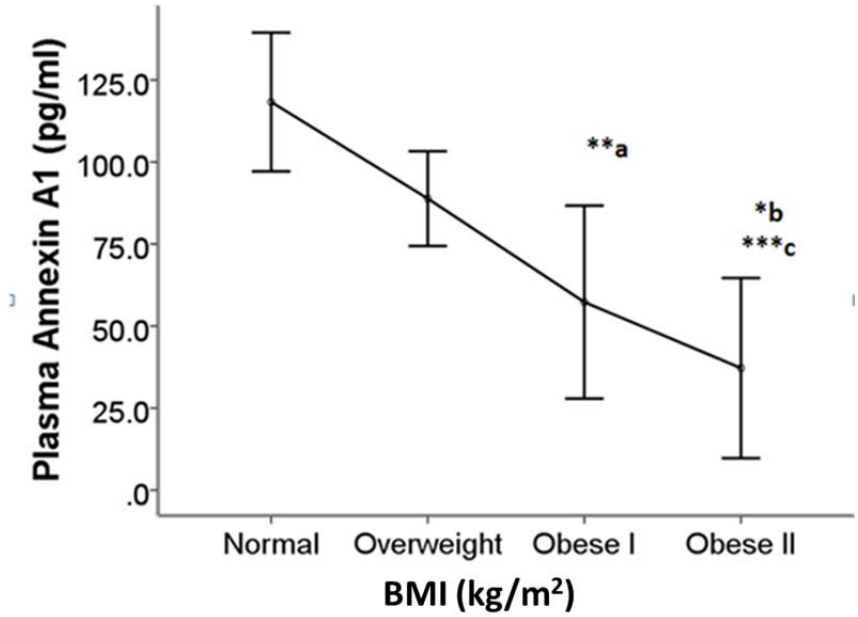
The figure shows a significant correlation (R = -0.192*, P = 0.037) between the plasma annexin A1 concentration plotted against the total body fat % (N =118).

Figure 54: Correlation between plasma annexin A1 (pg/ml) and waist to hip ratio.



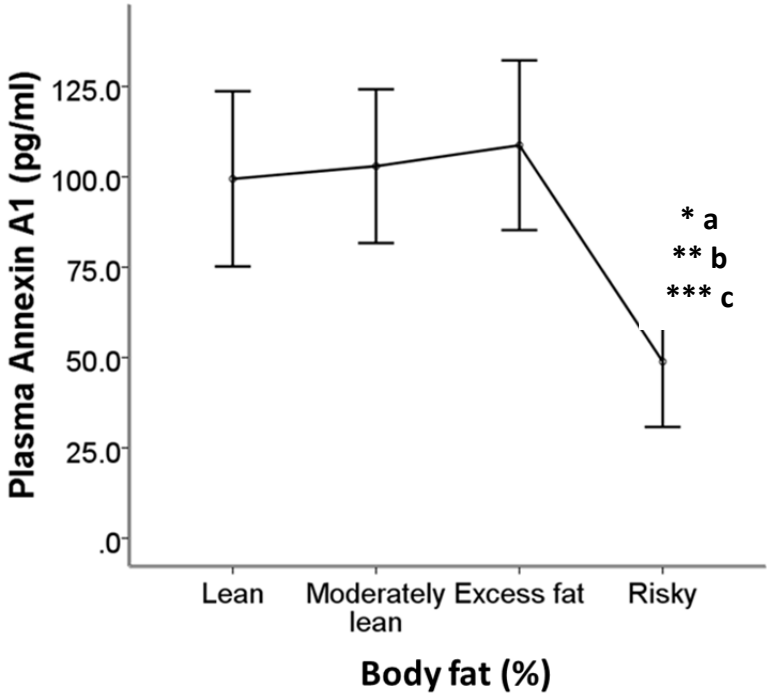
The figure shows a significant correlation (R = -0.390**, P < 0.001) between plasma annexin A1 concentration plotted against waist to hip ratio (N =118).

Figure 55: Mean plasma annexin A1 (pg/ml) across BMI range (kg/m^2).



Relationship between mean AnxA1 concentration and BMI categories. **a)** Normal vs. obese class I; **b)** Overweight vs. Obese class I; **c)** Normal vs. obese II. Values are expressed as means ± SEM. Statistical significance was determined by 1-way ANOVA followed by Tukey's post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001; 2-tailed (N = 118).

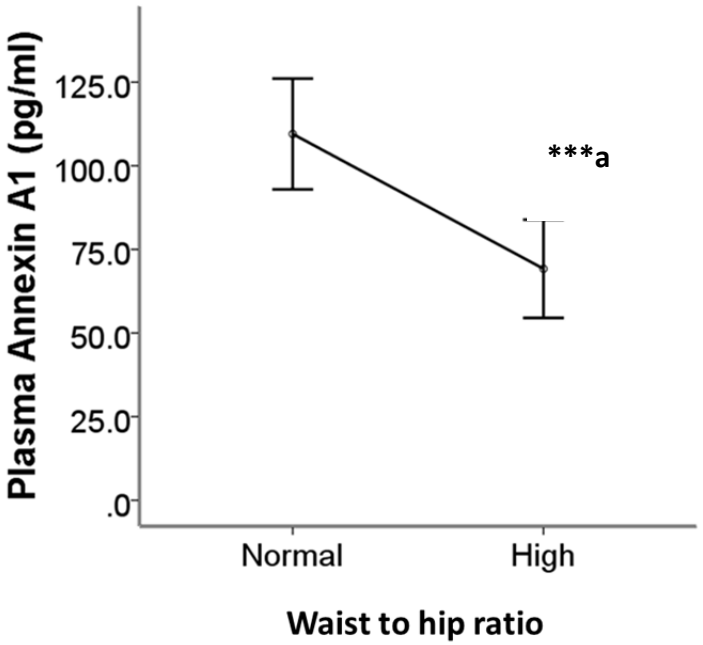
Figure 56: Mean plasma annexin A1 (pg/ml) across total body fat level range.



Relationship between plasma AnxA1 and total %BF range: lean, < 12.9 %; moderately lean, 13–19.9 %; excess fat, 20–29.9 %; risky, > 30 %. **a)** Lean vs. risky; **b)** Moderately lean vs. risky; **c)** Excess fat vs. risky. Values are expressed as means ± SEM. Statistical significance was determined by 1-way ANOVA followed by Tukey's post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001; 2-tailed (N = 118).

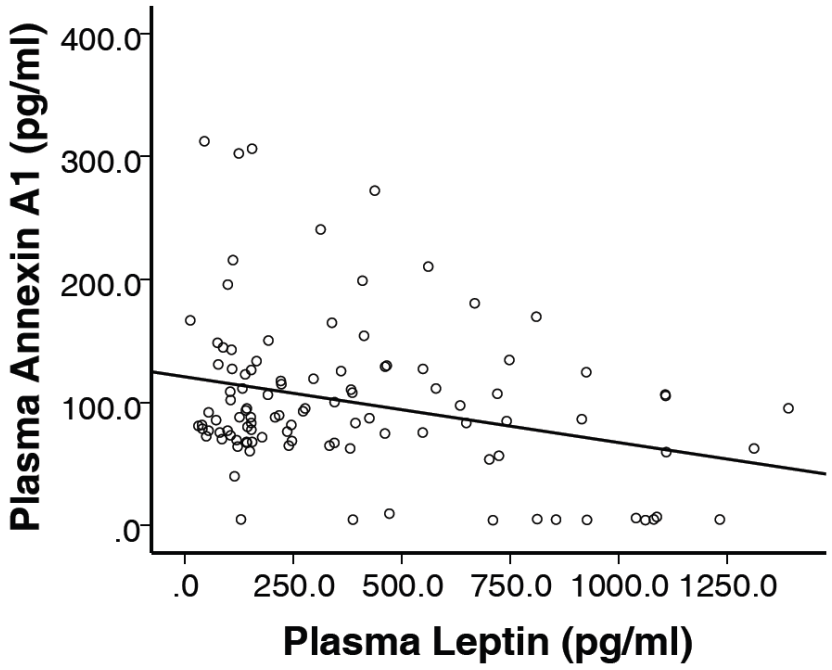
We looked at the association between plasma AnxA1 and adipokines secreted from the WAT and found an inverse correlation between plasma leptin concentration and plasma AnxA1 ($p = 0.002$, $R = -0.294$, Figure 58). There was no correlation between plasma adiponectin nor plasma cortisol and AnxA1. Whilst there was a significant inverse correlation between plasma AnxA1 and plasma CRP level ($p = 0.002$, $R = -0.288$, Figure 59). No associations were found with IL-6, IL-10, and TNF α (see Table 18).

Figure 57: Mean plasma annexin A1 (pg/ml) across waist to hip ratio range.



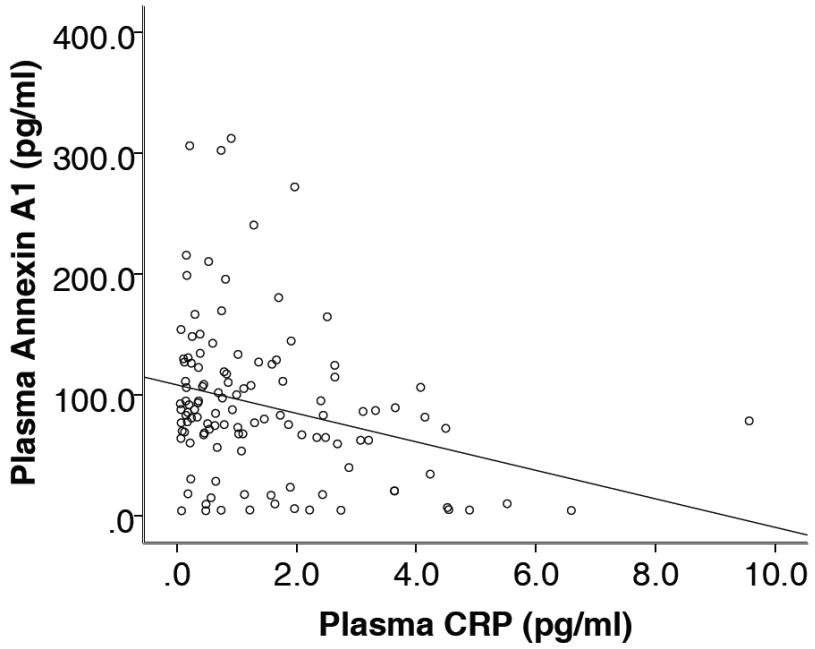
Relationship between plasma AnxA1 and WHR range: normal, < 0.95; high, > 0.95. **a)** Normal vs. High. Values are expressed as means ± SEM. Statistical significance was determined by 1-way ANOVA followed by Tukey's post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001; 2-tailed (N = 118).

Figure 58: Correlation between plasma annexin A1 (pg/ml) and plasma leptin concentration.



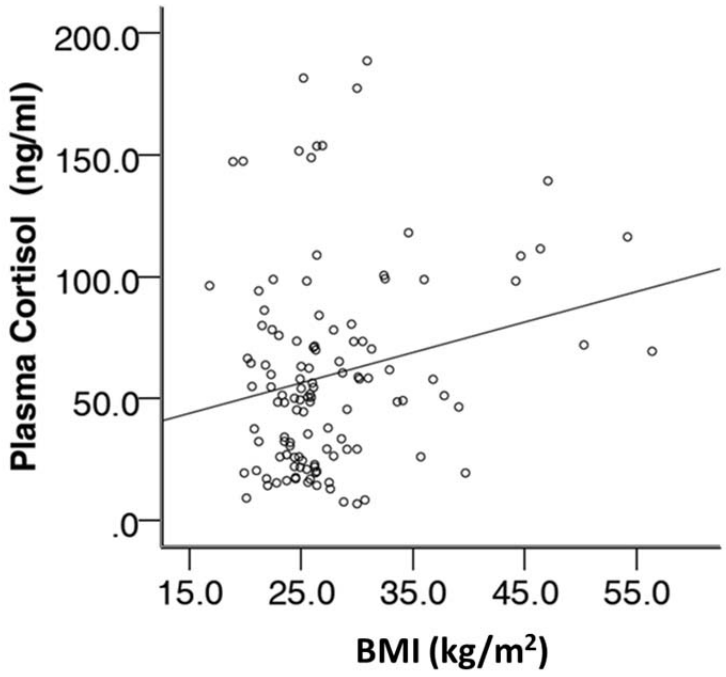
The figure shows a significant correlation (R = -0.294**, P = 0.002) between plasma annexin A1 concentration plotted against plasma leptin concentration (pg/ml) (N = 118).

Figure 59: Correlation between plasma annexin A1 (pg/ml) and plasma CRP level.



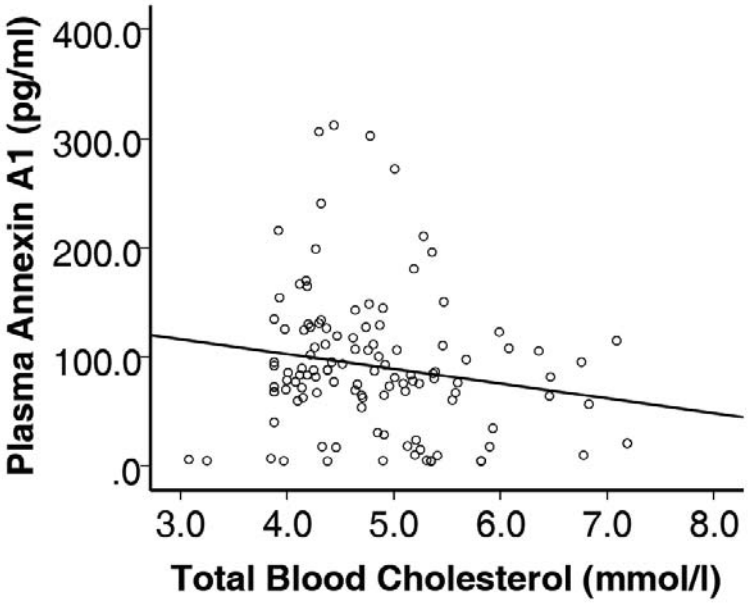
The figure shows a significant correlation (R=-0.288**, P = 0.002) between plasma annexin A1 concentration plotted against plasma CRP concentration (pg/ml) (N = 118).

Figure 60: Correlation between plasma cortisol (ng/ml) and BMI (kg/m^2).



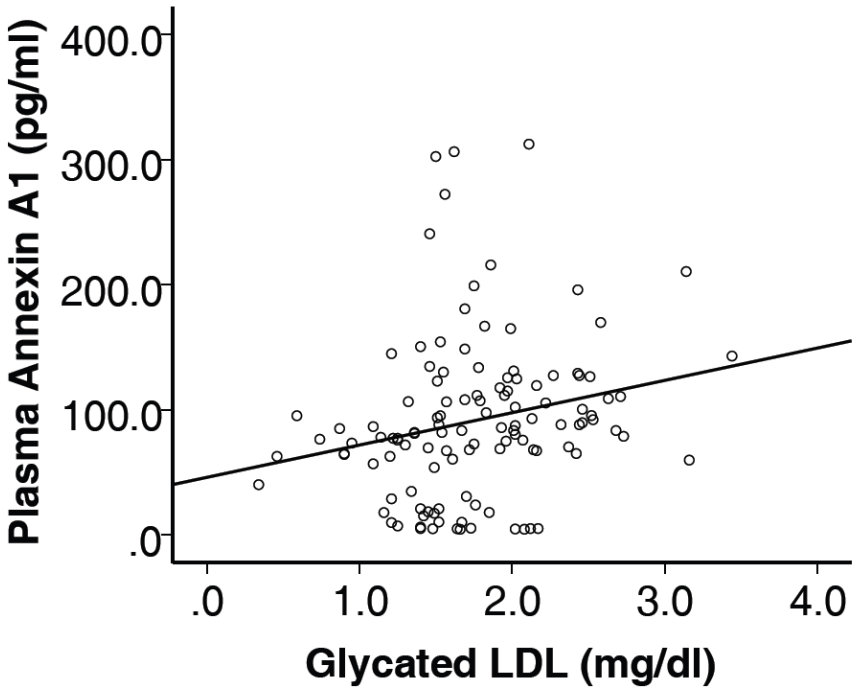
The figure shows a significant correlation (R=0.211, P=0.02) between plasma cortisol (ng/ml) plotted against BMI (kg/m^2) (N = 118).

Figure 61: Correlation between plasma annexin A1 and total blood cholesterol.



The figure shows a trend (R= -0.173, P=0.060) between plasma annexin A1 concentration (pg/ml) plotted against BMI (kg/m^2) (N = 118).

Figure 62: Correlation between plasma annexin A1 (pg/ml) and glycated LDL (mg/dl).



The figure shows a significant correlation (R=0.22*, P = 0.017) between the plasma annexin A1 concentration plotted against plasma glycated LDL concentration (mg/dl) (N = 118).

3.4 Discussion

To the best of our knowledge, this is the most extensive human study investigating plasma AnxA1. We examined the relationship between the pro-resolution mediator, plasma AnxA1 and body composition, total body fat level and body fat distribution. We also investigated the relationship between plasma AnxA1 and metabolic and inflammatory parameters as well as the plasma concentration of specific adipokines. 118 healthy volunteers aged between 19 and 61 years with varied BMI and body fat levels took part in this cross sectional study (see Table 15). Our results confirmed that plasma leptin concentration is significantly correlated with BMI ($P < 0.001$), total BF % ($P = 0.037$) and WHR ($P < 0.001$) (see Figure 52, 53, 54). Whilst plasma adiponectin level was significantly negatively correlated with total %BF and WHR, it failed to reach significance with BMI (Figure 46, 47 and 48) as a previous study reported (Kuo and Halpern, 2011). In agreement with previously published data (Tsuriya et al., 2011, Firat Guven et al., 2011, DeLoach et al., 2011), we also found a significant positive correlation ($P < 0.001$) between BMI, %BF and WHR with plasma CRP concentration as shown in Figure 49, 50 and 51.

The present study indicates that plasma AnxA1 is significantly reduced with increasing BMI, total body fat and increasing centrally located body fat (Figure 55, 56, and 57). The negative correlation of decreasing plasma AnxA1 was strongest statistically when compared with WHR, rather than total body fat, suggesting that centrally located fat may be more influential at reducing plasma AnxA1 concentrations. Extensive evidence shows that the specific distribution of body fat is strongly correlated with increased risks for the development of type 2 diabetes and cardiovascular disease (Gallagher et al., 1996, Gallagher et al., 2000, Ko et al., 1999, Vazzana et al., 2011, Doyle et al., 2011, Lam et al., 2011, Barac et al., 2012, Indulekha et al., 2011).

Our data show that plasma AnxA1 level decreases in subjects characterized by endomorphic body type and therefore, AnxA1 may be a potential depot specific biomarker and a predictor of future health risks and complications. Our data may also infer that AnxA1 could potentially have a protective role in the development of

obesity. When mean plasma AnxA1 protein levels are presented for each total body fat group (defined by WHO as lean, moderately lean, excess fat and risky (WHO, 1995), mean plasma AnxA1 levels are not statistically different across the lean, moderately lean and excess fat groups. However, there is a significant decrease in the mean plasma AnxA1 concentration in the risky group compared to all other groups (see Figure 56). Similarly, when categorized by BMI, the obese I and obese class II groups expressed significantly lower plasma AnxA1 compared to individuals with normal BMI (Figure 55). It is tempting to speculate therefore, that AnxA1 may have a protective role at the onset of central obesity but becomes overwhelmed as central fat mass continues to rise. Taking into account AnxA1's anti-inflammatory role in both the innate and adaptive immune systems, this may well explain the fact that there is a mild chronic inflammation associated with obesity (Trayhurn et al., 2008a, Wang et al., 2007, Trayhurn et al., 2008b, Do et al., 2006, Trayhurn and Wood, 2004, Rai and Sandell, 2011, Siervo et al., 2011, Pruller et al., 2011).

A previous study demonstrated that AnxA1 gene deletion in mice led to epididymal fat pad mass reduction. Whilst adipocyte size was not altered, the study suggested that adipocyte number was decreased (Warne et al., 2006), therefore demonstrating that AnxA1 may have an important role in the regulation of fat mass and in particular adipogenesis/proliferative events. In a study investigating the effect of long-term calorie restriction (LER) in male C57/Bl6 mice, it was reported that AnxA1 gene was expressed abundantly in the epididymal WAT (Higami et al., 2006). The LER treatment led to a significant reduction in a number of inflammation-related genes, amongst which, AnxA1 was found to be suppressed markedly (68 %).

Emerging evidence shows that obesity is associated with mild, chronic, sub-clinical inflammation and studies on lean mice show that the gene expression of inflammatory markers is suppressed in WAT and therefore anti-inflammatory markers are likewise reduced (Perretti and Gavins, 2003, Wu and Ballantyne, 2009). This leads to the hypothesis that adipose tissue is a major contributor to this inflammation. The role of AnxA1 has been investigated using an AnxA1 null mouse and demonstrate the inhibitory properties of this mediator in the context of

inflammation and/or tissue-injury. AnxA1 has been shown to act as a pivotal homeostatic mediator, where if absent, inflammation would overshoot and be prolonged (Perretti and Dalli, 2009). Pivotal to this anti-inflammatory action of AnxA1 protein is the anti-migratory action it exerts over the process of leukocyte extravasation (Perretti and Gavins, 2003). WAT has been shown to be infiltrated with macrophages in obese individuals (Weisberg et al., 2003). Recent evidence also suggests that T cells are key regulators of adipose tissue inflammation and promote the recruitment and activation of macrophages in WAT (Nishimura et al., 2009b). It is therefore; plausible to hypothesize that adipocyte derived AnxA1 protein may be involved in the control of leukocyte infiltration into adipose tissue, a process trigged by obesity.

It is well established that AnxA1 found in leukocytes of the innate immune system, such as phagocytic cells, is positively regulated by glucocorticoids. Conversely, lymphocyte AnxA1 mRNA and protein is decreased upon exposure to glucocorticoids (D'Acquisto et al., 2008a), suggesting a different role for this protein in each branch of the immune system. Our finding in this study, suggests that there was a positive correlation (P = 0.022, R=0.211*, Figure 60) between plasma cortisol and BMI. No direct correlation between plasma cortisol and plasma AnxA1 may indicate that the source of plasma AnxA1 may be contributed to by a number of different sources, or at least that plasma AnxA1 is not positively regulated by plasma glucocorticoids as in the case of phagocytic cells of the innate immune system.

Our study in humans shows a decrease in plasma AnxA1 concentration as the WAT expands in male subjects. Currently, it is not clear which tissues contribute to the plasma AnxA1 levels and further studies are needed to investigate the degree of expression of AnxA1 protein from visceral and subcutaneous fat in human subjects.

Further evidence suggests that AnxA1 administration may improve insulin sensitivity. It was demonstrated that treatment with rosiglitazone, a member of thiazolidinedione class of anti-diabetic agents used to enhance insulin sensitivity, led to an induction of abdominal subcutaneous adipose tissue AnxA (1 and 2) proteins

(Ahmed et al., 2010) suggesting that perhaps AnxA1 may also be involved in the regulation of glucose metabolism. Additionally, our data also showed a trend between plasma AnxA1 concentration and fasting total blood cholesterol (P = 0.06, R = - 0.150, Figure 61) and a significant correlation between glycated LDL and AnxA1 (P = 0.017, R = 0.22, Figure 62) suggesting that there may be a link between the function of AnxA1 and plasma lipids and consequently cardiovascular health risk. Glycated LDL is also used as a marker of glucose metabolism over a preceding week. It is therefore, tempting to speculate that the anti-inflammatory role of AnxA1 may be important in the glycation process of apoB protein. Moreover, a recent study investigated the expression of AnxA1 in patients with significant carotid stenosis (Cheuk and Cheng, 2011). Their data showed that high AnxA1 expression may bring about a stabilising outcome in asymptomatic patients who display less atherosclerotic deposits. Since atherosclerosis is an inflammatory process, AnxA1 may play an essential role in preventing plaque complications or disease progression in these patients.

3.5 Conclusion

Overall, these data suggest that AnxA1 may indeed be related to a range of key physiochemical body systems. Our study demonstrates that AnxA1 protein may have a protective role at the onset of central obesity as plasma AnxA1 is significantly negatively correlated with BMI, %BF and WHR. These findings indicate that dysregulation in the concentration of plasma AnxA1 with continued expansion of WAT and associated weight gain, may contribute to the aetiology of metabolic syndrome and diseases associated with mild chronic inflammation. Data, now widely accepted in the existing literature, linking omental fat deposition patterns to disease states has already been mechanistically associated with inflammatory processes (Wood et al., 2009, Trayhurn et al., 2008b). Furthermore, plasma AnxA1 may offer some promise as a depot specific biomarker of adipose tissue and/or therapeutic potential to control adipose tissue inflammation via its actions as a pro-resolution factor. Future studies investigating AnxA1's role in adipogenesis and WAT macrophage infiltration may elucidate the aetiology of obesity syndrome associated maladies.

4 AnxA1 peptide reduces inflammation in an *in vitro* model of obesity

Abstract

Context: Previous studies demonstrated that the N-terminal peptide of AnxA1 (peptide Ac2-26) can mimic the anti-inflammatory actions of the full-length protein in various systems. In the current study, we report the effectiveness of the peptide Ac2-26 as an anti-inflammatory agent in a model of human Simpson Golabi Behmel Syndrome (SGBS) adipocytes. We have previously demonstrated that plasma AnxA1 protein is significantly inversely correlated with BMI, body fat level and waist to hip ratio.

Objective: Given that low-level systemic inflammation is seen in metabolic syndrome-associated chronic pathologies, here, we investigate if Ac2-26 peptide alters or influences the inflammatory markers in human SGBS cells.

Design and intervention: To mimic the relative hypoxia found in the white adipose tissue of obese humans, mature adipocytes (14 days post-confluent) were treated with 4, 8 and 24 hour hypoxia treatment (1% O_2). The cells were also treated with 10^{-5} M Ac2-26 peptide or vehicle (DMEM/F12 medium supplemented with insulin [20 nM]). Cells were collected and SYBR green PCR analysis performed for CRP, IL-6, adiponectin and leptin genes which have been all shown previously to be altered by hypoxia (1% O_2) in SGBS cells.

Results: We have shown that ANXA1 gene is expressed in human SGBS adipocytes and hypoxia reduces the expression of ANXA1 gene showing that AnxA1 may act as a counter regulator of adipose tissue inflammation.We found that CRP expression was significantly down-regulated following 4 (P=0.015), 8 (P=0.035) and 24 (P=0.037) of hypoxia treatment in the cells also treated with Ac2-26 peptide compared to vehicle alone. IL-6 was also found to be significantly down-regulated after 24 hour hypoxia treatment in the Ac2-26 treated cells compared to vehicle (P=0.022).

Conclusion: Here, we demonstrate for the first time that an AnxA1 mimetic, Ac2-26 peptide, regulates pro-inflammatory markers in human SGBS adipocytes. These data show the effectiveness of the peptide Ac2-26 as an anti-inflammatory therapeutic agent in a human SGBS adipocyte model. Furthermore, AnxA1 may be an important modulator of inflammatory and pro-resolution pathways necessary to restore homeostasis in the inflamed adipose tissue of the obese.

4.1 Introduction

Following on from our characterisation study of inflammatory markers and their association with BMI, distribution and level of adiposity we have set out an investigation into the potential role of the anti-inflammatory molecule AnxA1 in adipocytes. The benefit of an *in vitro* model include examination of the effects of various interventions (e.g. mimicking tissue hypoxia) or treatment (e.g. stimulation with an anti-inflammatory agent) on the expression of genes and proteins. We have previously found that AnxA1 plasma protein is inversely correlated with BMI, %BF and W:H ratio. We speculated that AnxA1 may play a role in adipose tissue function and set out a study to investigate if ANXA1 gene is expressed in human WAT as this has not been previously measured. Previous studies demonstrated that the N-terminal peptide of AnxA1 (peptide Ac2-26) can mimic the anti-inflammatory actions of the full-length protein in various systems. In the current study, we investigate the effectiveness of the peptide Ac2-26 as an anti-inflammatory agent in a model of human Simpson Golabi Behmel Syndrome (SGBS) adipocytes. Given that low-level systemic inflammation is seen in metabolic syndrome-associated chronic pathologies, here, we investigate if Ac2-26 peptide can have an effect on the inflammatory markers in human SGBS cells.

4.1.2 *In vitro* models of adipogenesis

Most of the current knowledge describing molecular mechanisms and signal transduction pathways involved in adipogenesis emerged from studies on pre-adipocyte cell lines. Whilst primary pre-adipocytes represent the most suitable model to investigate adipocyte metabolism, their application is limited by their low proliferative capacity and the rapid decline in differentiation potential during subculturing (Wabitsch et al., 2001). Consequently, adipose tissue function has been widely studied using pre-adipocyte cell lines derived from rodents. Numerous studies used a rodent pre-adipocyte cell line known as 3T3-L1 and 3T3-F442A from a heterogenous Swiss 3T3 cell line that had been derived from mouse embryos (Bullo et al., 2005, Chen et al., 2006a). In recent years, there have been advances in obtaining immortalised human pre-adipocyte cell lines. A novel human pre-adipocyte cell line was established (Wabitsch et al., 2001) and it was derived from an

infant with SGBS characterised with a retained capacity for adipogenic differentiation. SGBS is an X-linked congenital overgrowth disease described by abnormalities in growth development including macroglossia, macrosomia and renal and skeletal abnormalities (Ng et al., 2009). The aetiology of this syndrome is not completely understood and it appears to arise because of either deletions or point mutations within the glypican-3 (GPC3) gene at Xq26. The cell GPC3 gene of SGBS pre-adipocytes was sequenced for detection of any abnormalities and none were detected (Fischer-Posovszky et al., 2008). There have been around 70 publications to date on studies using SGBS pre-adipocyte cell line. Functionally, SGBS adipocytes behave like primary human adipocytes (obtained from subcutaneous tissue) differentiated *in vitro* expressing comparable gene pattern (Wabitsch et al., 2001). The SGBS cell line has also been verified against the well-established 3T3-L1 murine adipocyte cell line showing comparable adipocyte-specific gene expressions and therefore, indicating its relevance to *in vitro* models for adipocyte biology and function research in humans (Allott et al., 2012).

4.1.3 AnxA1, FPR1 and FPR2/ALX function in adipose tissue

To date there is very limited amount of research on the role of AnxA1 and its two receptors in a human adipocyte and their relation to obesity. We postulated that that AnXA1 may be a new potential target for anti-inflammatory pathway to control inflammation in the adipose tissue of obese individuals. Following the publication of our study findings (Kosicka et al., 2013) that AnxA1 is inversely correlated with BMI, %BF and W:H ratio, Akasheh et al., (2013) demonstrated that AnxA1 may indeed, be involved in modulation of levels of adiposity. They demonstrated that ANXA1 knock-out mice are more susceptible to weight gain and diet-induced insulin resistance when compared to wild-type mice (Akasheh et al., 2013). Furthermore, AnxA1 protein extracted from subcutaneous adipose tissue of obese individuals was increased in the old obese individuals when compared to young obese individuals (Alfadda et al., 2013).

4.1.4 Hypoxia inducible factor-1 alpha (HIF-1α)

There are two models of hypoxia that mimic the low oxygen tension found in the adipose tissue of obese individuals. Low oxygen tension within adipocytes induces HIF-1 α. HIF-1 α is a heterodimer comprising of the O_2 modulated HIF-1 α subunit and the O_2-independent HIF-1 β subunit (Wang et al., 1995). HIF-1 α protein is hydroxylated at proline-402 and proline-564, the O_2 dependent degradation (ODD) sites and it is unstable in normoxia. HIF-1 α can be degraded by three different prolyl-hydroxylases named PHD1, PHD2 and PHD3 using a variety of co-factors including oxygen, 2-oxoglutarate, iron and ascorbate (Biswas et al., 2013). However, in hypoxic conditions HIF-1 α does not undergo hydroxylation. Instead of being degraded the molecule becomes stabilised (Semenza, 2004, Maxwell, 2005). HIF-1 α is cleaved with HIF-1 β during nuclear translocation and gene transcription is initiated. Once activated by the low oxygen tension HIF-1 α binds to target genes involved in energy regulation, angiogenesis, cell death and cancer biology (Goda et al., 2003, Semenza, 2010) via the action of NF-κB (Bonello et al., 2007). HIF-1 α can also be induced in normoxic conditions by various endocrine and inflammatory stimuli including thrombin (Gorlach et al., 2001), IL-1 β and TNF α (Hellwig-Burgel et al., 1999), insulin (Zelzer et al., 1998). Increased expression of HIF-1 α has also been linked with the chronic inflammatory status of adipocytes of obese individuals where insulin is a key mediator of adipogenesis (He et al., 2011). He et al., (2011) found that insulin induces HIF-1 α mRNA in adipose tissue of mice. However, the underlying mechanisms of this action are not currently understood.

SGBS cell lines have been extensively used to study the biology and function of human adipocytes (Allott et al., 2012, Bao et al., 2005, Geiger et al., 2011). A recent study for example investigated the effect of resveratrol on an *in vitro* model of inflamed adipose tissue (Zagotta et al., 2013). Treatment of SGBS cells with resveratrol decreased the expression of plasminogen activator inhibitor-1 (PAI-1) potentially via inhibition of the NF-κB pathway. PAI-1 is an inflammatory marker and is linked with an increased risk factor for cardiovascular complications. The expression of adipocytokines has been also studied using this cell cell in hypoxic conditions using either a modular incubator chamber to create hypoxic tissue culture

environment (defined as 1 % O_2, 94 % nitrogen (N) and 5 % CO_2) or using $CoCL_2$. $CoCL_2$ is a chemical inducer of HIF-1 α (Piret et al., 2002). Adiponectin expression was found to be down-regulated by hypoxia in SGBS cells, whilst leptin expression was induced in this model (Hosogai et al., 2007, Wang et al., 2007, Wood et al., 2007, Ye et al., 2007). Mimicking hypoxic conditions *in vitro* led to an up-regulation of pro-inflammatory markers including CRP, IL-6 and TNFα (Geiger et al., 2011, Mack et al., 2009).

4.2 Objectives

In the current research project, SGBS cells were used as an adipocyte model to study the following:

1. The expression of AnxA1, AnxA1 receptors, formyl peptide receptor 1 (FPR1) and FPR 2 whose acronym is FPR2/ALX in mature adipocytes.
2. The expression of ANXA1, FPR1, FPR2/ALX, adiponectin, leptin, CRP, and IL-6 genes in hypoxia (1% O_2).
3. The effect of the N-terminal peptide of AnxA1 (peptide Ac2-26) on the expression of inflammatory markers (CRP and IL-6) and adipose tissue specific hormones (adiponectin and leptin) in hypoxic conditions (1% O_2).

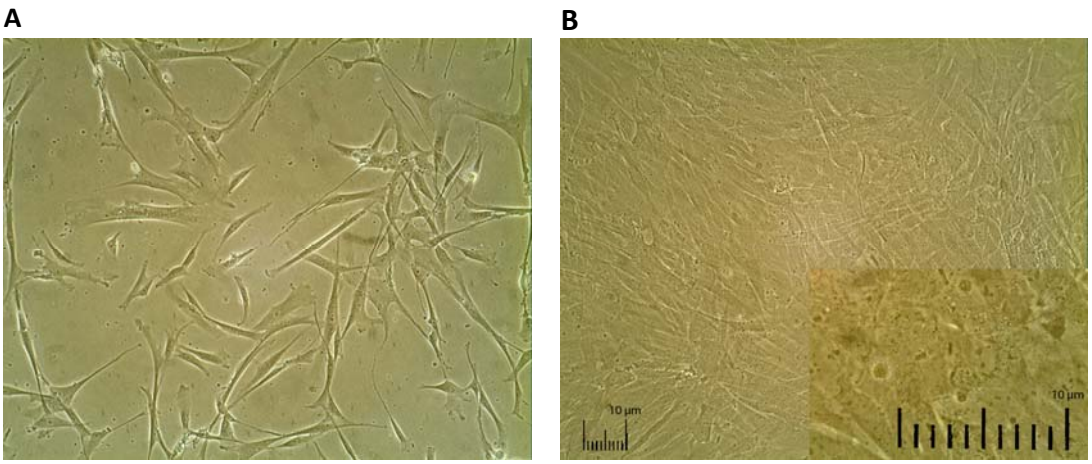
4.3 Results

4.3.2 Preadipocytes and mature adipocytes experiments in normoxia

SGBS preadipocytes were grown under normoxic conditions under they reached 70 % confluence following methods described in Section 2.3.4 and as shown in Figure 63 and 64.

4.3.2.1 Sub-culture of preadipocytes

Figure 63: Pre-adipocytes at day 1 and 14 after seeding.



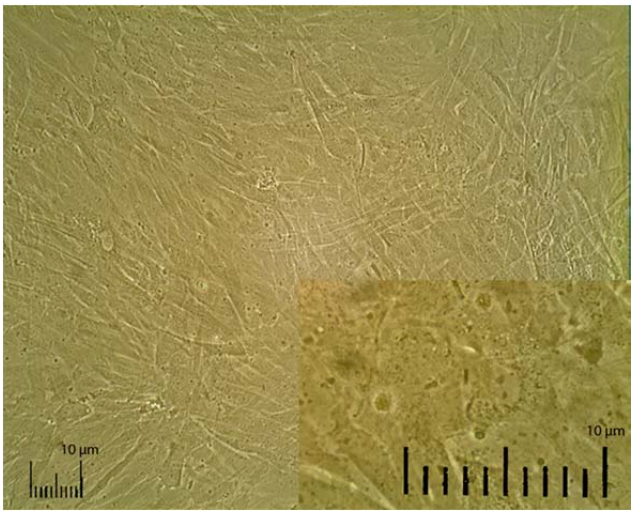
SGBS preadipocytes **(A)** were grown to confluence (**B)** using culture media. x 10 and 40 magnification.

Viability of adipocytes was assessed using oil red O. As preadipocytes mature and differentiate their capacity to store fat develops. The amount of the oil red O absorbed by the cells is proportional to the fat content within adipocytes. The amount of dye absorbed by the differentiating preadipocytes and then fully mature adipocytes was expressed as mean density as mesured using NIS-Elements software was **B**: 0.43 (SD=0.08), **C**: 0.57 (SD=0.03), **D**: 0.73 (SD=0.06), **E:** 1.18 (SD=0.14), see Figure 65.

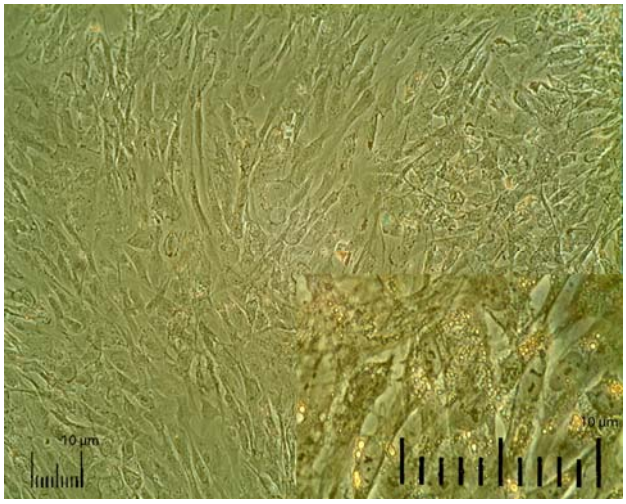
4.3.2.2 Oil red O staining and quantification

Figure 64: Oil red O staining of SGBS preadipocytes differentiation.

A: 4 DAYS PRE-INDUCTION

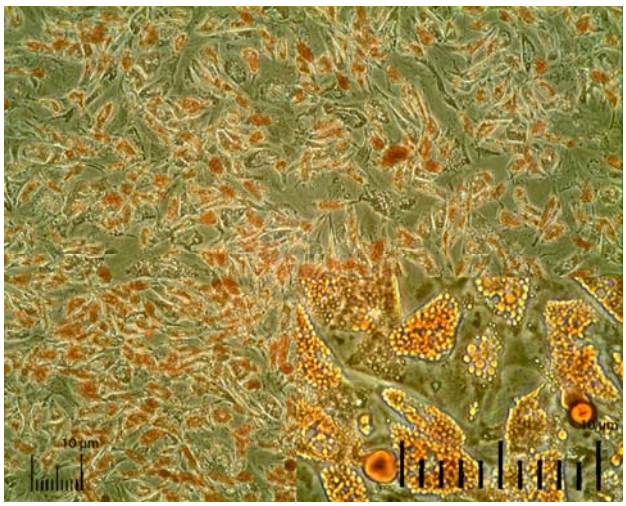


B: 4 DAYS POST INDUCTION

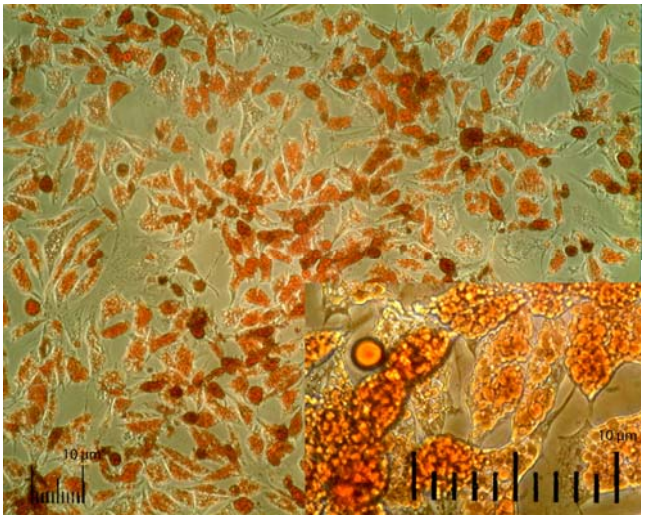


C: 7 DAYS POST INDUCTION

D: 10 DAYS POST INDUCTION



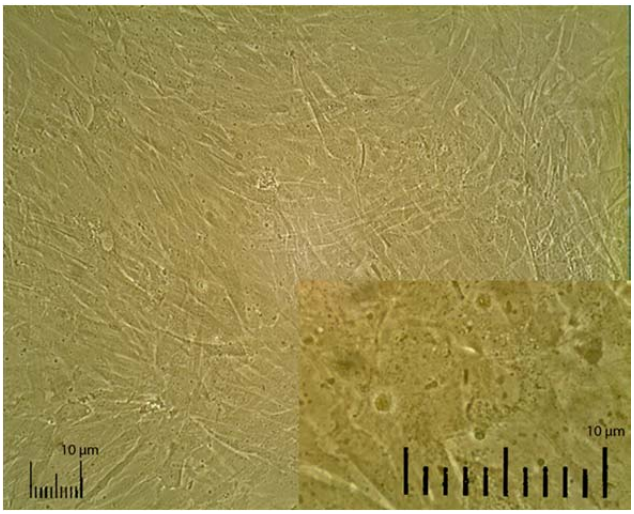
E: FULLY MATURE ADIPOCYTES



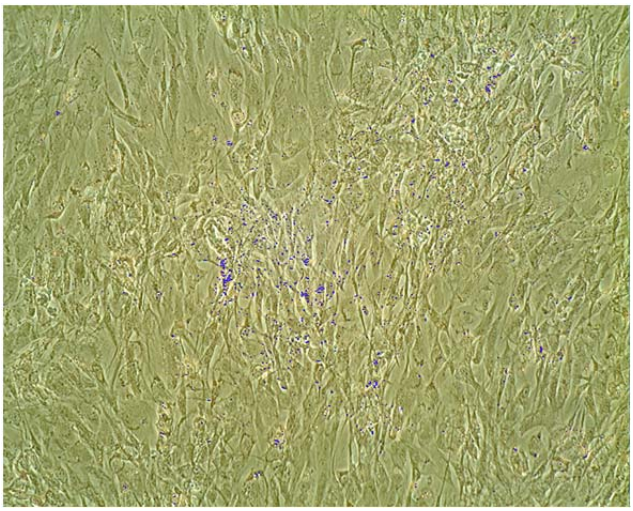
SGBS preadipocytes were grown to confluence and induction media was added to stimulate differentiation of preadipocytes **(A)** into mature adipocytes **(E).** As the adipocytes develop the amount of fat droplet accumulated increases (stained red). **A**: Fully confluent preadipocytes, **B**: Differentiating preadipocytes 4 days post induction, **C**: Differentiating preadipocytes 7 days post induction, **D**: Differentiating preadipocytes 10 days post induction, **E**: Fully mature adipocytes 14 days post induction. x 10 and 40 magnification

Figure 65: Oil red O quantification using NIS-Elements.

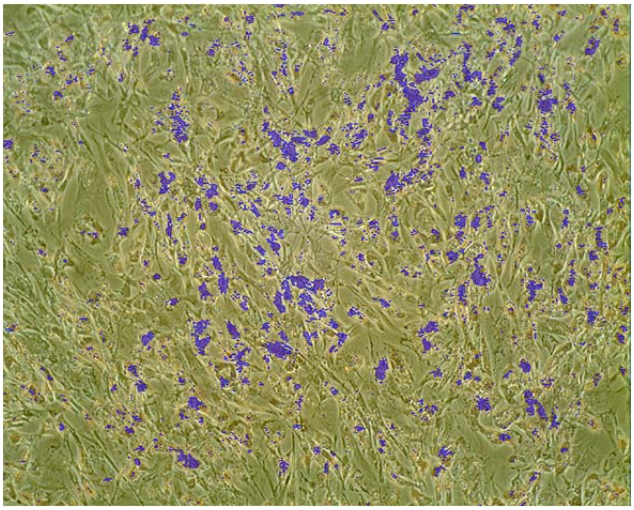
A: 4 DAYS PRE-INDUCTION



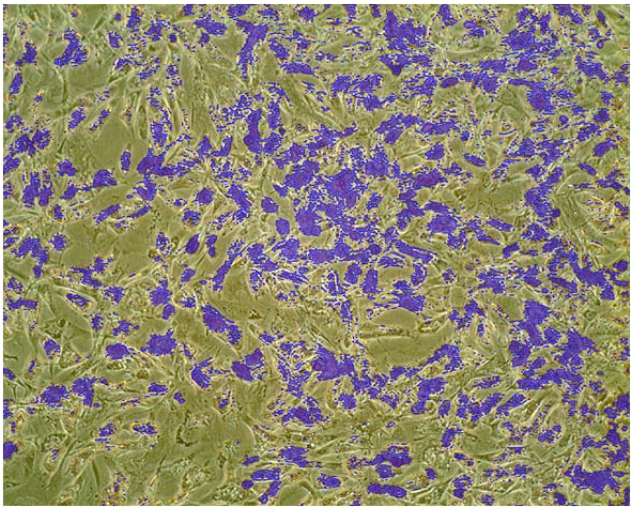
B: 4 DAYS POST INDUCTION



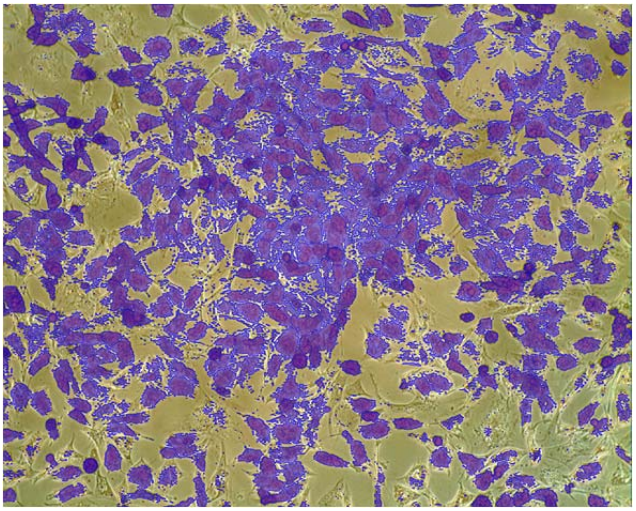
C: 7 DAYS POST INDUCTION



D: 10 DAYS POST INDUCTION



E: FULLY MATURE ADIPOCYTES

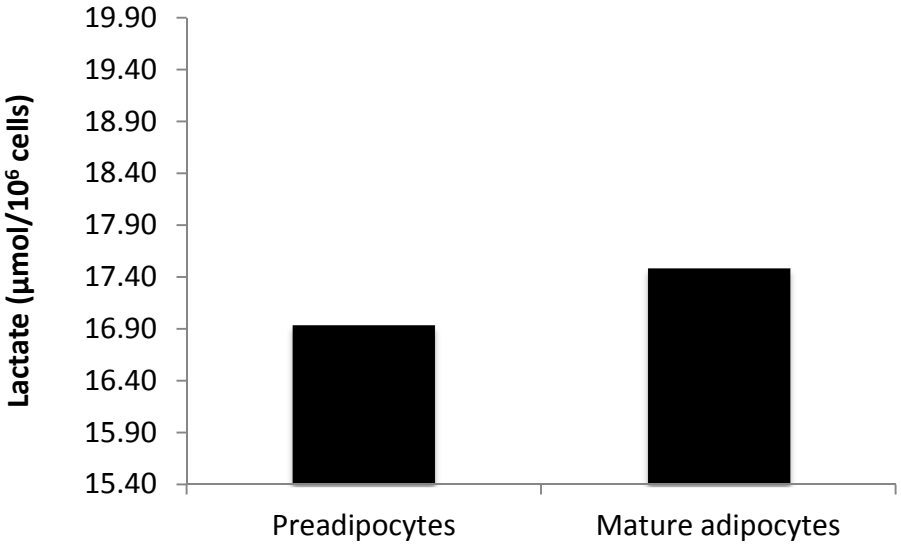


SGBS adipocytes were stained using oil red O dye. The amount of dye is proportional to the size and amount of lipid droplets formed during differentiation of preadipocytes. The size and density of adipocytes was measurred using NIS-Elements. The area of interest was marked in purple. x 10 magnification. Mean density as mesured was **B**: 0.43 (SD=0.08), **C**: 0.57 (SD=0.03), **D**: 0.73 (SD=0.06), **E:** 1.18 (SD=0.14). NIS Elements, Nikon (Surrey, UK).

4.3.2.3 Lactate assay

Lactate production was measured in fully confluent preadipocytes and mature adipocytes under normoxic conditions. The mean lactate concentration was higher in mature adipocytes (17.5 µmol/10^6 cells) when compared to preadipocytes (16.9 µmol/10^6 cells) (see Figure 66) but not statistically different. Lactate is released during glycolysis and therefore, can be used a marker of glucose utilisation.

Figure 66: Lactate release from preadipocytes and mature adipocytes in normoxia.

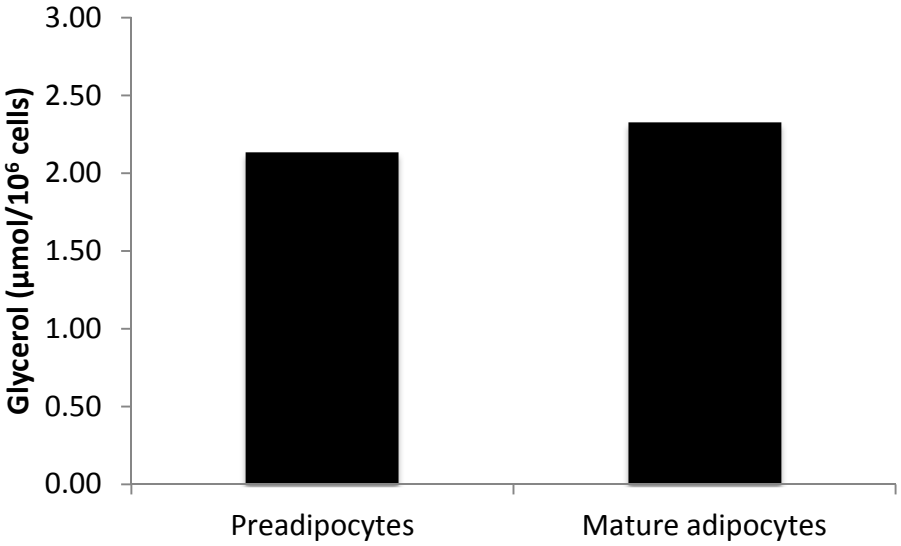


Lactate production (µmol/10^6 cells) as measured in the supernatant of preadipocytes and mature adipocytes (N = 2). Results are means (±SEM).

4.3.2.4 Glycerol assay

Glycerol production was measured in fully confluent preadipocytes and mature adipocytes under normoxic conditions. The mean glycerol concentration was marginally higher in mature adipocytes (2.3 µmol/10^6 cells) when compared to preadipocytes (2.1 µmol/10^6 cells) (see Figure 67) but not statistically different (P=0.09). Glycerol is released during lipolysis and therefore, can be used a marker of TAGs break down.

Figure 67: Glycerol release from preadipocytes and mature adipocytes in normoxia.

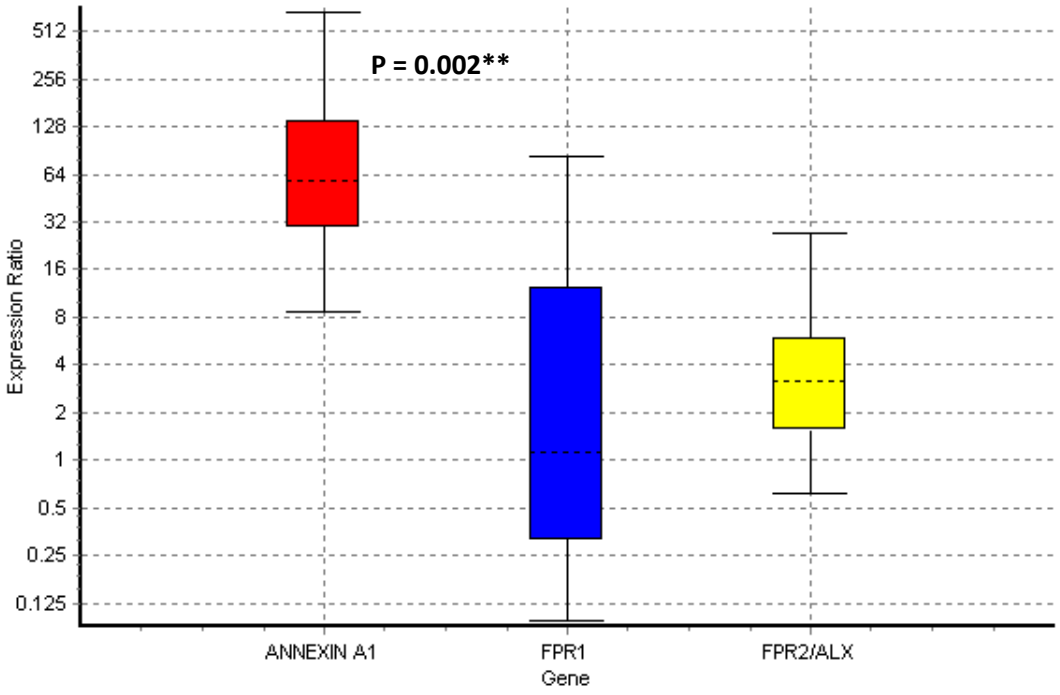


Glycerol production (µmol/10^6 cells) as measured in the supernatant of preadipocytes and mature adipocytes (N = 2). Results are means (±SEM)

4.3.2.5 RT-PCR gene expression

In this study we investigated whether AnxA1 was expressed in human adipose tissue. Our data suggests that there is a link between the level of plasma AnxA1 protein and BMI, and total body fat and fat distribution (Kosicka et al., 2012). AnxA1 protein has been demonstrated previously in murine adipocytes (Rhodes, 2010, Warne et al., 2006, Zhao et al., 2010) and the current data demonstrate for the first time that AnxA1 is also present in a human subcutaneous fat cell-line, the SGBS cell. We found that AnxA1 gene expression is significantly increased (66-fold increase, P=0.002**) during adipogenesis (see Figure 68), as AnxA1 protein is secreted from SGBS cell supernatants during adipogenesis (see Figure 70). We did not find any significant change to AnxA1's receptors, the FPR1 and FPR2/ALX when we compared the expression between mature adipocytes and preadipocytes. These findings suggest that AnxA1 plays a role in the adipocyte function. It is not currently understood if AnxA1 is linked with e.g. regulation of adiposity and how much it contributes to the chronic inflammation found in WAT of overweight and obese individuals. We have set out further experiments to find out if AnxA1 expression within SGBS cells is affected by hypoxia (1% O_2) *in vitro*.

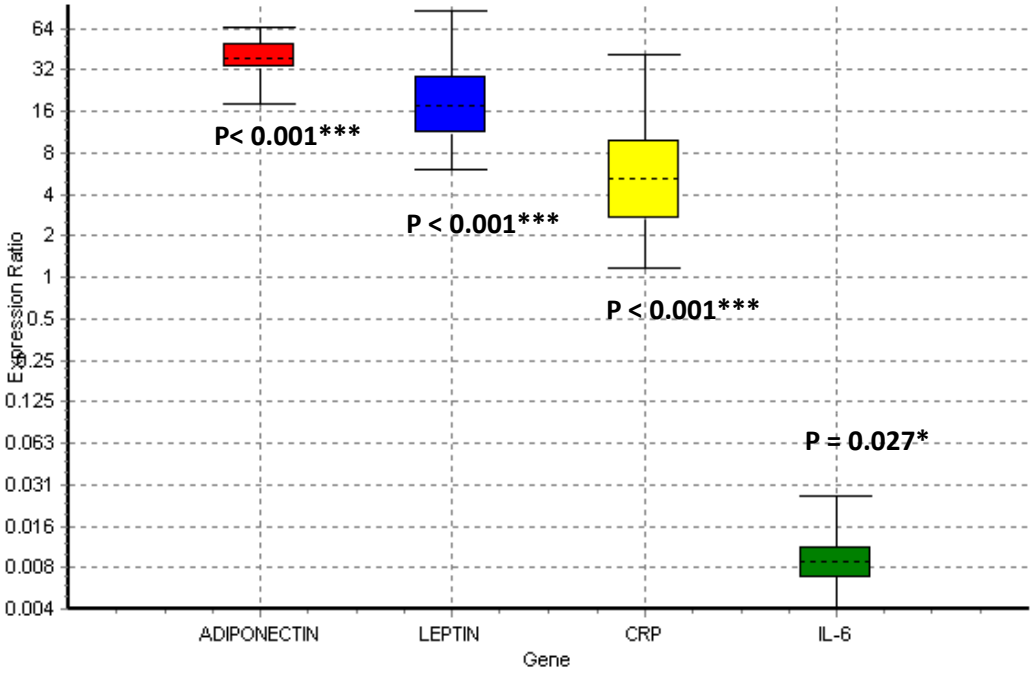
Figure 68: RT-PCR gene expression of annexin A1 and the receptors FPR1 and FPR2/ALX. Pre-adipocytes to mature adipocytes in normoxia



Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1. Values represent the mean expression, AnxA1 was significantly up-regulated (66-fold) where P = 0.002** (N = 4). RT-PCR analysis was carried out using REST 2009 © Copyright: 2009, Qiagen.

We have measured the expression of WAT specific hormones in mature adipocytes and compared these to the expression within preadipocytes. We found that both adiponectin (39-fold, P<0.001***) and leptin (19-fold, P<0.001***) genes were up-regulated in mature human SGBS adipocytes as previously demonstrated (Wabitsch et al., 2001). The up-regulation of adiponectin and leptin gene expression within mature adipocytes is linked with a greater role of these hormones in fully differentiated adipocytes, and therefore can be used as a marker of adipogenesis. CRP was up-regulated (6-fold) where significant was P<0.001*** and IL-6 was down-regulated (0.01-fold) where significance was P=0.027** (see Figure 69).

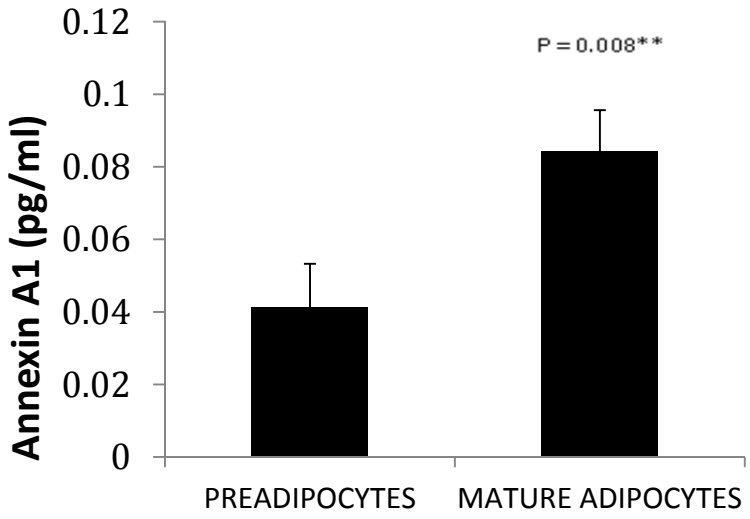
Figure 69: RT-PCR gene expression of adiponectin, leptin, CRP and IL-6. Pre-adipocytes to mature adipocytes in normoxia.



Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1. Adiponectin was significantly up-regulated (39-fold) where $P < 0.001$, leptin was up-regulated (19-fold) where $P<0.001$, CRP was up-regulated (6-fold) where $P<0.001$ and IL-6 was down-regulated (0.01-fold) where $P=0.027$ ($N = 4$).

4.3.2.6 Protein expression

Figure 70: Protein expression of annexin A1 (pg/ml). Pre-adipocytes to mature adipocytes in normoxia.

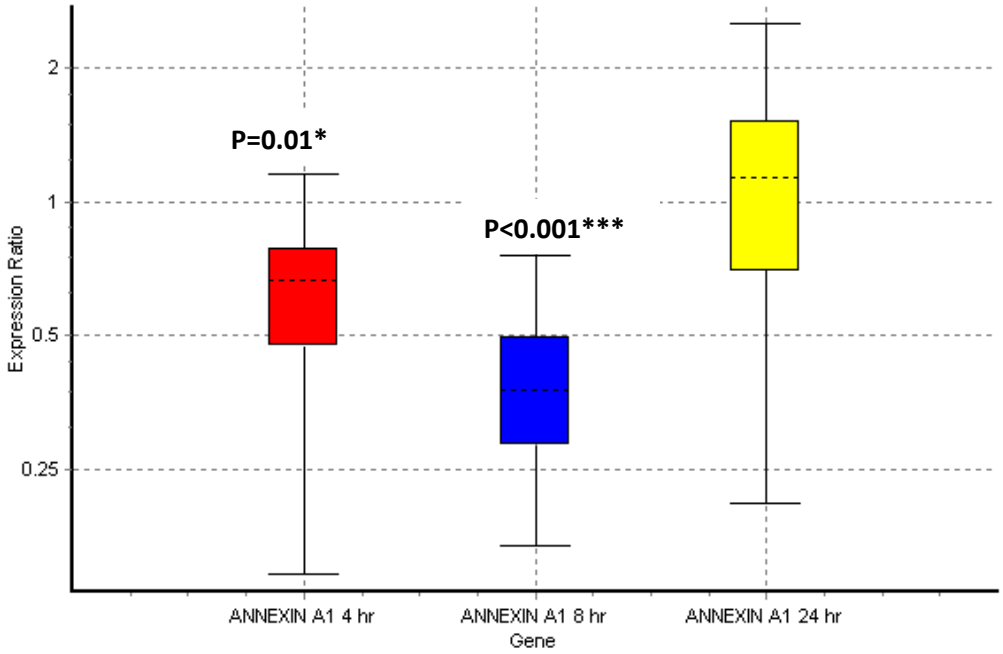


Immunoreactive AnxA1 protein was measured by ELISA from unstimulated cell supernatants fractions from pre-adipocytes and mature adipocytes. A significant increase in AnxA1 protein was measured in the supernatant following adipogenesis (P = 0.008**) (N = 4).

4.3.3 Pre-adipocytes - mature adipocytes during 4, 8 and 24 hour hypoxia treatments - RT-PCR gene expression.

Once we confirmed that AnxA1 and FPR1 and FPRL1/ALX receptors are expressed in human SGBS cell line we investigated how these genes may be affected by mimicking hypoxic conditions (1 % O_2)*in vitro*. We compared the expression of genes between mature adipocytes and pre-adipocytes after 4, 8, and 24 hour hypoxia incubation. We found that AnxA1 gene expression was down-regulated in mature adipocytes when compared to preadipocytes after 4 hour (0.6-fold, P=0.01*) and 8 hour (0.4-fold, P < 0.001***) hypoxia incubation. The expression of AnxA1 after 24 hour hypoxia incubation was not statistically different when compared to the 4 and 8 hour hypoxia treatment (see Figure 71).

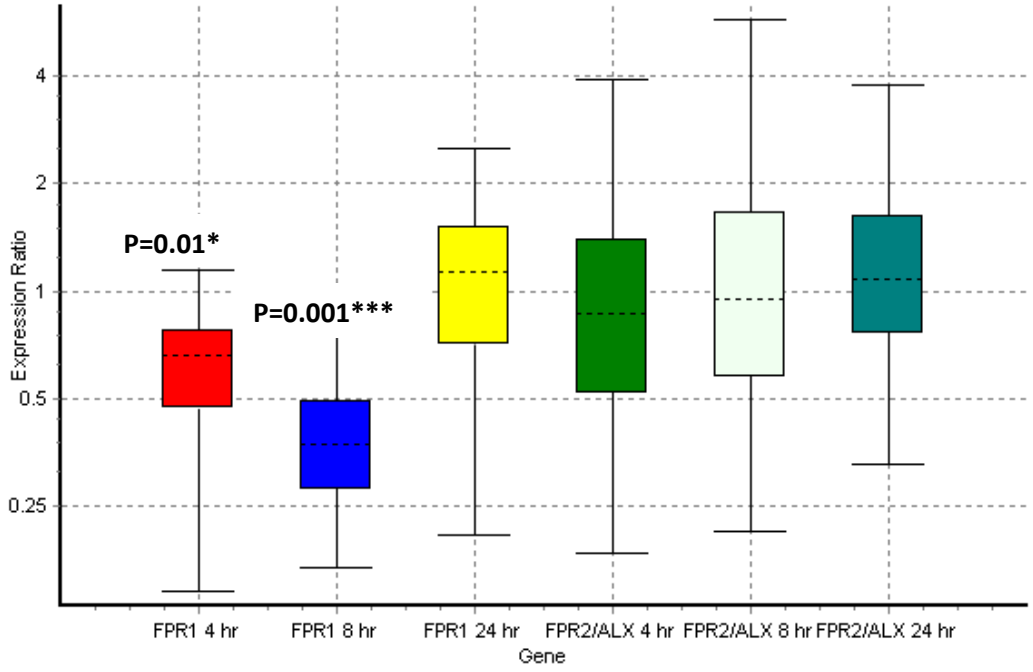
Figure 71: RT-PCR gene expression of annexin A1 during 4, 8 and 24 hour treatment of hypoxia. Pre-adipocytes compared to mature adipocytes.



Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1. AnxA1 was significantly down-regulated after 4 hours (0.6-fold, P=0.01*) and 8 hours (0.4-fold, P<0.001) of hypoxia treatment (N = 4).

We have also found that the FPR1 receptor was down-regulated after 4 hour (0.6-fold, P=0.01*) and 8 hour (0.4-fold, P=0.001***) hypoxia treatment in mature adipocytes when compared to preadipocytes after the same treatment (see Figure 72). There were no significant changes to the expression of FPR2/ALX receptor after 4, 8 and 24 hour hypoxia treatment in mature adipocytes when compared to preadipocytes. These data further suggest that AnxA1 has a role in adipocyte biology and it is affected by hypoxia *in vitro*.

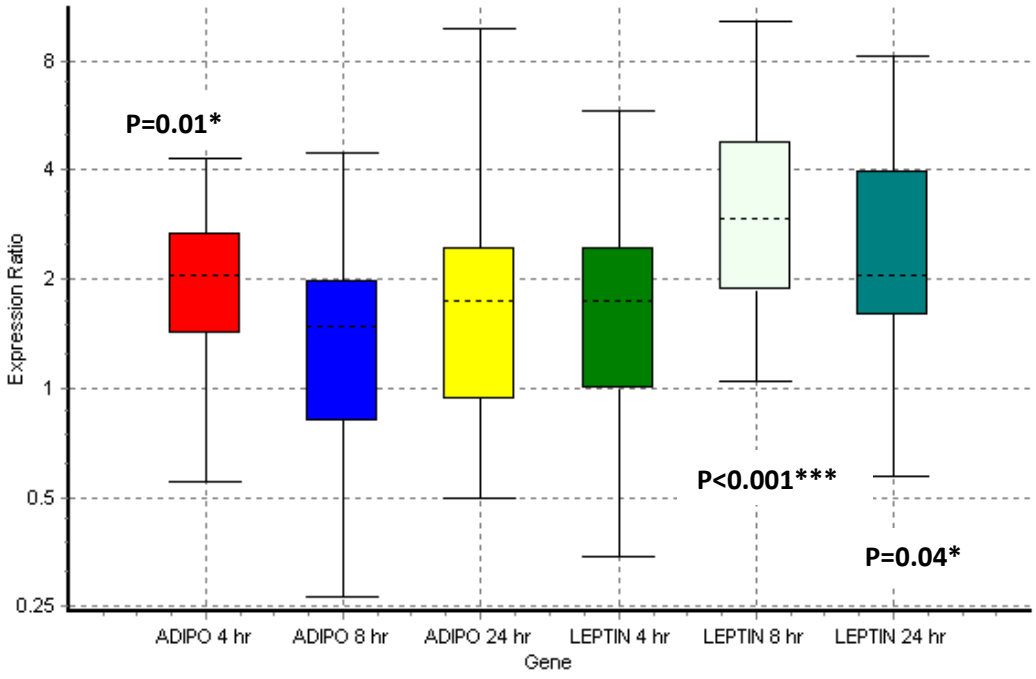
Figure 72: RT-PCR gene expression of AnxA1's receptors, FPR1 and FPR2/ALX during 4, 8 and 24 hour treatment of hypoxia. Pre-adipocytes compared to mature adipocytes.



Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1. FPR1 was significantly down-regulated after 4 hours (0.6-fold, P=0.01*) and 8 hours (0.4-fold, P=0.001) of hypoxia treatment (N = 4).

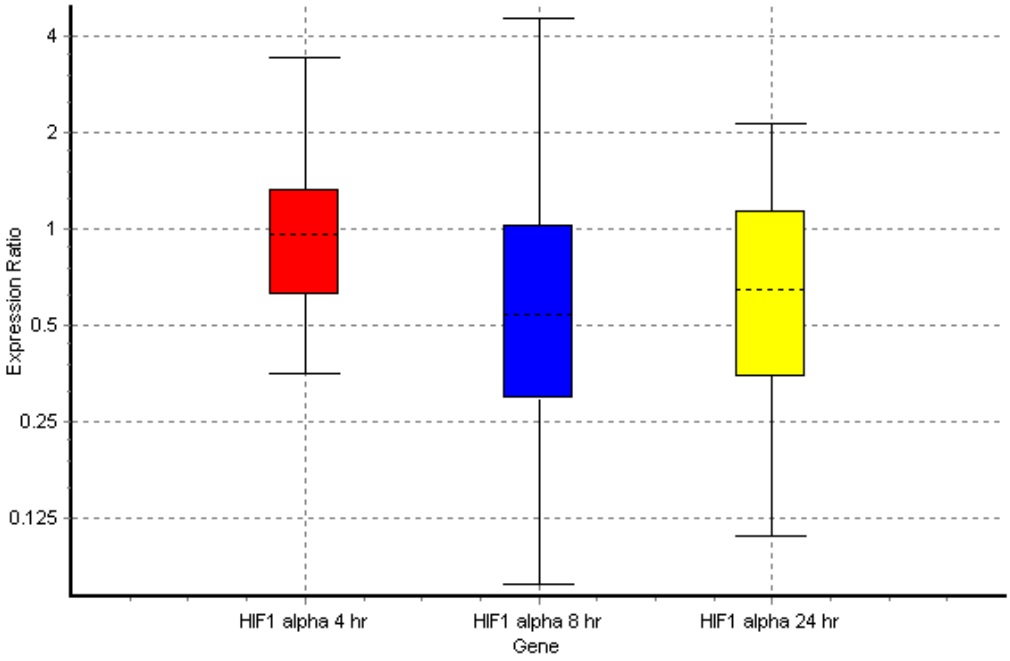
Interestingly, we found that the expression of adiponectin was decreased after 4 hour hypoxia treatment (1.9-fold, P=0.01*), however the level of expression was not significantly different after 8 and 24 hour hypoxia treatment. Figure 73 demonstrates there was a downward trend in the mean adiponectin expression during 4, 8 and 24 hour hypoxia incubation in mature SGBS adipocytes when compared to preadipocytes. Meanwhile, leptin gene expression peaked significantly at 8 hours of hypoxia (3-fold, P<0.001***) and continued to be statistically up-regulated after 24 hour of hypoxia (2.2-fold, P=0.04*) see Figure 73). We did not find a significant difference in the expression level of HIF-1α in mature adipocytes when compared to preadipocytes after 4, 8, or 24-hour hypoxia incubation (see Figure 74). This could be because the controls (preadipocytes) underwent the same hypoxic incubation, and HIF-1α is thought to be induced by hypoxia regardless of cell type. We investigate later if HIF-1α expression varies during hypoxia treatment versus normoxia.

Figure 73: RT-PCR gene expression of adiponectin and leptin during 4, 8 and 24 hour treatment of hypoxia. Pre-adipocytes compared to mature adipocytes.



Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1. Adiponectin was significantly up-regulated after 4 hours (1.9-fold, P=0.01*) and leptin was significantly up-regulated at 8 hours (3-fold, P<0.001***) and 24 hours (2.2-fold, P=0.04*) of hypoxia treatment (N = 4).

Figure 74: RT-PCR gene expression of HIF-1 alpha during 4, 8 and 24 hour treatment of hypoxia. Pre-adipocytes compared to mature adipocytes.



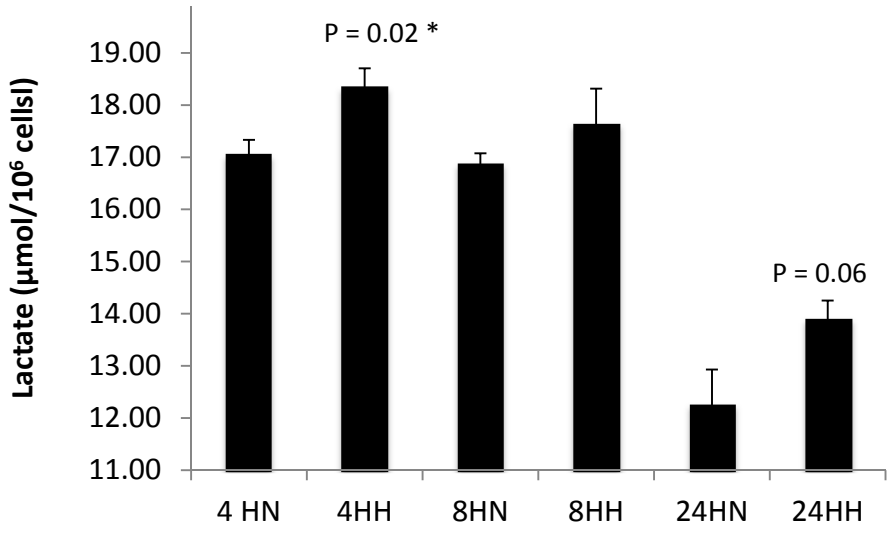
Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1. We did not find a significant change to the expression level of HIF-1 α at 4, 8 and 24 hours of hypoxia treatment (N = 4).

4.3.4 Mature adipocytes during hypoxia treatment

4.3.4.1 Lactate assay

Lactate production increases during anaerobic metabolism and therefore we suggested that we can use lactate release as a marker of hypoxia. We would expect lactate production to be increased after 4, 8, and 24 hours of hypoxia treatment. Our results show that incubation of mature adipocytes in hypoxic conditions induced on average a greater release of lactate when compared to mature adipocytes cultured in normoxic conditions (see Figure 75). The level of lactate as measured in the supernatant of cultured adipocytes was significantly higher after 4 hour of hypoxia treatment (P=0.02*) and it was nearly significant after 24 hour of hypoxia treatment (P=0.06).

Figure 75: Lactate production during 4, 8 and 24 hour treatment of hypoxia. Mature adipocytes.



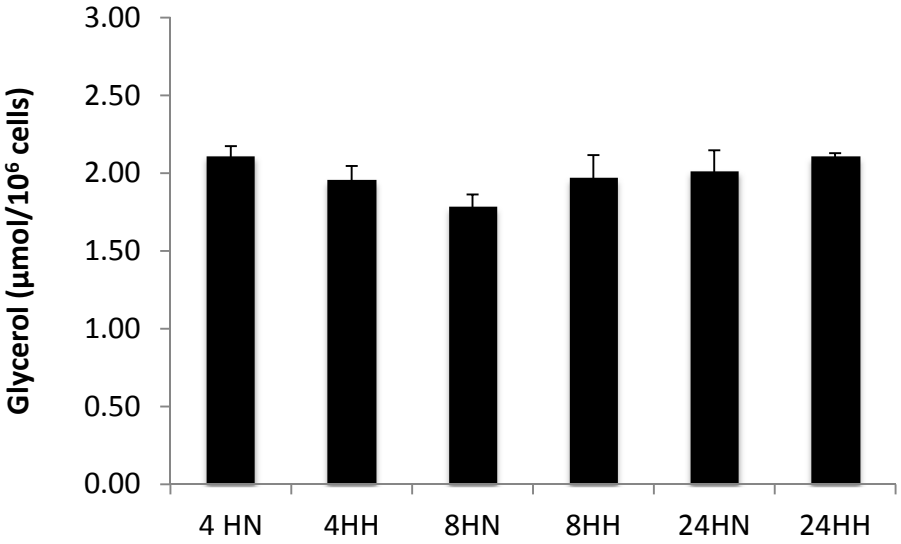
Lactate (µmol/10^6 cells) production during 4, 8 and 24 hour normoxia (HN) and hypoxia (HH) treatment. Lactate concentration was significantly increased during 4 hour hypoxia (P=0.02*) and it reached near significance after 24 hour hypoxia (P=0.06) treatment when compared to their normoxia controls (N = 6).

4.3.4.2 Glycerol assay

Glycerol production was measured in fully mature adipocytes under hypoxic conditions. The mean glycerol concentration was not statistically significantly

different between the 4 (P=0.29), 8 (P=0.39) and 24 (P=0.58) hour hypoxia treatments when compared to their normoxic controls (see Figure 78).

Figure 76: Glycerol production during 4, 8 and 24 hour treatment of hypoxia. Mature adipocytes.

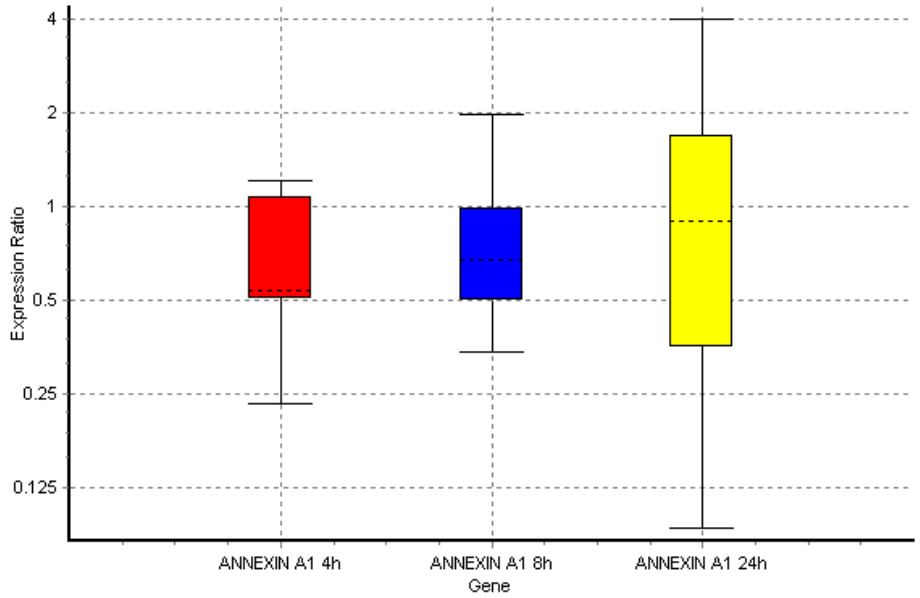


Glycerol (µmol/10^6 cells) production during 4 (P=0.29), 8 (P=0.39) and 24 (P=0.58) hour normoxia (HN) and hypoxia (HH) treatment (N = 6).

4.3.4.3 RT-PCR gene expression

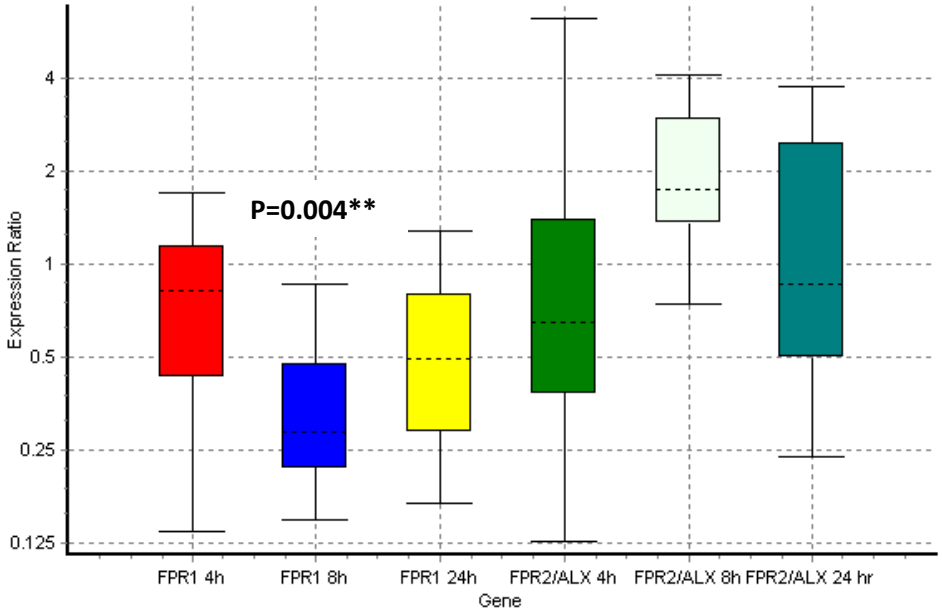
To test the hypothesis that AnxA1 may play a role in the inflammatory state of WAT induced by obesity, we further tested if AnxA1 is affected by hypoxia in mature adipocytes when compared to mature adipocytes in normoxic conditions. We found no difference in the level of AnxA1 expression after 4, 8, and 24-hour hypoxia treatment when compared to mature adipocytes cultured in normoxic conditions (see Figure 77). We did find that FPR1 receptor was down-regulated after 8 hour of hypoxia treatment (0.3-fold, P=0.004**) as shown in Figure 78 but there were no significant changes to the FPR2/ALX receptor. Hypoxia treatment further induced the expression of leptin gene at 8 hours of hypoxia treatment (2.1-fold, P<0.001*** and at 24 hours of hypoxia treatment (12-fold, P<0.001***) when compared to mature adipocytes grown in normoxic conditions. We found no further change to the expression of adiponectin gene level.8 hour hypoxic treatment up-regulated HIF-1α gene (2-fold, P=0.005**) but no significant changes were observed at 4 and 24-hour hypoxia treatment when compared to normoxia (see Figure 80).

Figure 77: RT-PCR gene expression of annexin A1 during 4, 8 and 24 hour treatment of hypoxia in mature adipocytes



We did not find a significant change to the expression level of AnxA1 at 4, 8 and 24 hours of hypoxia treatment (N = 4)[1].

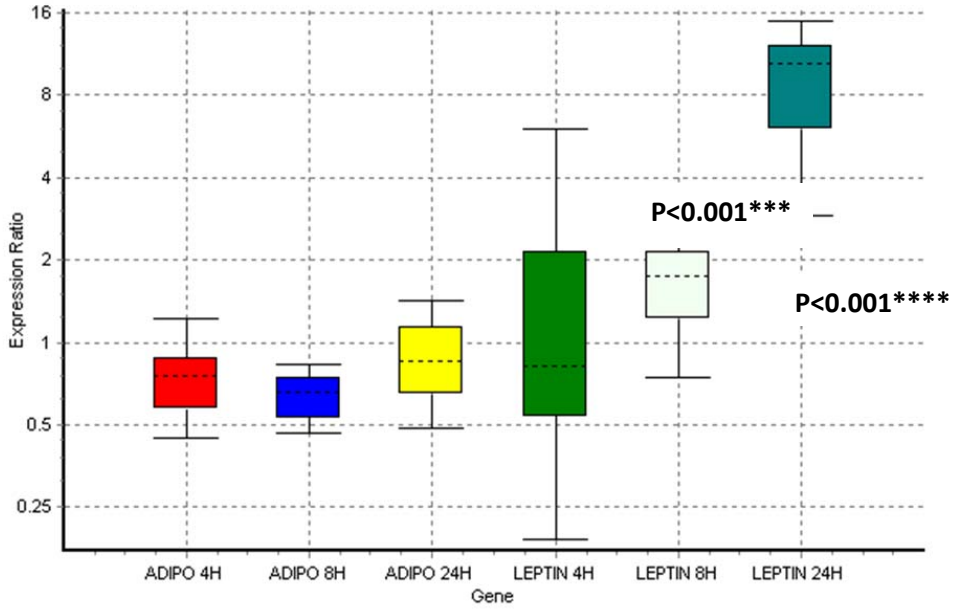
Figure 78: RT-PCR gene expression of FPR1 and FPR2/ALX during 4, 8 and 24 hour treatment of hypoxia in mature adipocytes



FPR1 was significantly down regulated at 8 hrs of hypoxia (0.3-fold, P=0.004**) (N = 4)[1].

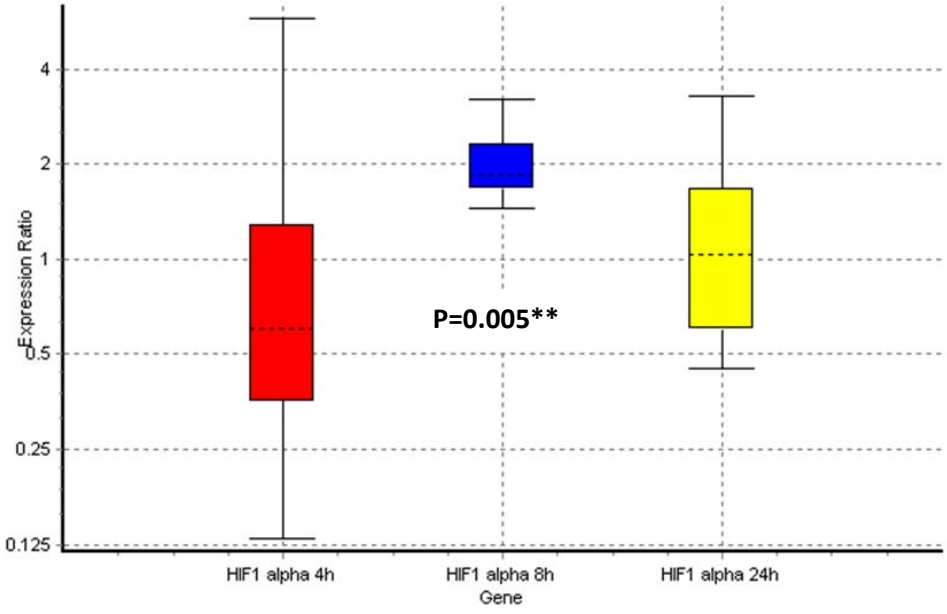
[1] Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1.

Figure 79: RT-PCR gene expression of adiponectin and leptin during 4, 8, and 24-hour treatment of hypoxia in mature adipocytes.



Leptin was significantly up-regulated at 8 hrs of hypoxia (2.1-fold, P<0.001*** and at 24 hours of hypoxia treatment (12-fold, P<0.001***) (N = 4)[2].

Figure 80: RT-PCR gene expression of HIF1 alpha during 4, 8, and 24 hour treatment of hypoxia in mature adipocytes.



HIF-1 α was significantly up-regulated at 8 hours of hypoxia treatment (2-fold, P=0.005**) (N = 4).

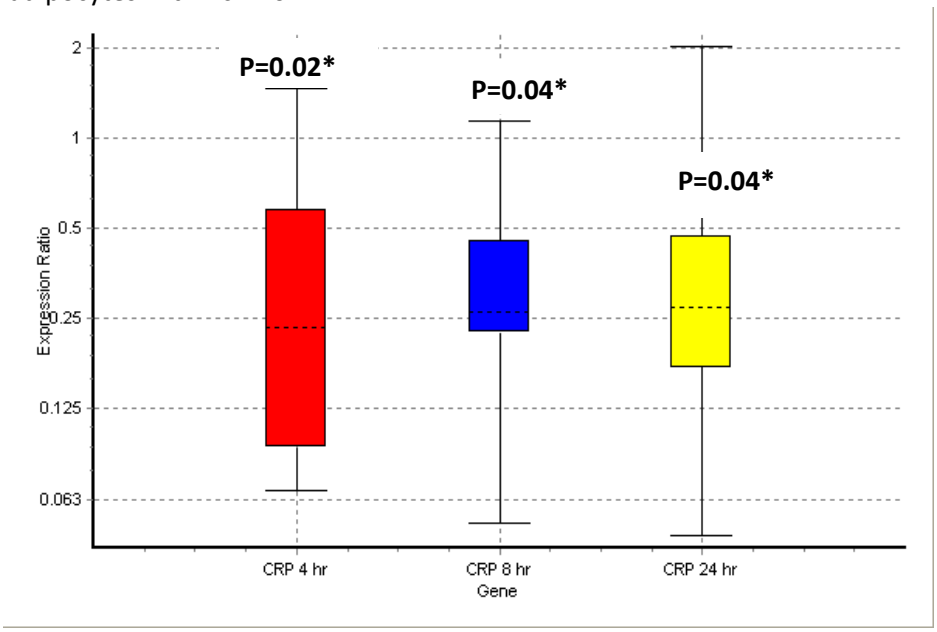
[2] Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1.

4.3.5 Mature adipocytes in hypoxia stimulated with Ac 2-26 peptide.

Previous studies demonstrated that the N-terminal peptide of AnxA1 (peptide Ac2-26) can mimic the anti-inflammatory actions of the full-length protein in various systems. In the current study, we report the effectiveness of the peptide Ac2-26 as an anti-inflammatory agent in a model of human Simpson Golabi Behmel Syndrome (SGBS) adipocytes. To mimic the relative hypoxia found in the white adipose tissue of obese humans, mature adipocytes (14 days post-confluent) were treated with 4, 8 and 24 hour hypoxia treatment (1% O_2). The cells were also treated with 10^{-5} M Ac2-26 peptide or vehicle (DMEM/F12 medium supplemented with insulin [20 nM]). WE found that the expression of CRP level was down-regulated at 4 hour (0.25-fold, P=0.02*), 8 hour (0.3-fold, P=0.04*) and 24-hour (0.29-fold, P=0.04*) of hypoxia treatment also supplemented with Ac2-26 peptide when compared to vehicle alone (see Figure 81).

4.3.5.1 RT-PCR gene expression

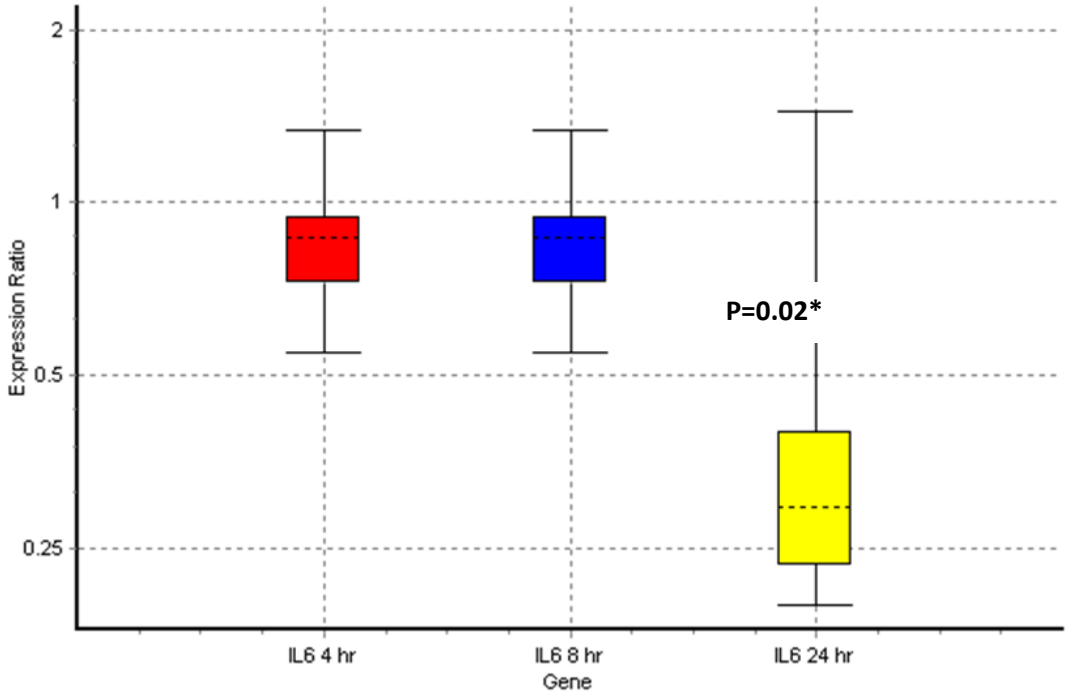
Figure 81: RT-PCR gene expression of CRP during 4, 8, and 24-hour treatment of hypoxia in mature adipocytes with Ac2-26.



Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1. CRP was significantly down-regulated at 4 hours (0.24-fold, P=0.02*), 8 hours (0.3-fold, P=0.04*) and 24 hours (0.29-fold, P=0.04*) of hypoxia treatment (N = 4).

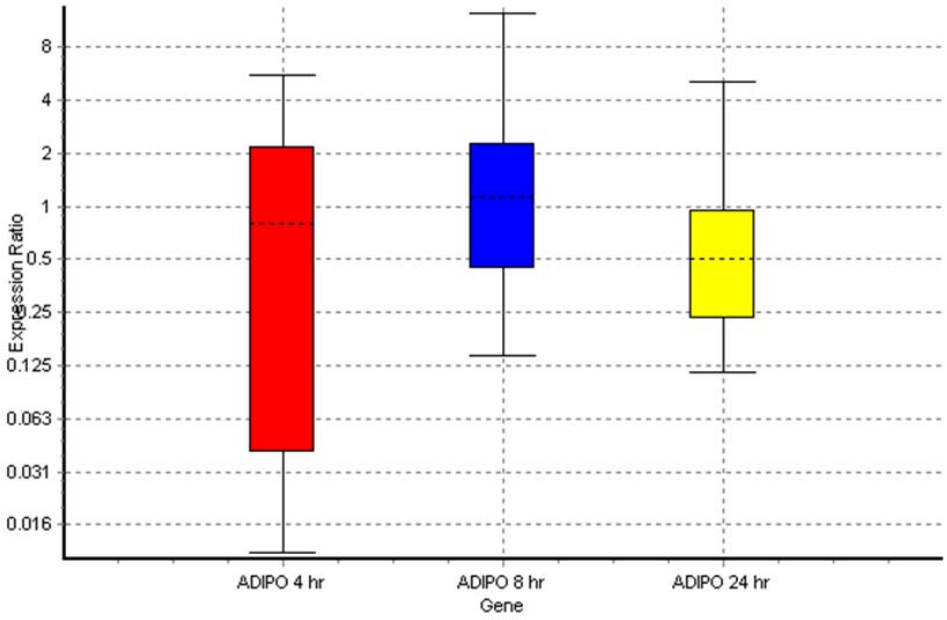
The expression of IL-6 gene was found to be down-regulated after 24-hour hypoxia treatment (0.4-fold, P=0.02*) with Ac2-26 peptide when compared to vehicle alone in mature adipocytes (see Figure 82). Exogenous Ac2-26 had no effect on the expression of adiponectin gene during 4, 8, and 24-hour hypoxia treatment (see Figure 81). There was an induction of leptin expression at 24 hour of hypoxia incubation with Ac2-26 (11-fold, P=0.01*) (see Figure 86), however no statistically significant change was observed after 4 and 8 hour of hypoxia incubation with Ac2-26. Ac2-26 treatment in hypoxic conditions down regulated the expression of HIF-1α at 24 hours (0.3-fold, P=0.02*) when compared to vehicle alone. These data suggest that treatment with Ac2-26 exerts anti-inflammatory effects as demonstrated by the reduced expression of pro-inflammatory markers, CRP and IL-6. Furthermore, Ac2-26 peptide altered the expression of leptin hormone suggesting that perhaps AnxA1 may also have a role in the modulation of adiposity.

Figure 82: RT-PCR gene expression of IL-6 during 4, 8, and 24-hour treatment of hypoxia in mature adipocytes with Ac2-26.



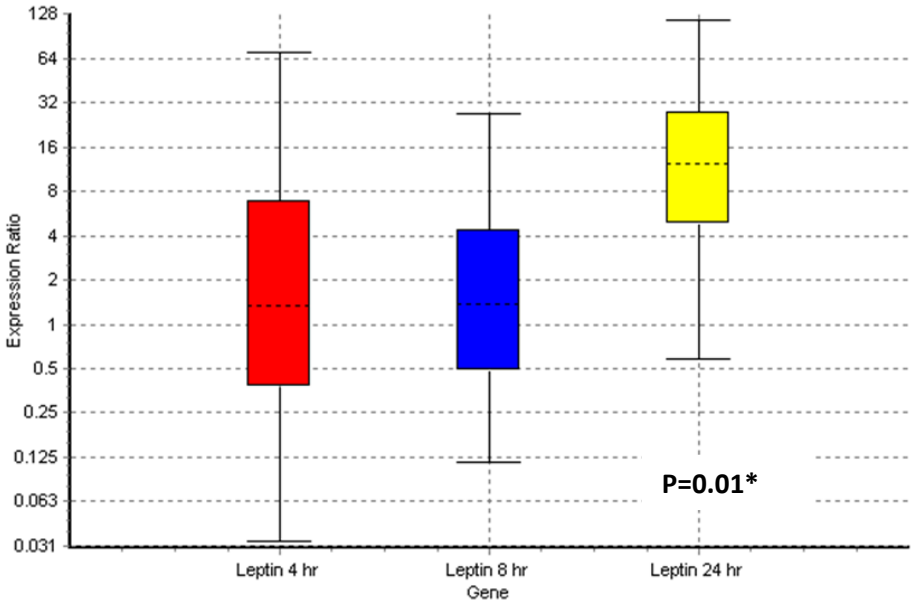
Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1. IL-6 was significantly down-regulated at 24 hours of hypoxia treatment (0.4-fold, P=0.02*) (N =4).

Figure 83: RT-PCR gene expression of adiponectin during 4, 8, and 24-hour treatment of hypoxia in mature adipocytes with Ac2-26.



Adiponectin expression was not significantly changed during the 4, 8, and 24 hour hypoxia (N = 4)[3].

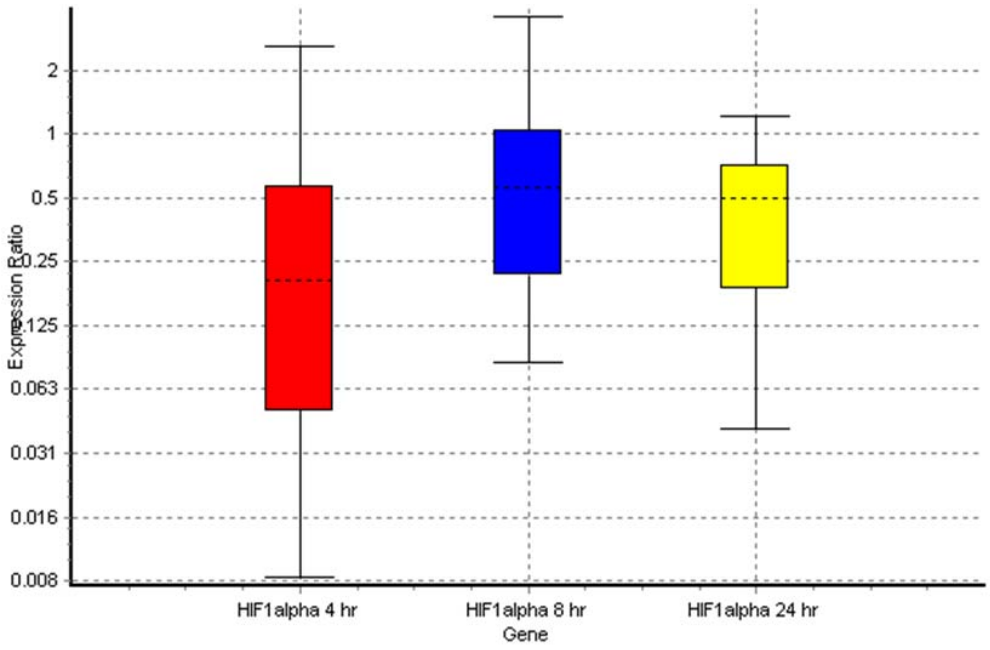
Figure 84: RT-PCR gene expression of leptin during 4, 8, and 24-hour treatment of hypoxia in mature adipocytes with Ac2-26.



Leptin expression was significantly up-regulated after 24 hour of hypoxia with Ac2-26 (11-fold, P=0.01*) (N = 4).

[3] Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1.

Figure 85: RT-PCR gene expression of HIF1 alpha during 4, 8, and 24-hour treatment of hypoxia in mature adipocytes with Ac2-26.



Boxes represent the interquartile range, or the middle 50 % of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Expression ratios of treated samples are normalised against housekeeping gene, GAPDH and compared to background level of 1. HIF-1α expression was significantly down-regulated after 24 hour of hypoxia treatment with Ac2-26 (0.3-fold, P=0.02*) (N = 4).

4.4 Discussion

We have demonstrated that plasma AnxA1 is inversely correlated with BMI, %BF and waist to hip ratio (Chapter 3). The cell culture studies of the role of AnxA1 in human SGBS cells presented in this chapter confirm for the first time that AnxA1, FPR1 and FPR2/ALX receptors are expressed in human subcutaneous adipocytes. The fact that the AnxA1 protein is derived from the supernatant fraction, suggests a possible function of AnxA1 protein within WAT, as it is not merely stored within the cell but released into the local environment. The exact source of plasma AnxA1 protein has not been determined to date and may represent the contribution of protein from a number of different sources. It may be assumed that leukocytes represent the largest source of plasma AnxA1 as these cells are known to store large quantities for their role as an anti-migratory agent for leukocytes (Rosales and Ernst, 1997).

However, given the current data, we cannot rule out the possibility that an adipocyte source of AnxA1 protein may significantly contribute to total plasma AnxA1 protein. It was interesting to note that AnxA1 gene expression was up-regulated (66-fold, P=0.002**, see Figure 68) in mature adipocytes when compared to preadipocytes in normoxic conditions. Addition of a hypoxic treatment on adipocytes when compared to preadipocytes down-regulated AnxA1 gene expression at 4 hours (0.6-fold, P=0.01*) and 8 hours (0.4-fold, P<0.001) (see Figure 71). However, we found no significant difference in the expression of AnxA1 during hypoxia when we compared the adipocytes to a normoxia control (see Figure 77). This finding may suggest that AnxA1 may be implicated in the adipocyte function irrespective of the inflammatory status of adipocytes. A study by Mack et al., 2009 revealed that preadipocytes have greater potency when compared with adipocytes as endothelial cell activator under normoxia, hypoxia, and TNFα exposure (Mack et al., 2009) and this may be another explanation why we did not find any significant changes in the expression of AnxA1 in hypoxic mature adipocytes when compared to normoxic controls . Furthermore, increasing adiposity may reach a critical point, beyond which AnxA1 protein from adipocyte and non-adipocyte sources are impaired leading to a collapse in plasma AnxA1 protein levels in affected individuals. By demonstrating a significant up regulation in both AnxA1 gene expression and secreted protein during adipogenesis, our current data further implicates AnxA1 in regulating adipocyte biology.

AnxA1 is induced by glucocorticoids and it is known to replicate the anti-inflammatory action of glucocorticoids. Extensive research demonstrated the anti-inflammatory role of AnxA1 in neutrophil function (Dalli et al., 2011) and emerging research suggests that this role may also involve the regulation of T-cell function and the adaptive immune responses (D'Acquisto et al., 2008b). Additionally our study showed for the first time that exogenous administration of the Ac2-26 peptide decreased the expression of CRP level and IL-6 in human SGBS adipocyte cell line under hypoxic conditions. CRP was significantly down-regulated at 4 hours (0.24-fold, P=0.02*), 8 hours (0.3-fold, P=0.04*) and 24 hours (0.29-fold, P=0.04*) of hypoxia treatment (see Figure 81) when treated with Ac2-26 compared to vehicle alone. Previous studies showed that Ac2-26 decreased neutrophil activity, levels of

PLA_2, PGE_2, nitric oxide and COX2 activity, therefore exerting an anti-inflammatory role (Perretti and D'Acquisto, 2009, Perretti and Dalli, 2009, Yang et al., 2013). Although AnxA1 is largely stored preformed in cytoplasmic granules ready for release into the extracellular space, after secretion, extracellular AnxA1 binds to its receptors, FPR1 and FPR2/ALX (Babbin et al., 2006, John et al., 2007) on adjacent cells and also in a juxtacrine manner. Similarly to the AnxA1 expression, we found that FPR1 receptor was significantly down-regulated after 4 hours (0.6-fold, P=0.01*) and 8 hours (0.4-fold, P=0.001) of hypoxia treatment in mature adipocytes when compared to preadipocytes (see Figure 72) but no significant changes were observed in normoxic conditions between mature adipocytes and preadipocytes (see Figure 68). FPR1 was also significantly down-regulated at 8 hours of hypoxia treatment (0.3-fold, P=0.004**) in mature adipocytes when compared to mature adipocytes in normoxic conditions (see Figure 78).

The FPR1 receptor gene data may suggest that AnxA1 signalling via the FPR1 receptor may be altered during hypoxia and if extrapolated, may be altered in the adipose tissue of obese humans. Although AnxA1 gene itself is not altered, its communication via its receptors may be a mechanism by which alterations/fine tuning in AnxA1 signalling is achieved in WAT during hypoxia. The implications of this are not known at this time. However, reduced FPR1 receptor expression may have implications for adipocyte recruitment. Our data on AnxA1 protein and gene expression in normoxic conditions suggests that this system is significantly increased during adipogenesis. If this process is disrupted by a reduced oxygen supply, then this may have implications for adipocyte recruitment. Furthermore, the recruitment of pre-adipocytes to mature adipocytes has been hypothesised to become dysregulated in a sub-population of obese patients who develop WAT fibrosis leading to systemic inflammation and metabolic disease (Sun et al., 2011). *In vitro* culture of WAT biopsies from lean, 'healthy' obese and obese patients with metabolic disease could be used to explore this area further in future studies.

4.5 Conclusion

We have shown for the first time that AnxA1 and the two receptors, FPR1 and FPR2/ALX are expressed in human SGBS cell line. We propose that AnxA1 plays a role in the biology of adipocytes and the regulation of inflammation within WAT. Our data suggest that AnxA1 may modulate adiposity independent of inflammation status and that exogenous administration of AnxA1 may have a beneficial effect on the mild chronic inflammation caused by low oxygen tension. In summary, AnxA1 presents a novel target as a mediator of adiposity and inflammation found in obese individuals.

5 Is there a free ride? Resveratrol as a calorie restriction mimetic

Abstract

Resveratrol (3, 5, 4'-trihydroxystilbene), a naturally occurring polyphenol, has attracted considerable interest for its beneficial potentials for human health. Beginning in the 1990s and continuing to date scientific studies *in vitro* and on animals have found that resveratrol has a wide range of desirable biological actions, including anti-oxidant, anti-inflammatory, cardioprotective and, anti-tumour activities and, prolongation of lifespan in several species. The metabolic effects of resveratrol are attributed to its ability to inhibit the oxidation of human low-density lipoprotein, while its suppression of cyclooxygenase-2 and inducible nitric oxide synthase activities also contribute to its anti-inflammatory and antioxidant effects. The compound used in this study was isolated from the root of *Polygonum cuspidatum.* Here, we treated 15 healthy, overweight and obese men with either 400 mg/day resveratrol or a placebo in a cross-over randomized double-blind study for 14 days. Resveratrol did not have a significant effect on neither the metabolic nor the inflammatory biomarkers we measured. There was no effect on total blood cholesterol, blood pressure, HOMA-IR, plasma AnxA1, C-reactive protein (CRP), interleukin 6 (IL-6), IL-17, tumour necrosis factor alpha (TNF-α), cortisol and adiponectin. There is lack of convincing evidence in human studies to support the role of resveratrol as a calorie restriction mimetic. The absence of metabolic and inflammatory effect disagrees with the strong and compelling data obtained from rodent models and some of the human studies questioning the use of resveratrol as a human nutritional supplement in metabolic disorders.

5.1 Introduction

As we observed behavioural and lifestyle changes in the modern society characterized by calorie-dense foods and low physical activity, we see an increase in the number of obesity-related diseases such as cancer, type 2 diabetes and cardiovascular complications. To date, the only non-pharmacological intervention to alleviate this constellation of diseases is calorie restriction. A substantial calorie reduction of 30 % – 50 % below the total energy expenditure or alternate day fasting and feeding practices are known to prolong longevity and the onset of chronic inflammatory diseases (Barger et al., 2003, Goodrick et al., 1990, McCay et al., 1989). Whilst restricting calorie intake exerts a wide a range of beneficial metabolic effects including increased insulin sensitivity (Lim et al., 2011) and improved cardiovascular disease risk (Lefevre et al., 2009), long-term adherence to calorie restriction depends on personal motivation and willingness of individuals (Moreira et al., 2011) and therefore, commonly a poor adherence to the prescribed diet is reported (Heymsfield et al., 2007). What is more, currently there is not enough evidence to prove that calorie restriction diet extends lifespan in humans making it even more difficult to gain widespread compliance towards eating less.

Alternative to calorie restriction dieting, are calorie restriction mimetics that evoke similar effects of calorie restriction without the actual decrease in energy consumption. Therein, sirtuins impose a key molecular modulator of the pathways involved in prolonging health span, for example regulating stress responses via deacetylate important transcription factors such as p53, forkhead homeobox type O proteins, nuclear factor κB, or PPARγ coactivator 1α. PPARγ coactivator 1α is involved in the transcription of pro- and antioxidant enzymes, by which the internal redox state is influenced(Radak et al., 2013).

The stilbene proanthocyanidin compound ‘resveratrol’ is found in high concentrations in mulberries, peanuts, the skins of grapes and has attracted attention in recent years as the dietary agent responsible for the ‘French paradox’(Burns et al., 2002). Resveratrol, is also a constituent of red wine, and drinking red wine in moderation has been associated with cardioprotective effects

(Lippi et al., 2010). Interest in resveratrol has been reawakened recently, after some studies showed it may have chemopreventive properties for skin cancer (Aziz et al., 2005), and later based on reports that resveratrol switches on sirtuin deacetylases and prolongs the lifespan of lower organisms (Boily et al., 2009, Vetterli and Maechler, 2011, Saini et al., 2012). Because resveratrol is an effective inhibitor of COX activity *in vivo* (Cianciulli et al., 2012), its anti-inflammatory properties have been investigated. Further, resveratrol was found to be the most potent activator of sirtuin proteins (Howitz et al., 2003). Amongst its suggested actions are hypoglycaemic and anti-cancer activities. Of particular interest to the present study is the reported anti-inflammatory (Yousuf et al., 2009) action of this plant derived compound. One of the possible mechanisms for its anti-inflammatory properties include down-regulation of cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2) and therefore, nuclear factor kappaB (NFkappaB) or activator protein-1 (AP-1) (Das and Das, 2007, Kraft et al., 2009).

Resveratrol significantly reduces both acute and chronic chemically induced oedema (Cao et al., 2011), lipopolysaccharide induced airway inflammation (Birrell et al., 2005) and osteoarthritis (Lei et al., 2012), and helps to prevent allograft rejection (Hsieh et al., 2007). Intravenously administered resveratrol decreases inflammation induced by ischaemia/reperfusion, oxidants generated by hypoxanthine/xanthine oxidase (HX/XO) or platelet activating factor, but not leukotriene B4 in rats. The first three conditions are all associated with superoxide formation, whereas leukotriene B4 induces inflammation via a superoxide-independent mechanism, suggesting that resveratrol treatment could have anti-oxidising effects. There is no known toxicity for resveratrol (Boocock et al., 2007a, Boocock et al., 2007b).

Resveratrol is known to have anti-cancer and oxidizing effects and it can induce cell apoptosis in a dose- and time-dependent manner. Treatment with resveratrol of human promyelocytic leukaemia (HL-60) cells for example resulted in smaller cells showing less refraction, and nuclear fragmentation; and it induced AnxA1, Bax, GADD45α and caspase-3 protein whilst suppressing the Bcl-2 protein (Li et al., 2011). Bcl-2 protein is involved in controlling the mitochondrial membrane permeability

exerting a blocking effect on the release of cytochrome C and prevents the activation of the caspase-3 apoptotic cascade (Zhan et al., 1994). A link was found between resveratrol's action and AnxA1 function in a study where AnxA1 was shown to be involved in apoptosis of cancer cells such as the HL-60 cells and pre-monomyelocytic U937 cells (Lim and Pervaiz, 2007, Debret et al., 2003, Alldridge et al., 1999). The induction of AnxA1 promotes apoptosis via association with caspase-3 activation, the key executioner of cell death (Alldridge et al., 1999, Solito et al., 2001). GADD45α gene has pleiotropic biological functions i.e. it was found to suppress cell growth (Zhan et al., 1994), it has a role in reconstruction of DNA damage (Smith and Gadd, 2000), it modulates the cell cycle at the G2/M stages (Wang et al., 1999), however, its most important role is considered in apoptotic signal pathways (Liebermann and Hoffman, 2002, Carrier et al., 1999, Kastan et al., 1992).

Resveratrol has been associated with a range of health beneficial effects, including antioxidant and anti-inflammatory actions, cardioprotective and anti-cancer properties and prevention of diabetes, thereby establishing itself as a chief nutritional aid for anti-aging with the endorsement of more than 5,000 scientific papers and reviews (Escola-Gil et al., 2013, Nakata et al., 2012, Turan et al., 2012, Xu and Si, 2012, Chung et al., 2012, Hu et al., 2011, Calamini et al., 2010, Kraft et al., 2009).

Herein, we proposed to conduct an *in vivo* study in individuals with elevated levels of pro-inflammatory adipocytokines. Resveratrol has no known toxicity at levels up to 700 mg/kg body weight (Williams et al., 2009) and the possibility of reducing inflammatory status in 'at-risk' individuals would demonstrate a novel neutraceutical approach to the potential prophylaxis of obesity related disorders. In view of our previous study on AnxA A1 in overweight humans, we were particularly interested if supplementation of resveratrol exerts any effects on plasma AnxA1 concentration.

5.2 Objectives

The aim of this study was to investigate the effect of two-week resveratrol supplementation on glucose control, blood cholesterol, and blood pressure; and pro and anti-inflammatory markers found in systemic circulation. To date *in vitro* data suggest that resveratrol has cardioprotective properties and this study was set out to investigate the acute effects of resveratrol on cardiovascular biomarkers *in vivo*. Alongside inflammatory markers and adipokines, blood glucose, insulin, cholesterol, glycated LDL and blood pressure were measured. The aim of this study was to investigate possible natural alternatives to reduce systemic inflammation, possibly via modulation of plasma AnxA1 levels.

5.3 Results

5.3.2 Study design

15 overweight healthy male volunteers without a family history of diabetes or any other endocrine disorders took part in this study. Baseline characteristics of the participants are shown in Table 19. Volunteers participated in two experimental trials: (1) a placebo and (2) a 250 mg/day resveratrol treatment in a randomized, double-blind, cross-over design with a 2 week wash-out period. Volunteers were asked to take the first supplement on the day after the baseline measurements (day 1) and the last supplement on day 14 preferably with a meal. Previous studies reported that 150 mg of resveratrol supplementation results in even higher plasma resveratrol levels in humans (i.e. 231 ng/ml) (Timmers et al., 2011) than those obtained in mice (10-120 ng/ml) with a high dose of exogenous resveratrol (200-400 mg/kg/day) (Baur et al., 2006, Lagouge et al., 2006). Volunteers were asked to inform the principal investigator as soon as possible if any adverse effects took place. To ensure no side effects resulted from the supplementation of resveratrol, we screened the blood and plasma of the volunteers for several general health parameters (see Table 19). No adverse events were communicated during the course of this study.

5.3.3 Participants' anthropometric and metabolic profile

We recruited 15 healthy but overweight (means± SEM; BMI 28.5 ± 0.93 kg/m^2) males for this study of average age 45.6 ± 2.4 years (see Table 19). The volunteers had excess body fat level (26.0 ± 1.9 %) and a high WHR (0.98 ± 0.02), therefore their anthropometric profile was within the risky category for metabolic syndrome associated diseases. The participants were healthy showing a normal systolic (118.3 ± 3.5 mmHg) and diastolic (76.6 ± 1.8 mmHg) blood pressure (see Table 19), a normal fasting blood glucose (4.97 ± 0.42 mmol/L) and insulin (9.10 ± 1.77 µU/ml) with the exception of fasting blood cholesterol which we found to be in the slightly high range at a mean value of 5.53 ± 0.23 mmol/L.

Table 19: Subjects' characteristics at the start of the intervention (day 0).

	Subjects' baseline characteristics		
	Placebo	**Resveratrol**	**P value**
Age (years)	45.6 ± 2.4	45.6 ± 2.4	-
Body weight (kg)	87.1 ± 2.6	86.8 ± 2.5	0.35
BMI (kg/m^2)	28.5 ± 0.93	28.3 ± 0.89	0.37
Body fat (%)	26.0 ± 1.9	25.8 ± 1.8	0.66
WHR	0.98 ± 0.02	0.97 ± 0.02	0.38
Systolic blood pressure	118.3 ± 3.5	118.1 ± 3.0	0.95
Diastolic blood pressure	76.6 ± 1.8	77.7 ± 2.1	0.59
Glucose (mmol/L)	4.97 ± 0.42	5.24 ± 0.34	0.28
Insulin (µU/ml)	9.10 ± 1.77	9.06 ± 0.90	0.97
Cholesterol(mmol/L)	5.53 ± 0.23	5.09 ± 0.17	0.02
Glycated LDL (mg/dL)	1.22 ± 0.21	1.14 ± 0.13	0.33

This table shows subjects' baseline characteristics before placebo and resveratrol course. Values are given as means ± SEM (N = 15).

Calorie restriction regime is linked with a decrease in body weight and a consequent reduction of 24-hour energy expenditure (Heilbronn et al., 2006). A study carried out at a later date investigated the effect of resveratrol supplementation on total body weight and energy expenditure. They found that 150 mg/day resveratrol supplementation for a period of 30 days significantly decreased sleeping metabolic rate, however 24 hr energy expenditure was unchanged (Timmers et al., 2011). Volunteers in our study did not have a significant change in their body weight, total body fat level, and WHR when placebo was compared with the resveratrol intervention (see Table 20).

Table 20: Subjects' characteristics after 14 days of intervention.

	Subjects' characteristics after 14 days of intervention		
	Placebo	**Resveratrol**	**P value**
Body weight (kg)	86.9 ± 2.6	86.6 ± 2.6	0.16
BMI (kg/m^2)	28.4 ± 0.93	28.3 ± 0.92	0.17
Body fat (%)	26.8 ± 2.07	28.9 ± 4.4	0.53
WHR	0.97 ± 0.02	0.97 ± 0.02	0.40
Systolic blood pressure	122 ± 3.0	120.5 ± 2.66	0.56
Diastolic blood pressure	78.3 ± 2.3	80.5 ± 2.2	0.29

This table presents subjects' characteristics after 14 days of placebo and resveratrol. Values are given as means ± SEM (n = 15).

The effects of long-term calorie restriction were investigated in healthy, normal weight humans over a period of 6 months and it was found that calorie restriction alone improved lipid profile and blood pressure with or without exercise thereby reducing cardiovascular risk factors (Lefevre et al., 2009). A randomized, double-blind, crossover, placebo controlled study investigating calorie restriction mimetics in 11 obese but healthy volunteers confirmed that a 150 mg/day resveratrol supplementation led to a 5 mmHg decrease in systolic blood pressure (placebo systolic blood pressure 130.5 ± 2.7, resveratrol systolic blood pressure 124.7 ± 3.1, P + 0.006), whilst no significant change was found in diastolic blood pressure(Timmers et al., 2011). A very recent double blind randomized and cross-over trial of 30 day resveratrol supplementation (500 mg/day) in healthy adult smokers found no statistically significant difference in metabolic parameters of interest including blood lipids and liver enzymes, blood sugar, insulin sensitivity, uric acid. The authors also did not find any significant changes in anthropometric measurements and blood pressure (Bo et al., 2013). Yet another recent double blind , randomized, high dose study investigating the effect of supplemental resveratrol found that it had no effect on blood pressure; resting energy expenditure; oxidation rates of lipid; ectopic or visceral fat content(Poulsen et al., 2013). Our results show a decrease in systolic blood pressure from 122 ± 3.0 to 120.5 ± 2.66 mmHg when placebo is compared with resveratrol treatment, respectively, however the change is not statistically significant. Our study found that diastolic blood pressure increased marginally but statistically not significantly so from 78.3 ± 2.3 to 80.5 ± 2.2 mmHg.

5.3.4 Clinical improvement or not after resveratrol supplementation

Calorie restriction dieting has profound beneficial effects on metabolic markers and therefore we set out to investigate if administration of supplemental resveratrol can exert similar effects. We measured the standard gold markers of risks for metabolic syndrome associated diseases including fasting blood glucose, OGTT glucose, fasting insulin, OGTT insulin, HOMA index, total blood cholesterol and glycated LDL and found no statistically significant change after 14 days of placebo or resveratrol treatment (see Table 21). A similar study investigating high-dose resveratrol supplementation in obese men measured endogenous glucose production, the turnover and glucose oxidation rate in a resveratrol trial and also found no change (Poulsen et al., 2013).

Table 21: Subjects' plasma biochemistry after placebo or resveratrol.

	Plasma biochemistry		
	Placebo	**Resveratrol**	**P value**
Glucose (mmol/L)	5.21 ± 0.35	5.34 ± 0.46	0.44
OGTT Glucose (mmol/L)	6.88 ± 1.04	6.56 ± 1.08	0.62
Insulin (µU/ml)	8.43 ± 1.67	9.07 ± 1.39	0.63
OGTT Insulin (µU/ml)	26.9 ± 8.79	25.8 ± 8.81	0.74
HOMA index	28.5 ± 0.93	28.3 ± 0.89	0.37
Cholesterol (mmol/L)	5.03 ± 0.36	4.89 ± 0.52	0.57
Glycated LDL (mg/dL)	1.31 ± 0.24	1.18 ± 0.34	0.39
Leptin (pg/mL)	118.3 ± 3.5	118.1 ± 3.0	0.95
Adiponectin (pg/mL)	76.6 ± 1.8	77.7 ± 2.1	0.59
Annexin A1 (pg/ml)	15.78 ± 6.44	14.65 ± 8.49	0.50
CRP (ng/mL)	9.10 ± 1.77	9.06 ± 0.90	0.97
IL-6 (pg/mL)	11.23 ± 5.25	10.91 ± 5.05	0.50

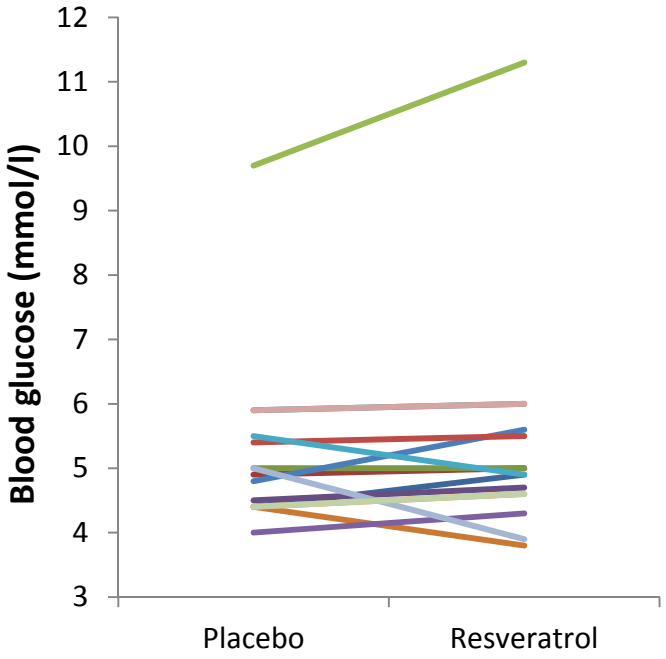
Plasma values after 14 days of placebo or resveratrol supplementation. Values are given as means ± SEM. The raw values for each subject are presented in Figures 86 – 92 (N = 15).

Due to the low number of subjects in this study, we represented the raw values of the concentrations of key markers of interest including fasting blood glucose, OGTT glucose, fasting insulin, OGTT insulin, cholesterol, glycated LDL and AnxA1. The Figures 86 to 92 illustrate that 250 mg/day of resveratrol supplementation over 14 days had no significant change on the parameters that we measured. The means and SD are also shown in Table 21.

We did not find a significant improvement in the concentration of the hormones, adiponectin (P = 0.59) and leptin (0.95) either (see Table 21).

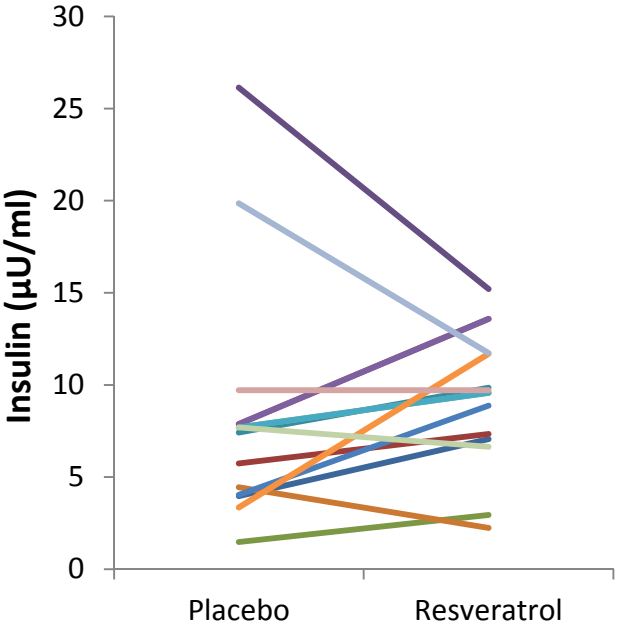
Whilst a study on resveratrol supplementation (30 days, 500 mg/day) in 50 male smokers found that resveratrol trial significantly reduced CRP and triglyceride levels (Bo et al., 2013) we did not find a change in neither CRP (P = 0.97) nor IL-6 (0.50) levels in our study.

Figure 86: Concentration of total blood glucose after 14 days of placebo or resveratrol supplementation.



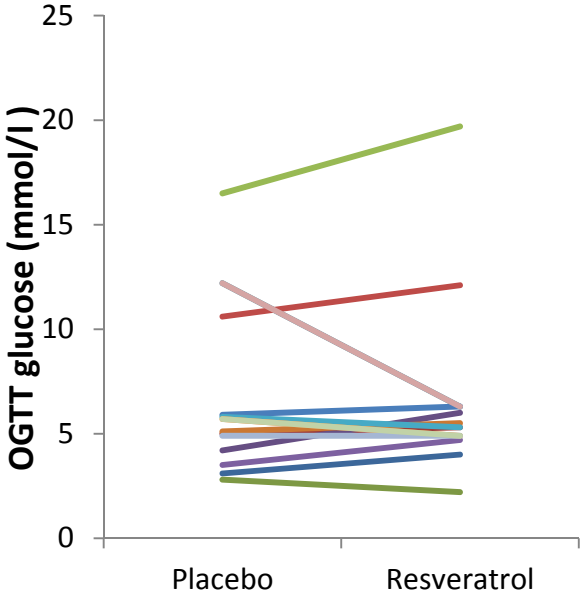
There was no statistically significant difference (P = 0.44) in the concentration levels of total blood glucose after 14 days of placebo or resveratrol treatment (N = 15).

Figure 87: Concentration of plasma insulin after 14 days of placebo or resveratrol supplementation.



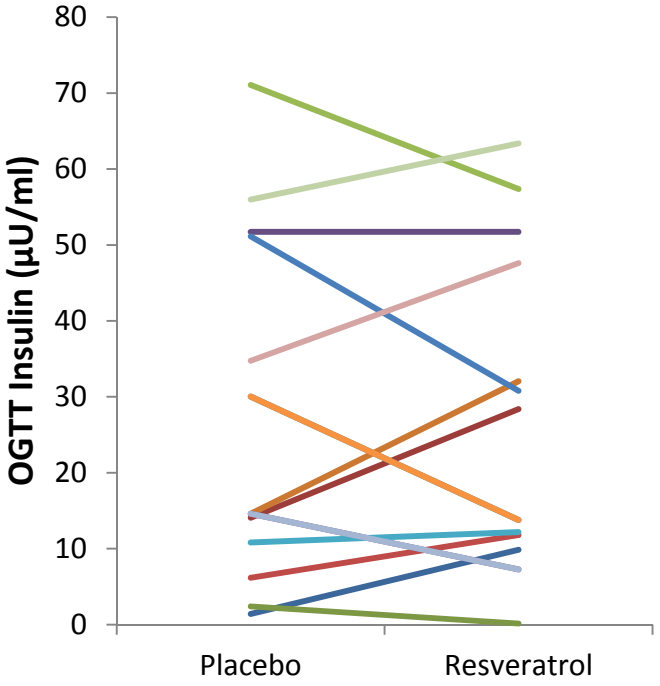
There was no statistically significant difference (P = 0.63) in the concentration levels of plasma insulin concentration after 14 days of placebo or resveratrol treatment (N = 15).

Figure 88: OGTT glucose result after 14 days of placebo or resveratrol supplementation



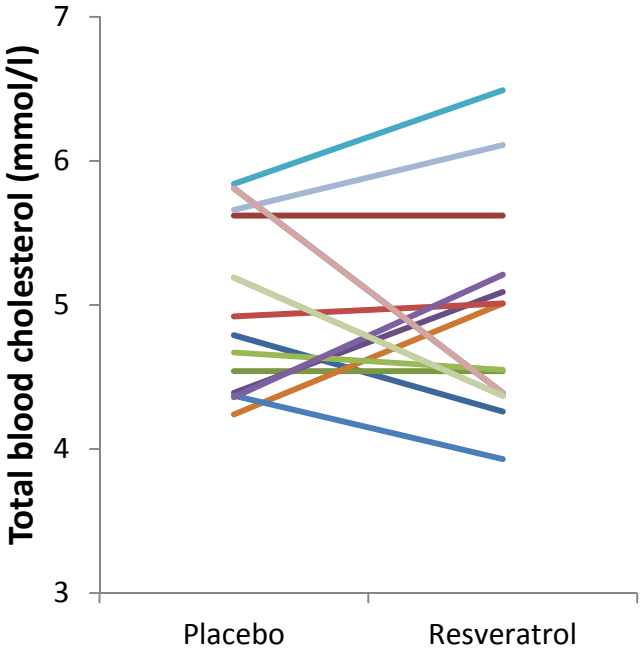
There was no statistically significant difference (P = 0.62) in the OGTT glucose result after 14 days of placebo or resveratrol treatment (N = 15).

Figure 89: OGTT insulin result after 14 days of placebo or resveratrol supplementation.



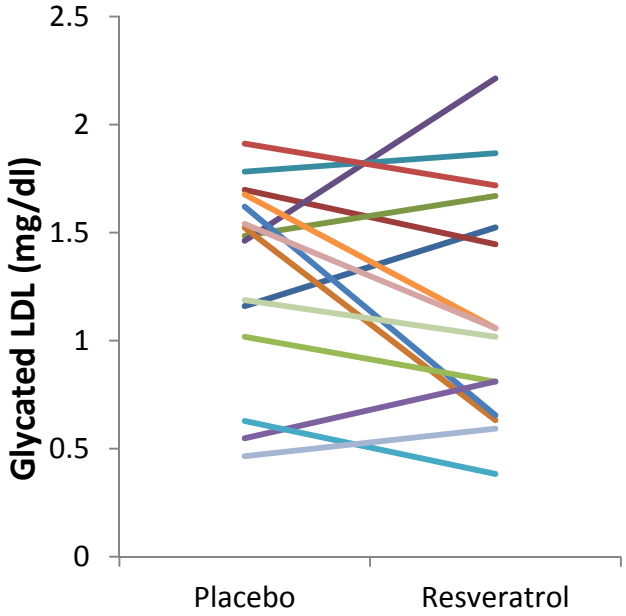
There was no statistically significant difference (P = 0.74) in the OGTT insulin result after 14 days of placebo or resveratrol treatment (N = 15).

Figure 90: Concentration of total blood cholesterol after 14 days of placebo or resveratrol supplementation.



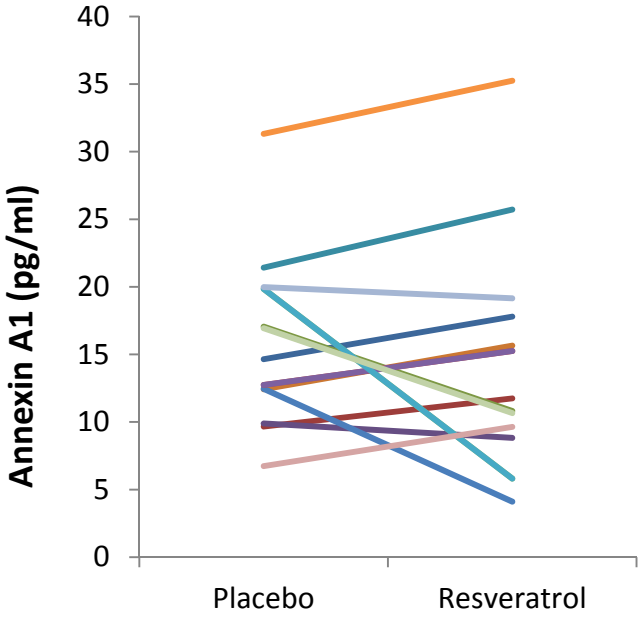
There was no statistically significant difference (P = 0.57) in the concentration levels of total blood cholesterol after 14 days of placebo or resveratrol treatment (N = 15).

Figure 91: Concentration of glycated LDL after 14 days of placebo or resveratrol supplementation.



There was no statistically significant difference (P = 0.39) in the concentration levels of glycated LDL after 14 days of placebo or resveratrol treatment (N = 15).

Figure 92: Concentration of plasma annexin A1 after 14 days of placebo or resveratrol supplementation.



There was no statistically significant difference (P = 0.50) in the concentration levels of plasma Annexin A1 after 14 days of placebo or resveratrol treatment (N = 15).

5.4 Discussion

At the time of designing this study in 2011 there was very little evidence of the effects of resveratrol supplementation on metabolic parameters in human subjects. Since then a number of double-blind placebo controlled studies have been carried out in humans providing some evidence for the role of resveratrol as a calorie restriction mimetic (Poulsen et al., 2013, Bhatt et al., 2012, Yoshino et al., 2012, Timmers et al., 2011, Rossi et al., 2012, Bode et al., 2013, Militaru et al., 2013, O'Connor et al., 2013, Rotches-Ribalta et al., 2012).

Whilst there is strong evidence that calorie restriction diet leads to a significant change in body weight, body composition, WHR and energy expenditure (Poehlman et al., 1991b, Heilbronn et al., 2006, Larson-Meyer et al., 2006), the attempts to bring about similar effects with the use of supplementary resveratrol failed (Poulsen et al., 2013, Bhatt et al., 2012, Yoshino et al., 2012, Timmers et al., 2011). Our study found no significant change in total body weight, body composition, or WHR as others reported.

We set out to investigate if the evidence from animal and *in vitro* studies (Kang et al., 2012, Lee et al., 2011a) supporting the role of resveratrol in reducing the risk factors for diabetes; hypertension and hyperlipidaemia can be replicated in humans. As confirmed in similar trials into the effect of resveratrol on metabolic function (Poulsen et al., 2013, Bo et al., 2013, Yoshino et al., 2012, Heger et al., 2012), our study showed no difference in the metabolic markers measured including blood pressure, blood glucose, blood cholesterol and glycated LDL. We speculated that perhaps this was due to the shorter length of our trial when compared with the period of trials of other studies (generally 30 days). However Bo et al., (2013) demonstrated that 500 mg/day (dose double of the one used in our study) resveratrol supplementation over 30 days brought about no change to glucose, insulin, cholesterol, liver enzyme concentrations and blood pressure. These findings were further supported by the high-dose resveratrol study in obese men where resveratrol treatment had no effect on blood pressure, resting energy expenditure, oxidation of lipid; ectopic and visceral fat content; or inflammatory and metabolic

biomarkers (Poulsen et al., 2013). Yet another evidence against the role of resveratrol as a calorie restriction mimetic was reported by Yoshino et al., (2012) in a study with non-obese women with normal glucose tolerance where they found resveratrol had no effect on metabolic or inflammatory markers.

Poulsen et al., 2011 demonstrated that 150 mg/day resveratrol supplementation over the period of 30 days significantly improved systolic blood pressure by ~ 5 mmHg but had no change on diastolic blood pressure. Whilst this finding was statistically significant, it is important to note here that inter-individual variability of blood pressure can deviate by 10-20 % and is influenced by an array of factors ranging from internal factors (e.g. neurohormonal regulation) and the extrinsic factors (physical activity, sleep deprivation or quality, and dietary sodium). Furthermore, behavioural factors (mental activity and emotional status) and lifestyle factors (alcohol drinking and smoking) can also affect the natural rhythm of blood pressure from measurement to measurement (Choi, 2012).

There is some data upholding the anti-inflammatory role of resveratrol where in a couple of resveratrol trials it was shown that resveratrol supplementation had significantly decreased CRP concentrations in 25 healthy smokers (Bo et al., 2013) and in patients with angina pectoris (Militaru et al., 2013). In the study by Militaru et al., (2013) this effect was further exaggerated by addition of calcium fructoborate (CF), a complex of calcium, fructose and boron naturally occurring in fresh and dried fruits and vegetables (Scorei and Rotaru, 2011). CF was proposed as a stabilizer for resveratrol degradation in the digestive tract by these authors but there is no published data to endorse this statement. Yet again, our study did not find a change in plasma CRP, IL-6 or AnxA1 level concentrations rejecting the hypothesis that resveratrol has anti-inflammatory effects in humans.

Resveratrol is contained in a variety of foods but the source that receives most press is the one found in the skins of grapes and therefore, red wine. It has been postulated that drinking a glass of red wine has beneficial effects on cancer and cardiovascular risk profile and glucose metabolism (Kraft et al., 2009, Turan et al., 2012, Wallenborg et al., 2009, Lippi et al., 2010). However, a study investigated if a

dose of 6 mg per day supplemental resveratrol equivalent to that found in a large glass of red wine (0.3 L), had an effect on oxidative DNA damage and redox status. The data showed that the intake of the supplement had no effect on the DNA stability and on the total antioxidant capacity (TAC) and oxidized low-density lipoprotein (oxLDL) biomarkers of the redox status (Heger et al., 2012). Furthermore, a trial measuring the effect of grape consumption on fitness, muscle injury, mood and perceived health in young active adults over a period of 45 days proved to have no effect on VO_{2max}, work capacity, mood, perceived health status, inflammation, pain, or physical-function responses to a mild injury induced by eccentric exercise (18 high-intensity actions of non-dominant elbow flexors) (O'Connor et al., 2013). This may not be surprising in view of the limited and variable content of resveratrol in foods (Rossi et al., 2012) and the difficulty in obtaining it as the biological availability of resveratrol is highly dependent on several factors including e.g. the food matrix (Rotches-Ribalta et al., 2012) and the gut microbiota (Bode et al., 2013). It is plausible to speculate that the lack of supporting evidence in human studies for the anti-inflammatory, anti-oxidant and cardioprotective role of resveratrol may be due to its low bioavailability and rapid metabolism and elimination (Walle et al., 2004). Future investigations into potential resveratrol synergistic enhancers and stabilizers e.g. CF, may shed some light on the conflicting data presented in animal and *in vitro* studies and human studies.

In conclusion, we demonstrate that resveratrol supplementation for 14 days has no beneficial effects on the metabolic and inflammatory profile in healthy overweight males. Therefore, it does not support its role as a cardioprotective, anti-inflammatory and anti-oxidant agent in humans.

To date there are only a handful of well-designed human studies supporting the role of resveratrol as a dietary aid bringing about beneficial health effects (Timmers et al., 2011, Bhatt et al., 2012). It is important to note that also the effects that were observed in these studies were modest and although statistically different, perhaps not significant in terms of physiological importance. In view of the recent numerous human studies and the findings from our study the role of both supplemental and

dietary source (from red wine and grapes) resveratrol as a calorie restriction mimetic exerting beneficial metabolic and anti-inflammatory actions is questionable.

5.5 Conclusion

The lack of metabolic and inflammatory effect disagrees with the strong and compelling data obtained from rodent models and some of the human studies questioning the use of supplemental resveratrol as a nutritional therapeutic for metabolic disorders. The worldwide retailing of active food ingredients as dietary supplements is developing faster than most other areas in the commercial food enterprise and epitomizes a behavioural and scientific hybridization between attitudes towards food and pharmacological agents. Health conscious customers should have access to the evidence for efficacy of nutritional supplements to avoid disappointment and manage their expectations.

6 Final discussion and future studies

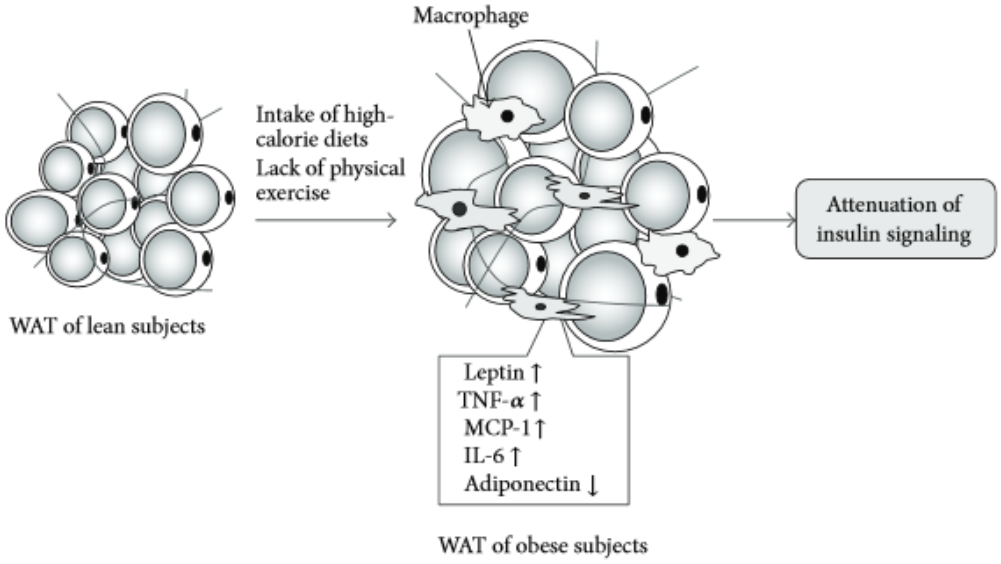
6.1 Final discussion

Obesity has become a serious global issue and requires actions and solutions on a global scale. Obesity is now a problem even in the poorest of countries, e.g. Sierra Leone, where people experience the double burden of diseases, including infectious diseases (e.g. malaria, pneumonia) and at the same time, obesity associated maladies. Obesity is linked with a number of degenerative disease states including cancers, type 2 diabetes, and hypertension. At large obesity is caused by chronic excess calorie consumption coupled with low physical activity. Excess food intake leads to a state of chronic systemic inflammation and WAT is thought to be the major source of increased level of pro-inflammatory molecules including CRP, IL-6 and TNFα. This PhD research study was set out to determine if there are any novel exogenous and/or endogenous molecular targets that can alter the chronic inflammation found in the WAT of obese individuals and therefore, in the systemic circulation.

Adipose tissue is vital for the normal function of the human body; however excess adiposity (Shils, 2006) is strongly linked with the cause of type 2 diabetes, cardiovascular disease and certain types of cancers (Cao, 2014). Certain metabolic, physiological and lifestyle factors such as dyslipidaemia, arterial hypertension, large waist size, BMI >25 kg/m^2 and low activity levels have been identified as increasing risk factors for metabolic syndrome. The pathology of disease states is strongly linked with qualitative aspects of dietary choices i.e. high added sugar and fructose corn syrup products (Johnson et al., 2013), low physical activity levels and poor mental health i.e. depression (Gallagher and Hannigan, 2013). There is good evidence that long-term inflammatory status may increase susceptibility to cancer via DNA damage and genomic instability, as multiple cancers have chronic inflammatory precursor lesions/tissues and therefore, any dysregulation in the inflammatory balance is of great significance (Ahmad et al., 2009, Kidane et al., 2014). There is evidence showing that the metabolic syndrome is characterized by a mild chronic inflammation found in the systemic circulation (Cao, 2014).

There are several theories about the causality of mild inflammation in the obese. Primarily, chronic positive energy balance leads to hypertrophy and hyperplasia of adipocytes (Trayhurn, 2005). As the fat mass expands in obesity, the levels of lipids increase proportionately in adipocytes. High lipid levels in adipocytes may lead to initiation of cellular stress (Maassen et al., 2008). It has been found that adipocyte necrosis is three times higher in the obese and it is relative to hypertropic increase of adipocytes (Wang et al., 2008). This in turn may lead to increased secretion of pro-inflammatory cytokines as shown in Figure 93. Chronic hypercaloric energy intake and low energy expenditure lead to hypertrophy of adipocytes. Enlargement of adipocytes induces macrophage chemoattractant protein-1 (MCP-1) which in turn results in adipose tissue infiltration with macrophages. There is a subsequent increased expression in pro-inflammatory markers including TNF-α, MCP-1, and IL-6 and decreased expression of anti-inflammatory adipokines (e.g. adiponectin).

Figure 93: Proposed model of inflammation process in the white adipose tissue of obese individuals.



Our proposed model of inflammation process in WAT of obese individuals. Chronic hypercaloric energy intake and low energy expenditure lead to hypertrophy of adipocytes. Enlargement of adipocytes induces macrophage chemoattractant protein-1 (MCP-1) which in turn results in adipose tissue infiltration with macrophages. There is a subsequent increased expression in pro-inflammatory markers including TNF-α, MCP-1, and IL-6 and decreased expression of anti-inflammatory adipokines (adiponectin). Chronic inflammatory imbalance leads to attenuation of insulin signalling.

Chronic inflammatory imbalance may lead to attenuation of insulin signalling.

To the best of our knowledge, we have carried out the most extensive human study investigating plasma AnxA1 levels and its association with body composition. We examined the relationship between the pro-resolution mediator, plasma AnxA1 and body composition, total body fat level and body fat distribution. We also investigated the relationship between plasma AnxA1 and metabolic and inflammatory parameters as well as the plasma concentration of specific adipokines which are up-regulated in obesity. We showed that plasma AnxA1 is significantly reduced with increasing BMI, total body fat and increasing centrally located body fat (Figure 55, 56, and 57). The negative correlation of decreasing plasma AnxA1 was strongest statistically when compared with WHR, rather than total body fat, suggesting that centrally located fat may be more influential at reducing plasma AnxA1 concentrations. Extensive evidence shows that the specific distribution of body fat is strongly correlated with increased risks for the development of type 2 diabetes and cardiovascular disease (Gallagher et al., 1996, Gallagher et al., 2000, Ko et al., 1999, Vazzana et al., 2011, Doyle et al., 2011, Lam et al., 2011, Barac et al., 2012, Indulekha et al., 2011).

Our data show that plasma AnxA1 level decreases in subjects characterized by endomorphic body type and therefore, AnxA1 may be a potential depot specific biomarker and a predictor of future health risks and complications. Our data may also infer that AnxA1 could potentially have a protective role in the development of obesity. When mean plasma AnxA1 protein levels are presented for each total body fat group (defined by WHO as lean, moderately lean, excess fat and risky (WHO, 1995), mean plasma AnxA1 levels are not statistically different across the lean, moderately lean and excess fat groups. However, there is a significant decrease in the mean plasma AnxA1 concentration in the risky group compared to all other groups (see Figure 56). Similarly, when categorized by BMI, the obese I and obese class II groups expressed significantly lower plasma AnxA1 compared to individuals with normal BMI (Figure 55). It is tempting to speculate therefore, that AnxA1 may have a protective role at the onset of central obesity but becomes overwhelmed as

central fat mass continues to rise. Taking into account AnxA1's anti-inflammatory role in both the innate and adaptive immune systems, this may well explain the fact that there is a mild chronic inflammation associated with obesity (Trayhurn et al., 2008a, Wang et al., 2007, Trayhurn et al., 2008b, Do et al., 2006, Trayhurn and Wood, 2004, Rai and Sandell, 2011, Siervo et al., 2011, Pruller et al., 2011).

Our study in humans shows a decrease in plasma AnxA1 concentration as the WAT expands in male subjects. Currently, it is not clear which tissues contribute to the plasma AnxA1 levels and further studies are needed to investigate the degree of expression of AnxA1 protein from visceral and subcutaneous fat in human subjects.

The cell culture studies of the role of AnxA1 in human SGBS cells presented in Chapter 4 confirm for the first time that AnxA1, FPR1 and FPR2/ALX receptors are expressed in human subcutaneous adipocytes. The fact that the AnxA1 protein is derived from the supernatant fraction, suggests a possible function of AnxA1 protein within WAT, as it is not merely stored within the cell but released into the local environment. The exact source of plasma AnxA1 protein has not been determined to date and may represent the contribution of protein from a number of different sources. It may be assumed that leukocytes represent the largest source of plasma AnxA1 as these cells are known to store large quantities for their role as an anti-migratory agent for leukocytes (Rosales and Ernst, 1997). However, given the current data, we cannot rule out the possibility that an adipocyte source of AnxA1 protein may significantly contribute to total plasma AnxA1 protein.

AnxA1 may be implicated in the adipocyte function irrespective of the inflammatory status of adipocytes. A study by Mack et al., 2009 revealed that preadipocytes have greater potency when compared with adipocytes as endothelial cell activator under normoxia, hypoxia, and TNFα exposure (Mack et al., 2009) and this may be another explanation why we did not find any significant changes in the expression of AnxA1 in hypoxic mature adipocytes when compared to normoxic controls. Furthermore, increasing adiposity may reach a critical point, beyond which AnxA1 protein from adipocyte and non-adipocyte sources are impaired leading to a collapse in plasma AnxA1 protein levels in affected individuals. By demonstrating a significant up

regulation in both AnxA1 gene expression and secreted protein during adipogenesis, our current data further implicates AnxA1's role in regulating adipocyte biology.

Additionally our study showed for the first time that exogenous administration of the Ac2-26 peptide decreased the expression of CRP level and IL-6 in human SGBS adipocyte cell line under hypoxic conditions. Previous studies showed that Ac2-26 decreased neutrophil activity, levels of PLA2, PGE2, nitric oxide and COX2 activity, therefore exerting an anti-inflammatory role (Perretti and D'Acquisto, 2009, Perretti and Dalli, 2009, Yang et al., 2013). Although AnxA1 is largely stored preformed in cytoplasmic granules ready for release into the extracellular space, after secretion, extracellular AnxA1 binds to its receptors, FPR1 and FPR2/ALX (Babbin et al., 2006, John et al., 2007) on adjacent cells and also in a juxtacrine manner. Similarly to the AnxA1 expression, we found that FPR1 receptor was significantly down-regulated after 4 hours (0.6-fold, P=0.01*) and 8 hours (0.4-fold, P=0.001) of hypoxia treatment in mature adipocytes when compared to preadipocytes (see Figure 72) but no significant changes were observed in normoxic conditions between mature adipocytes and preadipocytes (see Figure 68). FPR1 was also significantly down-regulated at 8 hours of hypoxia treatment (0.3-fold, P=0.004**) in mature adipocytes when compared to mature adipocytes in normoxic conditions (see Figure 78).

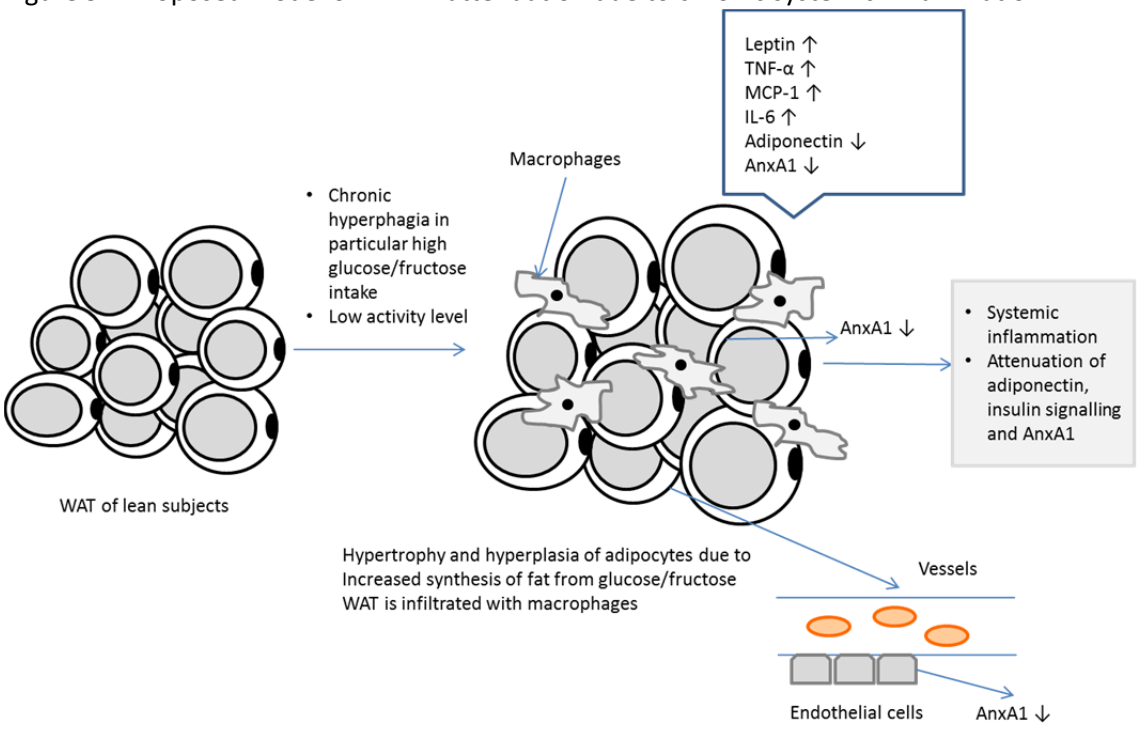
The FPR1 receptor gene data may suggest that AnxA1 signalling via the FPR1 receptor may be altered during hypoxia and if extrapolated, may be altered in the adipose tissue of obese humans. Although AnxA1 gene itself is not altered, its communication via its receptors may be a mechanism by which alterations/fine tuning in AnxA1 signalling is achieved in WAT during hypoxia. The implications of this are not known at this time. However, reduced FPR1 receptor expression may have implications for adipocyte recruitment. Our data on AnxA1 protein and gene expression in normoxic conditions suggests that this system is significantly increased during adipogenesis. If this process is disrupted by a reduced oxygen supply, then this may have implications for adipocyte recruitment. Furthermore, the recruitment of pre-adipocytes to mature adipocytes has been hypothesised to become dysregulated in a sub-population of obese patients who develop WAT fibrosis

leading to systemic inflammation and metabolic disease (Sun et al., 2011). In vitro culture of WAT biopsies from lean, 'healthy' obese and obese patients with metabolic disease could be used to explore this area further in future studies.

We propose that chronic hypercaloric energy intake in particular high glucose/fructose consumption and low energy expenditure lead to hypertrophy and hyperplasia of adipocytes via high circulating blood glucose stimulated increased lipogenesis. Enlargement of adipocytes induces macrophage chemoattractant protein-1 (MCP-1) which in turn results in adipose tissue infiltration with macrophages. There is a subsequent increased expression in pro-inflammatory markers including TNF-α, MCP-1, and IL-6 and decreased expression of anti-inflammatory adipokines (adiponectin). AnxA1 secretion is reduced both within WAT and within the endothelial cells and leukocytes of the capillaries. Chronic inflammatory imbalance leads to attenuation of adiponectin, AnxA1 and insulin signalling and increased vascular complications (see Figure 94).

We explored exogenous administration of a calorie restriction mimetic , resveratrol and its effect on systemic inflammation and metabolic profile of 15 men. Our findings showed that resveratrol supplementation for 14 days has no beneficial effects on the metabolic and inflammatory profile in healthy overweight males. Therefore, it does not support its role as a cardioprotective, anti-inflammatory and anti-oxidant agent in humans. To date there are only a handful of well-designed human studies supporting the role of resveratrol as a dietary aid bringing about beneficial health effects (Timmers et al., 2011, Bhatt et al., 2012). It is important to note that also the effects that were observed in these studies were modest and although statistically different, perhaps not significant in terms of physiological importance. In view of the recent numerous human studies and the findings from our study the role of both supplemental and dietary source (from red wine and grapes) resveratrol as a calorie restriction mimetic exerting beneficial metabolic and anti-inflammatory actions is questionable.

Figure 94: Proposed model of AnxA1 attenuation due to chronic systemic inflammation.



Chronic hypercaloric energy intake in particular high glucose/fructose consumption and low energy expenditure lead to hypertrophy and hyperplasia of adipocytes via High circulating blood glucose stimulated increased lipogenesis. Enlargement of adipocytes induces macrophage chemoattractant protein-1 (MCP-1) which in turn results in adipose tissue infiltration with macrophages. There is a subsequent increased expression in pro-inflammatory markers including TNF-α, MCP-1, and IL-6 and decreased expression of anti-inflammatory adipokines (adiponectin). AnxA1 secretion is reduced both within WAT and within the endothelial cells and leukocytes of the capillaries. Chronic inflammatory imbalance leads to attenuation of adiponectin, AnxA1 and insulin signalling and increased vascular complications.

6.2 Conclusion

These data demonstrate that AnxA1 could potentially represent a (fat) depot specific biomarker whose decline with increasing central adiposity may relate to the phenomena of increasing systemic inflammation and associated disease risk. We also demonstrate for the first time that an AnxA1 is expressed in human SGBS preadipocytes and mature adipocytes and AnxA1 mimetic, Ac2-26 peptide, regulates pro-inflammatory markers in human SGBS adipocytes. We showed that it may be difficult to improve the metabolic profile of individuals through supplementation of exogenous anti-inflammatory agent, resveratrol. Whilst anti-inflammatory agents

such as AnxA1 may propose novel therapeutics for metabolic syndrome associated diseases, to date regular exercise and weight loss remain the main interventions that significantly cut the risk of developing chronic long-term conditions and obesity-associated maladies.

6.3 Future studies

We have demonstrated that endogenous anti-inflammatory protein, AnxA1 may be implicated in the regulation of adiposity and that exogenous supplementation with the Ac2-26 peptide may alter the pro-inflammatory status of human adipocytes. We propose a number of studies that can help understand if AnxA1 is a potential novel target in the treatment of obesity related complications. We suggest that future studies are carried out in the specific following areas to further investigate AnxA1's role as a regulator of adiposity and inflammation in WAT:

1) Characterisation of the role of ANXA1 in WAT in the ANXA1 knock-out (KO) mice and wild type (WT) on high fat diets and measurement of the impact on the adiposity level over time. This study would enable us to understand if AnxA1 influences expansion of fat mass, the number of pre-adipocytes/adipocytes, the size of adipocyte lipid droplet. We would also investigate if WAT of KO mice is infiltrated with macrophages in the same way as WAT of WT mice. This study may help to find out why AnxA1 was increased during adipogenesis and if there are any defects in adipogenesis if AnxA1 is absent –in the null mice.

2) Investigate which factors from WAT modulate AnxA1 expression in various cell types including preadipocytes and adipocytes *in vitro* in isolation. The cells can be stimulated with a CRP, NFκB, IL-6 pro-inflammatory mediators and we would then measure the impact of such treatment on expression and production of AnXA1 gene protein from the cell types.

3) Finally, we proposed that plasma AnxA1 may have a protective effect on the development of metabolic syndrome related complications. Exogenous supplementation with AnxA1 could be studied *in vivo* in a murine atherogenic model

e.g. ApoE mouse (mice which are highly prone to atherosclerosis). The effects of high fat diet and daily treatment with AnxA1 could help understand if AnxA1 may be an effective molecular target for the treatment of inflammation and if supplementation improves health outcomes.

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