In Vitro And *In Vivo* Models Investigating Pharmacological Modulators Of The Metabolic Syndrome

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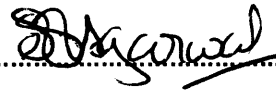
Signed .....  (candidate) Date ....20/09/10....

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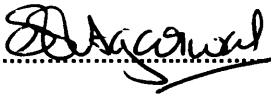
Signed .....  (candidate) Date 20/09/10.....

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CONTENTS

SUMMARY

BACKGROUND

Owing to the long-term health risks associated with increased adiposity and the limited efficacy of existing anti-obesity agents, there is an urgent need for further development of alternative therapeutic options to serve as an adjunct to lifestyle measures to address global cardiometabolic risk and thus reduce the risk of developing obesity-related illnesses such as type II diabetes and cardiovascular disease.

AIMS & METHODS

The studies presented in this thesis aimed to investigate the effects of novel (CB_1 antagonist) and existing (metformin) pharmacological agents on components of the metabolic syndrome using *in vitro* and *in vivo* models. This was undertaken with *in vitro* studies of the effect of CB_1 receptor modulation on white, brown and primary (subcutaneous and omental) pre-adipocyte proliferation, adipogenesis and adipokine production; as well as a randomised, double-blind cross-over trial of the effects of metformin on vascular function and metabolic profile in young women with polycystic ovary syndrome (*in vivo*).

RESULTS

CB_1 receptor antagonism inhibited white and primary pre-adipocyte proliferation and increased expression of terminal markers of adipogenesis while promoting adiponectin production. In young women with PCOS, short term metformin therapy improved arterial stiffness and endothelial function, resulted in modest weight loss and an elevation in serum adiponectin. There is increasing evidence for the key role the latter plays in metabolic processes and vascular health, and these increases in adiponectin levels may underlie some of the mechanisms mediating the changes observed. Therefore treatments targeting a switch inthe adipokine profile as the therapeutic goal represent a new approach to addressing global cardiometabolic risk.

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PUBLICATIONS AND PRESENTATIONS

Publications Arising From Research

Agarwal N, Rice SP, Bolusani H, Luzio SD, Dunseath G, Ludgate M, Rees DA: **Metformin reduces arterial stiffness and improves endothelial function in young women with polycystic ovary syndrome: a randomized, placebo-controlled, crossover trial.**
Journal of Clinical Endocrinology & Metabolism 2010, **95**(2):722-730.

Agarwal N, Rice SP, Bolusani H, Luzio SD, Dunseath G, Ludgate M, Rees DA: **Metformin reduces arterial stiffness and improves endothelial function in young women with polycystic ovary syndrome: a randomized, placebo-controlled, crossover trial.**
Obsterical & Gynaecological Survey 2010, **65**(6):381-382

Presentations To Learned Societies

N Agarwal, SPL Rice, H Bolusani, SD Luzio, G Dunseath, ME Ludgate, DA Rees. **The effect of metformin on vascular function in PCOS.**
Society for Endocrinology, April 2009. Harrogate. (Oral presentation/abstract)

N Agarwal, SPL Rice, H Bolusani, SD Luzio, G Dunseath, ME Ludgate, DA Rees. **The effect of metformin on vascular function in PCOS.**
Welsh Endocrine & Diabetes Society Meeting, May 2009, Newport. (Oral presentation/abstract – David Q Borsey prize)

N Agarwal, S Rice, L Zhang, DA Rees, and ME Ludgate. **Comparison of the effects of cannabinoid receptor modulation on adipogenesis in human primary preadipocytes and a murine cell line.**

Society for Endocrinology, April 2008. Harrogate. (Poster/abstract)

N Agarwal, DA Rees, ME Ludgate. **Cannabinoid Receptors in proliferation and differentiation of white and brown adipose tissues.** Welsh Endocrine and Diabetes Society, Newport. May 2007. (Oral presentation)

N Agarwal, DA Rees, ME Ludgate. **Comparison of the effects of cannabinoid receptor modulation in human primary preadipocytes and a murine cell line.**
Society for Endocrinology, Birmingham. March 2007. (Poster/Abstract)

N Agarwal, DA Rees, ME Ludgate. **Cannabinoid Receptors in proliferation and differentiation of white and brown adipose tissues.**
Society for Endocinology, London. November 2006. (Poster/Abstract)

This work has has also been presented locally at Cardiff University Annual Research Days and at Cardiff University Interdisciplinary Research Group meetings.

ABBREVIATIONS

µg	Microgrammes
µl	Microlitres
17-OHP	17-hydroxyprogesterone
2-AG	2-arachidonylglycerol
5-HT	5-hydroxytryptamine
A	Adenosine
A_{260}	Absorbance at wavelengths of 260 nanometres
A_{280}	Absorbance at wavelengths of 280 nanometres
AACE	American Association of Clinical Endocrinology
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropic hormone
AEA	Anandamide
AIx	Augmentation index
AMPK	Adenosine monophosphate-activated protein kinase
Amp^r	ampicillin resistance
AP	Active – Placebo
Apo B	Apoprotein B
APRT	adenosine phosphoribosyltransferase
aPWV	Aortic pulse wave velocity
ARP	acidic ribosomal phosphoprotein
ATII	angiotensin-II
BAT	Brown adipose tissue
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
bp	Base pairs
BP	Blood pressure

bPWV	Brachial pulse wave velocity
BSA	bovine serum albumin
Buffer AW	Wash buffer
C	Cytosine
C/EBP	CCAAT-enhancer-binding proteins
C/EBPβ	CCAAT-enhancer-binding protein β
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
cDBP	Central diastolic blood pressure
cDNA	complementary DNA
CHD	Coronary heart disease
CHOP	C/EBP homologous protein
CiAP2	cellular inhibitor of apoptosis2
CIMT	Carotid intima media thickness
cm	centimetre
CNP	C-type natriuretic peptide
COX	cyclooxygenase
CRP	C-reactive protein
cSBP	Central systolic blood pressure
Ct	threshold cycle
CVD	Cardiovasular disease
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphophate
dGTP	Deoxyguanosine triphosphate
DHEA	Dehydroepiandrosterone
DM	Differentiation medium

DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
dNTPs	deoxynucleotide triphosphates
DPP	Diabetes prevention programme
dTTP	Deoxythymidine triphosphates
E. Coli	*Escherichia coli*
ECS	Endocannabinoid system
EDHF	endothelium-derived hyperpolarizing factor
EDTA	Ethylenediaminetetraacetic acid
EGIR	The European Group for the study of Insulin Resistance
EMA	European medicines agency
eNOS	endothelium NOS
ET-1	endothelin-1
FA	Fatty acid
FAAH	Fatty acid amide hydrolase
FAH	Functional adrenal hyperandrogenism
FAI	Free androgen index
FAS	Fatty acid synthase
FCS	Fetal calf serum
FDG PET	2-[^{18}F]fluoro-2-deoxy-glucose positron emission tomography
FDPT	Finnish diabetes prevention trial
FFA	Free fatty acids
FOH	Functional ovarian hyperandrogenism
FSH	Follicle stimulating hormone
G	Grammes

G	Guanine
G_0	Gap 0 cell cycle phase
$G_{1/2}$	Gap 1 or 2: cell growth phase
GATA	glutamyl amino transferidase subunit A
GH1	Growth Hormone 1
GHR	Growth Hormone Receptor
GLUT4	glucose transporter 4
GnRH	Gonadotrophin releasing hormone
GPDH	glycerol-3-phosphate dehydrogenase
GTN	Glyceryl trinitrite
H20	Pure water
HAART	highly active antiretroviral therapy
HBSS	Hank's balanced salt solution
HDL	High density lipoprotein
HIV	Human Immunodeficieny Virus
HOMA	Homeostasis model assessment
HOMA-IR	Homeostasis model assessment - Insulin Resistance
HRP	Horseradish-peroxidase
hsCRP	High sensitivity C-reactive protein
IBMX	3-isobutyl-1-methylxanthine
IDF	International Diabetes Federation
IGF	Impaired fasting glucose
IGF-1	Insulin-like growth factor 1
IGT	Impaired glucose tolerance
IL-6	Interleukin-6
IMP	investigational medicinal product
iNOS	inducible NOS
IP3	inositol triphosphate 3

IQR	Inter Quartile Range
IR	Insulin resistance
IRS	Insulin receptor substrate
ISRCTN	International Standard Randomised Controlled Trial Number
K^+_A	A-type potassium channels
Kb	Kilo base
kg	Kilogram
KLF	Krupple-like factors
L	Litre
*Lac*Z	β-galactosidase
LB	Luria Bertani
LDL	Low density lipoprotein
LH	Luteinising hormone
LPL	Lipoprotein lipase
M	Molar
MAP	mitogen-activated protein
mg	Milligrams
$MgCl_2$	Magnesium chloride
MHRA	Medicines and Healthcare Products Regulatory Authority
ml	Millilitres
mM	Millimolar
MMLV	Moloney murine leukemia virus
mmol	Millimoles
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
NCEP-ATP III	National Cholesterol Education Program Third Adult Treatment Panel
ng	Nanograms
NHANES	National Health and Nutrition Examination Survey

nM	Nanomolar
NO	Nitric oxide
NOS	NO synthases
OD_{49}	Optical density at 49 nm
PA	Placebo Active
PAI-1	Plasminogen activator inhibitor-1
PBS	Phophate buffered saline
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
pDBP	Perioheral diastolic blood pressure
pg	Picogrammes
PGI_2	Prostacyclin
PGK1	phosphoglycerate-1-kinas
PI3K	phosphoinositide 3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	phopholipase C
pmol	Picomoles
pmol	Picomoles
PPARγ	Peroxisome proliferator-activated receptor-γ
Pref-1	Pre-adipocyte factor 1
pSBP	Peripheral systolic blood pressure
PWA	Pulse wave analysis
PWV	Pulse wave velocity
QPCR	Quantitative real time polymerase chain reaction
RIO	Rimonabant in obesity
RNA	Ribonucleic acid
RNAse	Ribonuclease

RPA	ribonuclease protection assay
rpm	Revolutions per minute
RSq	correlation coefficient
S	DNA synthesis phase
SAE	Serious adverse event
SEM	Standard error of mean
SHBG	Sex-hormone binding globulin
siRNA	small interfering RNA
SNP	sodium nitroprusside
SREBP1c	Sterol response element binding protein 1c
$T_{10}E_1$	(10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
T2DM	Type II diabetes mellitus
TAE	Tris-Acetate-EDTA
TC	Total cholesterol
TG	Triglycerides
TGF-β	transforming growth factor-β
Tm	melting temperature
TNF-α	Tumour necrosis factor-alpha
t-PA	Tissue plasminogen activator inhibitor
T_R	Time to inflection point
Tris.HCl	Tris(hydroxymethyl)aminomethane hydrochloride
U	Units
UCP-1	uncoupling protein-1
UHW	University Hospital of Wales
UV	Ultraviolet
VLDL	Very low-density lipoprotein
v/v	Volume/volume
w/v	Weight/volume

WAT	White adipose tissue
WHO	World Health Organisation
WHR	Waist hip ratio
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Δ9-THC	Δ^9-tetrahydrocannabinol
μm	Micrometre
μM	Micromolar

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CHAPTER 1: GENERAL INTRODUCTION - MODELS OF THE METABOLIC SYNDROME

1.1 Historical Perspective

The concept of the metabolic syndrome, or a clustering of metabolic abnormalities arising from the increasing prevalence of obesity, has existed since the 1920s, when the Swedish physician Kylin first described an association between hypertension, hyperglycaemia and hyperuricaemia [1]. A correlation with upper body adiposity (android or male-type obesity) was acknowledged by Vague in the 1940s [2] while the association with insulin resistance was made towards the end of the 1980s [3]. Since then numerous labels have been applied including the "deadly quartet," "syndrome X" and the "insulin resistance syndrome" eventually leading to today's terminology of the metabolic syndrome referring to the coexistence of glucose intolerance and insulin resistance, central obesity, dyslipidaemia and hypertension [4]. These are all well recognised risk factors predisposing individuals to the development of type II diabetes and cardiovascular disease, both associated with increased morbidity and mortality. With a growing worldwide prevalence, addressing the metabolic syndrome has therefore become a global public health challenge.

1.2 Definition Of The Metabolic Syndrome

Over recent years numerous expert groups have attempted to develop an internationally accepted and unified definition of the metabolic syndrome (Table 1.1), including the World Health Organisation (WHO), the European Group for the Study of Insulin Resistance (EGIR), and the National Cholesterol Education Program Expert Panel on the Detection, Evaluation and Treatment of High Blood Cholesterol in Adults - Third Adult Treatment Panel (NCEP-ATP

III), as well as position statements from the American Association of Clinical Endocrinology (AACE) and the International Diabetes Federation (IDF) [4, 5].

Table 1.1: Definitions Of The Metabolic Syndrome

	WHO 1999 [6] Impaired glucose tolerance or diabetes and/or insulin resistance AND two of the following:	EGIR 1999 [7] Insulin resistance AND two of the following:	NCEP ATP III 2001 [8] Three or more of the following risk factors:	AACE 2003 [9]
Central obesity	Waist hip ratio ≥ 0.9 ♂ ≥ 0.85 ♀ or BMI > 30 kg/m²	Waist circumference ≥ 94 cm ♂ ≥ 80 cm ♀	Waist circumference ≥ 102 cm ♂ ≥ 88 cm ♀	BMI > 25 kg/m²
Dysglycaemia (mmol/l)	Fasting glucose ≥ 6.1 or 2 hours post glucose challenge ≥ 7.8	Fasting glucose ≥ 6.1	Fasting glucose ≥ 6.1 (modified to ≥ 5.6 in 2004)	Fasting glucose ≥ 6.1 or 2 hours post glucose challenge ≥ 7.8
Insulin resistance	Glucose uptake below lowest quartile measured under hyperinsulinaemic euglycaemic conditions	Top 25% of fasting insulin values among the non-diabetic population	Not applicable	Not applicable
Blood Pressure (mmHg)	≥ 140/90	≥ 140/90 or treated hypertension	> 130/85 or treated hypertension	> 130/85
Triglycerides (mmol/l)	≥ 1.7	> 2.0	≥ 1.7	≥ 1.7
HDL cholesterol (mmol/l)	♂ < 0.9 ♀ < 1.0	< 1.0 or on treatment	♂ < 1.03 ♀ < 1.29	♂ < 1.03 ♀ < 1.29
Other factors	Microalbuminuria (urinary albumin excretion rate > 20 µg/min or albumin: creatinine ratio > 30 mg/g)	Not applicable	Not applicable	Family history of type II DM Polycystic Ovary Syndrome Sedentary lifestyle Increasing age Ethnicity

While all groups are in agreement over the core components of obesity, hyperglycaemia, hypertension and dyslipidaemia, considerable confusion and controversy exists regarding the specific criteria and cut-off points for each individual component, and the applicability of these criteria to differing populations. The assessment of obesity has been most difficult, with older definitions failing to account for ethnic variations in body mass index (BMI) and waist circumference, though this has been addressed in the 2005 IDF consensus statement recommending an updated global definition for the metabolic syndrome (Table 1.2). By accounting for ethnic variations in the measurement, and implications, of central obesity, the IDF definition should enable physicians internationally to identify and treat at risk individuals, whilst also allowing comparison of the prevalence and health outcomes of this syndrome in differing populations [5]. This definition also requires the presence of central obesity for the diagnosis, thus recognising the strong correlation with other features of the metabolic syndrome.

Table 1.2: IDF Worldwide Definition Of The Metabolic Syndrome [5]

Clinical Parameter	Eligibility Criteria (Ethnicity specific central obesity plus 2 other factors)	
Triglycerides (mmol/l)	≥ 1.7 or treated hypertriglyceridaemia	
HDL cholesterol (mmol/l)	♂ < 1.03 ♀ < 1.29	
Blood pressure (mmHg)	Systolic ≥ 135 or diastolic ≥ 85	
Dysglycaemia (mmol/l)	Fasting glucose ≥ 5.6 or previously diagnosed type II DM	
Ethnicity specific central obesity (waist circumference - cm)	Europeans	♂ ≥ 94 cm ♀ ≥ 80 cm
	USA (Caucasians)	♂ ≥ 102 cm ♀ ≥ 88 cm
	South Asians	♂ ≥ 90 cm ♀ ≥ 80 cm
	Chinese	♂ ≥ 90 cm ♀ ≥ 80 cm
	Japanese	♂ ≥ 90 cm ♀ ≥ 80 cm
	South & Central Americans	Use South Asian values till further data available
	Sub-Saharan Africans Eastern Mediterraneans Middle Easterns	Use European values till further data available

1.3 Pathogenesis Of The Metabolic Syndrome

The complex pathogenesis of the metabolic syndrome and its key components is still to be fully elucidated. Both insulin resistance together with central or visceral adiposity are the dominant causative factors recognised to date. The global obesity epidemic is thought to be the most important driving force for the increasing prevalence of both insulin resistance and the metabolic syndrome [5]. Environmental factors which include cigarette smoking and sedentary lifestyles also contribute to the development of many of the major metabolic disturbances [4].

1.3.1 Obesity & Abnormal Body Fat Distribution

Epidemiological studies have demonstrated a clear association between increasing body weight, measured by the body mass index (BMI), and all-cause mortality [10]. Furthermore, amongst individuals with similar BMI values, those with greater abdominal adiposity (as assessed by measurement of waist circumference or waist-hip ratio), are at greater risk of developing type II diabetes mellitus (DM), hypertension and cardiovascular disease (CVD) [11, 12]. This excess in visceral, as compared to subcutaneous, accumulation of adipose tissue has been shown to be associated with adverse and potentially atherogenic metabolic disturbances in both men and women, including fasting hypertriglyceridaemia, reduction in high-density lipoprotein (HDL), and elevations in both fasting and post-prandial insulin and glucose levels [13].

The hypotheses which link central obesity with components of the metabolic syndrome have arisen from the recent understanding of the function of adipose tissue as not only an energy store, but an endocrine organ capable of secreting adipocytokines, growth factors and other bio-active substances (Table 1.3) known to impair the action of insulin. These include free fatty acids (FFA) and the inflammatory mediators tumour necrosis factor-alpha (TNF-α) and

interleukin-6 (IL-6) [14]. TNF-α has been shown to promote insulin resistance, stimulate lipolysis and thus increase FFA release, and inhibit peripheral glucose uptake while IL-6 increases hepatic glucose production & triglyceride synthesis. Excess visceral adipose tissue is also associated with a reduction in the insulin-sensitising cytokine adiponectin, and thus a loss of its anti-diabetic, anti-inflammatory, and anti-atherosclerotic properties [14]. These pathogenic processes are illustrated further in Figure 1.1.

Table 1.3: Proteins Secreted By Adipocytes [15]

Molecule	Effect
Adiponectin	Insulin sensitisation Improved fatty acid transport and utilisation
Leptin	Hypothalamic energy regulation Maturation of reproductive function
Resistin	Impairs insulin sensitivity
Tumour necrosis factor alpha	Mediator of the acute phase response Increased insulin resistance (↓ lipogenesis, ↑ lipolysis, ↓insulin-induced glucose uptake)
Interleukin 6	Increases hepatic glucose production & triglyceride synthesis
Plasminogen activator inhibitor 1	Potent inhibitor of the fibrinolytic system
Angiotensinogen	Regulator of blood pressure & electrolyte homeostasis
Transforming growth factor beta	Regulator of growth & differentiation
Insulin like growth factor 1	Stimulates cell proliferation and mediates many effects of growth hormone
Adipsin	? link between activation of the complement pathway and adipose tissue metabolism
Acylation Stimulating Protein	Activates diacylglycerol acyltransferase Inhibits lipase Stimulates Glucose transporter 4 translocation to cell surface
Tissue factor	Initiator of the coagulation cascade
PGI2 PGF2alpha	Implicated in blood clotting, ovulation & menstruation
Migration inhibitory factor	Involved in proinflammatory processes & immunoregulation
Adipocyte protein 2	Involved in intracellular trafficking and targeting of fatty acids
Agouti protein	? induces insulin resistance through increasing intracellular free calcium concentrations Increases appetite & decreases energy expenditure

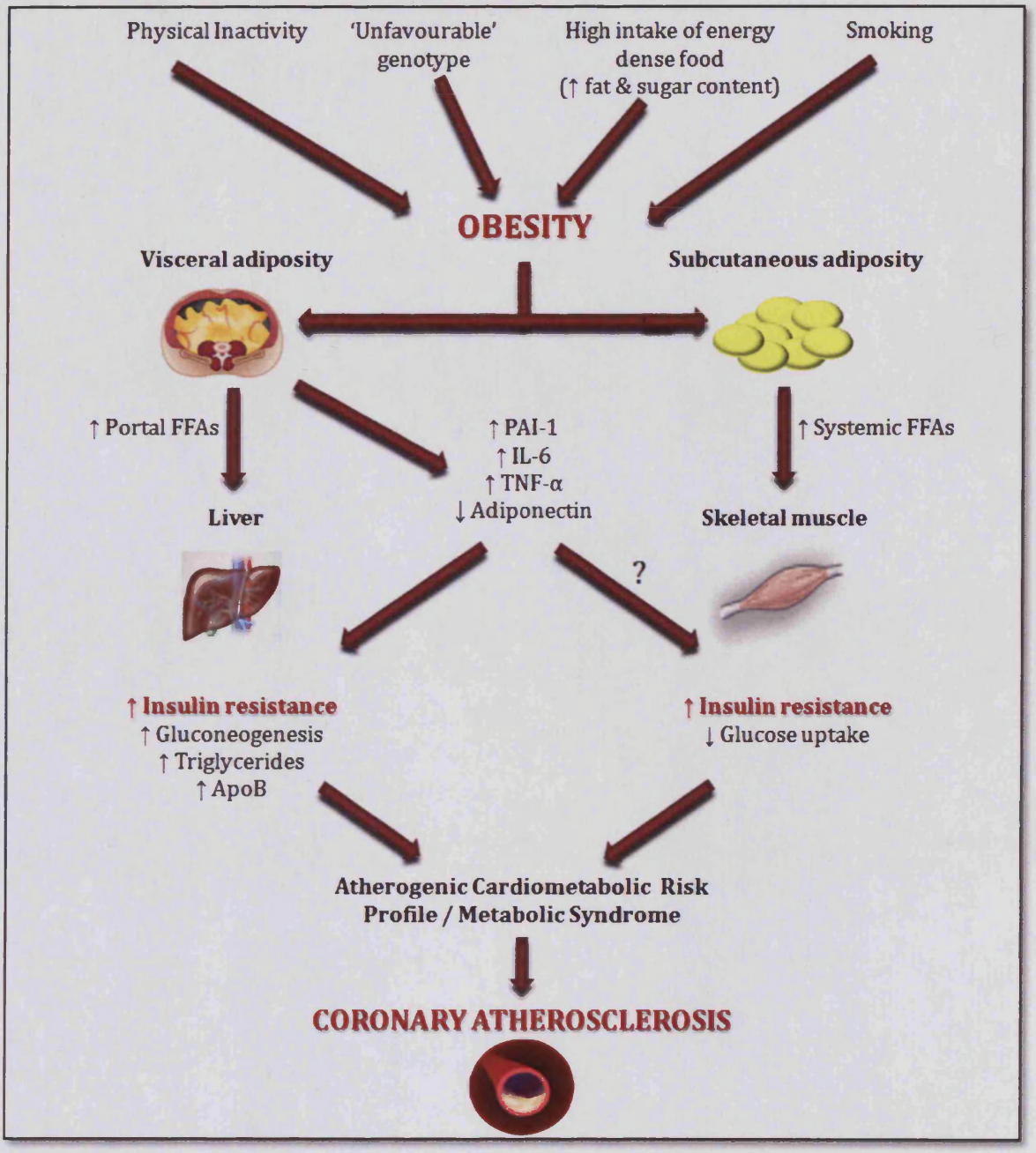
1.3.2 Insulin Resistance & Glucose Intolerance

There is clear evidence supporting a major role of insulin resistance in the development of the metabolic syndrome, though the exact underlying mechanism is not yet fully understood. It is present in the majority of individuals meeting the criteria for the metabolic syndrome with a strong association with the other dominant components, as well as the correlation with the development of type II DM and CVD [5]. The Bruneck study examined the prevalence of insulin resistance in random subjects from the general population aged between 40 and 79 years by the homeostasis model assessment (HOMA), and demonstrated a clear correlation between increasing degrees of insulin resistance and the number of co-existing metabolic abnormalities [16]. Meta-analysis of previous data has shown hyperinsulinaemia in non-diabetic men and women to be an independent risk factor for CVD, especially in non-white populations, though this does still require further study [17].

1.3.2.1 The Role Of Obesity In The Pathogenesis Of Insulin Resistance

An overabundance of FFAs, derived predominantly from lipolysis of triglyceride stores within adipose tissue, is thought to be a main contributor to the development of insulin resistance [4]. Insulin itself has important antilipolytic properties, thus further compounding the problem in the setting of insulin resistance. The excess FFAs further antagonise the actions of insulin by stimulating hepatic gluconeogenesis thus contributing to hyperglycaemia, whilst also suppressing peripheral skeletal muscle glucose transport leading to reduced muscle glycogen synthesis and glycolysis [18]. The increasing and prolonged exposure to the resultant elevated concentrations of insulin eventually leads to the development of frank type II DM, deterioration in pancreatic β-cell function and therefore a fall in insulin secretion with worsening hyperglycaemia [19]. These pathogenic processes are illustrated further in Figure 1.1.

Figure 1.1: The Role Of Obesity & Insulin Resistance In The Pathogenesis Of The Metabolic Syndrome [4, 20, 21]



1.3.2.2 Impaired Insulin Receptor Signalling In Insulin Resistance

Insulin binding induces tyrosine phosphorylation of the insulin receptor, resulting in the binding of a number of intracellular proteins such as the insulin receptor substrates (IRS-1

and IRS-2) [22]. Phosphorylated tyrosine residues on these proteins serve as docking sites for the recruitment and binding of downstream signalling proteins such as phosphoinositide 3 kinase (PI3K) and Akt, a family of genes encoding serine/threonine-specific protein kinases, eventually regulating responses including glucose transport, protein and glycogen synthesis [22].

In obese states, lipid accumulation can occur in ectopic sites outside of adipose tissue due to excess fat intake, fatty acid synthesis and reduced fatty acid disposal. Moreover lipid accumulation within myocytes and hepatocytes is strongly associated with increased insulin resistance [18]. Accumulation of excess lipid within skeletal muscle and the liver inhibits tyrosine phosphorylation of IRS-1, which in turn follows serine phophorylation therefore inhibiting PI3K binding and activation and thus reducing insulin-stimulated glucose transport [23]. Candidate proteins implicated in this serine phosphorylation include the protein kinase C family which may be activated by accumulation of lipid, as well as inflammatory intermediaries such as TNFα and IL-6 which may be activated within adipose tissue in obese states [23].

1.3.3 Dyslipidaemia

The dyslipidaemic profile seen in the metabolic syndrome tends to be characterised by the pro-atherogenic pattern of raised triglycerides (TG) and low HDL, as well as other lipoprotein changes including elevated small dense low-density lipoprotein (LDL) and apolipoprotein B (Apo B) [24]. As discussed previously, obesity gives rise to excess FFAs which in turn exacerbate insulin resistance. As well as stimulating hepatic gluconeogenesis, FFAs also increase hepatic TG synthesis and the production of Apo B-containing, triglyceride-rich very low-density lipoprotein (VLDL) [25]. TGs are also primarily responsible for an associated remodelling of HDL by a process of reverse cholesterol transport and cholesterol esterification leading to a reduction in the cholesterol content of HDL [26].

1.3.4 Elevated Blood Pressure

Hypertension is closely associated with obesity and insulin resistance and is associated with a two-fold increase in CVD risk for every 20/10 mmHg increment in blood pressure commencing at 115/75 mmHg [27, 28]. Several mechanisms have been postulated linking insulin resistance with the development of elevated blood pressure. For instance, there is evidence indicating increased renal sodium reabsorption in the metabolic syndrome, especially in Caucasian populations [29], as well a loss of vasodilatory effects of insulin observed in a normal physiological setting [30]. Furthermore insulin also stimulates sympathetic nervous activity [31] while FFAs themselves mediate a relative vasoconstrictive effect [32].

1.3.5 Proinflammatory Cytokines & Adiponectin

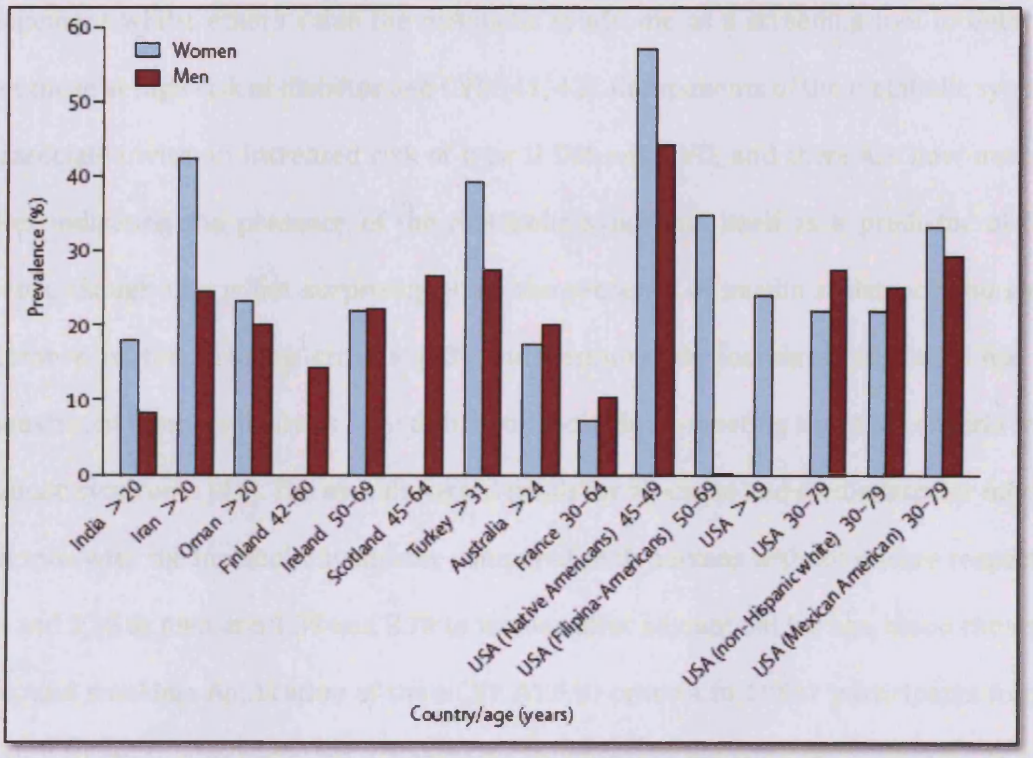
Numerous other parameters have also been demonstrated to feature in individuals with the metabolic syndrome including a pro-inflammatory state, as indicated by elevated plasma TNF-α, IL-6 and C-reactive protein (CRP), markers of a pro-thrombotic state, the most common being plasminogen activator inhibitor-1 (PAI-1), and adverse adipocytokine profiles, especially low circulating adiponectin [5]. The latter is a cytokine produced almost exclusively by adipocytes and enhances insulin sensitivity and inhibits numerous steps in the inflammatory process [33]. In the liver it suppresses endogenous glucose production while improving glucose transport and fatty acid oxidation in skeletal muscle [34, 35]. Furthermore, TNFα is also over expressed in adipose tissue and induces insulin resistance through acute and chronic effects on insulin-sensitive tissues [36]. As discussed previously, the increase in proinflammatory cytokines probably reflects over-production by the expanded adipose tissue mass seen in these individuals. However, these features have not been incorporated into current definitions of the metabolic syndrome to date.

1.4 Prevalence Of The Metabolic Syndrome

Due to differences in the existing definitions of the metabolic syndrome, there are varying reports of its prevalence depending on the definition used and the ethnic group studied. Most studies suggest a prevalence of 10-30% in the general adult population in developed countries and urban areas of developing countries (Figure 1.2) [37].

Figure 1.2: Prevalence Of The Metabolic Syndrome From NCEP ATP III Definition

Adapted from Cameron et al [4, 37]



The prevalence of the metabolic syndrome, as defined by the NCEP ATP III criteria, was evaluated in 8814 adults in the United States in the third National Health and Nutrition Examination Survey (NHANES III). The overall prevalence was shown to be 22 percent, with an age-dependent increase and higher prevalence rates seen in Mexican and African subgroups [38]. Data from NHANES 1999-2000 confirms that the prevalence has continued to increase, particularly in women [39]. The prevalence of the metabolic syndrome by NCEP ATP III criteria was also assessed in a middle-aged cohort of 3323 Framingham Heart Study

participants who did not have diabetes or cardiovascular disease at an initial examination in the early 1990s. At baseline, the prevalence of the metabolic syndrome was 26.8 percent in men and 16.6 percent in women. Following an eight year period there was an age-adjusted 56 percent increase in prevalence among men and a 47 percent increase among women [40].

1.5 Consequences Of The Metabolic Syndrome

There is considerable controversy and dispute about the concept of the metabolic syndrome – some believe that this label adds little practical clinical value to individual patient management whilst others value the metabolic syndrome as a screening tool to detect and target those at high risk of diabetes and CVD [41, 42]. Components of the metabolic syndrome are associated with an increased risk of type II DM and CVD, and there are now numerous studies indicating the presence of the metabolic syndrome itself as a predictor of future diabetes, though this is not surprising given the presence of insulin resistance and glucose intolerance in the defining criteria [43]. Furthermore, an increased mortality has been demonstrated by meta-analysis in non-diabetic individuals meeting the WHO criteria for the metabolic syndrome [44]. The overall hazard ratios for all-cause and cardiovascular mortality in persons with the metabolic syndrome compared with persons without it were respectively 1.44 and 2.26 in men and 1.38 and 2.78 in women after adjustment for age, blood cholesterol levels, and smoking. Application of the NCEP ATP III criteria to 10537 participants from the NHANES III study also indicated a significant relationship between the syndrome and both myocardial infarction and stroke in men and women [45].

Apart from type II DM and CVD, the metabolic syndrome is also associated with several other disorders all of which demonstrate a clear link with obesity, including non-alcoholic steatohepatitis [46], chronic kidney disease [47], polycystic ovary syndrome [48], obstructive sleep apnoea [49] and cognitive decline, though this is by no means an exhaustive list.

Other studies have disputed whether application of the metabolic syndrome provides any additional information over and above the individual well-established CVD risk factors which may in part reflect the inadequacies and inconsistencies in the definitions used [50]. It is not clear whether the metabolic syndrome simply reflects the increasingly sedentary lifestyle and dietary habits of today's society or if accumulating excess central fat deposits was associated with survival benefits in the past. There is however clear evidence that the metabolic syndrome, considered either as a separate disease entity or a constellation of risk factors, predisposes to cardiovascular risk [51-53]. These individuals represent an at-risk population and require further attention.

1.6 Management Of The Metabolic Syndrome

The metabolic syndrome represents a common cluster of metabolic disturbances associated with increased risk of type II DM and CVD, predominantly arising from the increasing prevalence of obesity. In 2003 a report from the American Heart Association and the National Institutes of Health recommended two main therapeutic goals for the management of the metabolic syndrome [54]. Firstly this involves lifestyle modification to treat the underlying causes of obesity and physical inactivity by intensifying weight management and increasing physical activity. This should be followed by treatment of cardiovascular risk factors if they persist despite lifestyle modification, and includes treatment with insulin sensitising agents, anti-hypertensive and lipid-lowering drugs. However, at present there is no direct evidence supporting the attempts to prevent type II DM and CVD by treating the metabolic syndrome and no trials comparing the lifestyle modifying vs. pharmacological approaches in improving outcome.

Weight loss, regardless of how this is accomplished, improves metabolic parameters, reduces cardiovascular risk and improves disease outcome (Table 1.4). However, sustained weight loss is challenging to achieve, and most individuals find lifestyle modifications difficult to

adhere to long-term. The few existing drugs that have been used for the treatment of obesity do help achieve modest weight loss in the short-term but many patients return to their pre-treatment body weights within a few years of treatment discontinuation. Although the core approach to the treatment of obesity, and thus the metabolic syndrome, encompasses weight reduction and increased physical activity, there may also be a role for pharmacotherapy in reducing the burden of diabetes and cardiovascular disease; hence the adipocyte represents a major molecular target for novel therapeutic agents.

Table 1.4: Benefits Of 10kg Weight Loss [55]

Clinical Parameter	Effect
Mortality	20-25% ↓ total mortality 30-40% ↓ diabetes-related deaths 40-50% ↓ obesity-related cancer deaths
Blood pressure	10 mmHg ↓ systolic pressure 20 mmHg ↓ diastolic pressure
Lipid Profile	10% ↓ total cholesterol 15% ↓ LDL cholesterol 30% ↓ triglycerides 8% ↑ HDL cholesterol
Glycaemia	>50% ↓ risk of developing diabetes 30-50% ↓ fasting blood glucose 15% ↓ HbA1c
Angina	91% ↓ symptoms 33% ↑ exercise tolerance

1.7 Models Of The Metabolic Syndrome

We have chosen to study the effect of pharmacomodulation in two models representing features of the metabolic syndrome: firstly, the endocannabinoid system, as an *in vitro* model of obesity, and secondly, polycystic ovary syndrome (PCOS), as an *in vivo* model for the syndrome itself. We hypothesise that novel (CB_1 antagonist) and existing (metformin)

pharmacolotherapy targets may generate new benefits through changes in obesity, and in particular, central adiposity.

1.8 The Endocannabinoid System (ECS)

1.8.1 Role Of The ECS In Contributing To Obesity

The discovery of the ECS can be traced back almost 4000 years when the ancient Indians first described the pleasure-inducing medicinal properties of the plant *Cannabis sativa* [56], though the identification of Δ^9-tetrahydrocannabinol (Δ^9-THC) as the principal active compound of marijuana was not made until the late 20th century leading to further characterisation of the cannabinoid receptors and their endogenous ligands [57]. More recent research into the physiology of the ECS has aroused considerable interest, with the recognition of significant effects in the regulation of food intake as well as energy metabolism via central and peripheral mechanisms in both animals and humans [58, 59]. The ECS is therefore emerging as a novel therapeutic target for management of components of the metabolic syndrome.

The elements of the ECS comprise the cannabinoid receptors, the endogenous lipid ligands, the proteins required for their biosynthesis and inactivation, and the affected intracellular signalling pathways. In addition to its role in energy metabolism, the ECS also contributes to numerous other physiological functions including behaviour regulation, neuroprotection, nociception, and immunomodulation [60].

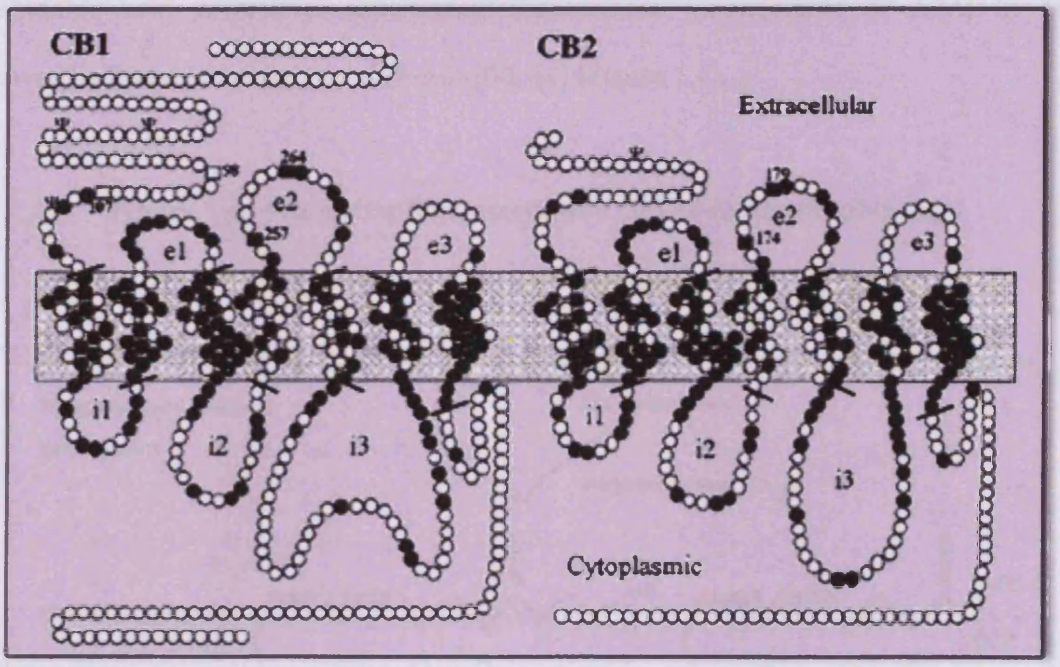
1.8.2 Cannabinoid Receptors

Two cannabinoid receptors, namely cannabinoid receptor type 1 (CB_1) and cannabinoid receptor type 2 (CB_2), have been identified to date, and form part of the seven transmembrane G protein-coupled family of receptors [60] (Figure 1.3).

Figure 1.3 : Schematic Representation of Human CB1 and CB2 Receptors

Adapted from Shire et al [61]

(where ● represents amino acids common to both CB1 & CB2)



CB_1 receptors are located extensively throughout the central nervous system particularly specific mesolimbic regions, the pituitary and the hypothalamus, especially in regions known to be involved in the control of calorie intake and satiety, as well as in other organs involved in energy homeostasis including adipose tissue, liver, gastrointestinal tract, pancreas and skeletal muscle. They are also found in the myocardium and vascular endothelium, while CB_2 receptors are predominantly expressed on immune and blood cells [62]. Therefore CB_1 receptor modulation appears to affect metabolic function whereas CB_2 activation has been

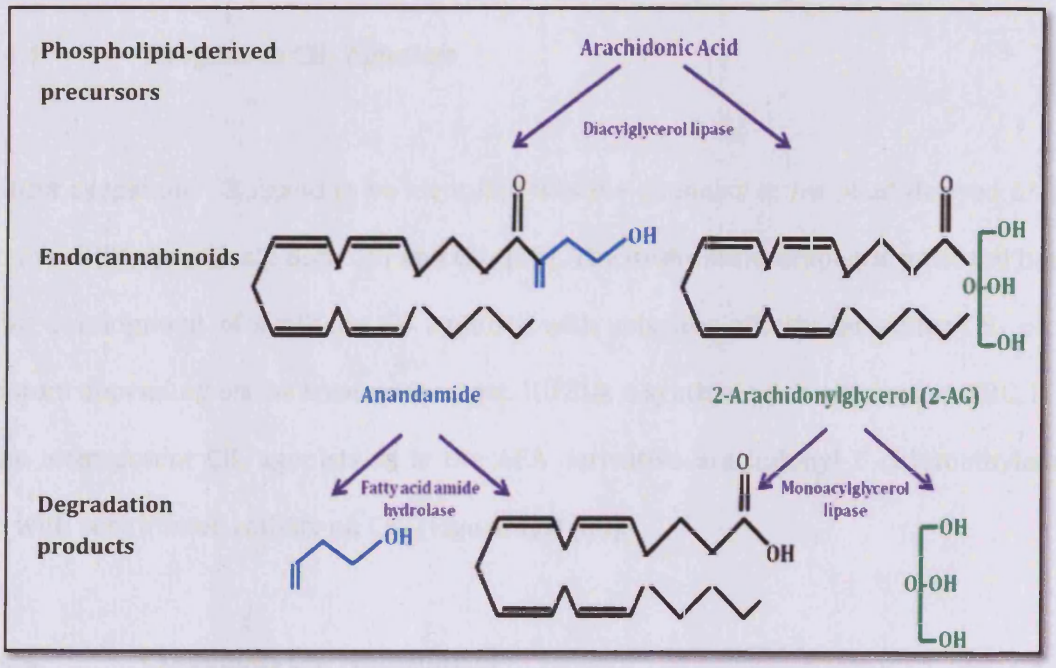
implicated in pain and immune modulation. Recent studies from CB_1 and CB_2 receptor knockout mice have also suggested the existence of non-CB_1, non-CB_2 endocannabinoid receptors but these remain the subject of ongoing study [63]. Given its role in energy homeostasis, this thesis will focus on the effects of CB_1 in the ECS.

1.8.3 Endogenous Endocannabinoids

The cannabinoid receptors are activated by a group of naturally occurring, cannabis-like lipids known as the endocannabinoids, the earliest of which to be identified were the arachidonic acid derivatives arachidonylethanolamide (anandamide or AEA) in 1992, followed by 2-arachidonylglycerol (2-AG) [60, 64] (Figure 1.4).

Figure 1.4 : Schematic Representation Of The Endocannabinoids

Adapted from Di Marzo et al [65]



More recently other bioactive mediators have also been described including noladin ether, virodhamine and oleamide, though their physiological functions have not yet been fully

established [60]. AEA synthesis occurs via the phosphodiesterase-mediated cleavage of the membrane phospholipid, N-arachidonoyl-phophatidylethanolamine [66]. Being lipophilic compounds the endocannabinoids cannot be stored in vesicles like other neurotransmitters and are therefore synthesised upon demand from membrane phospholipids by cells at the site of action [64]. Following release and local receptor activation, the products are then rapidly degraded by hydrolysis via fatty acid amide hydrolase (FAAH), thus acting primarily near their site of synthesis by binding to cannabinoid receptors on the neighbouring cell surfaces [64]. Both AEA and 2-AG are capable of binding to CB_1 and CB_2 but with differing affinities and activation efficiencies [60].

1.8.4 Exogenous CB_1 Receptor Ligands

The exogenous CB_1 receptor ligands can be further classified according to their ability to either activate (agonists) or inactivate (antagonists) CB_1 receptors.

1.8.4.1 Exogenous CB_1 Agonists

The first exogenous CB ligand to be identified was the *Cannabis sativa* plant-derived Δ^9-THC, with the ability to activate both CB_1 and CB_2 [57]. The desire for therapeutic potential has led to the development of synthetic CB agonists with selective affinity for either CB_1 or CB_2 receptors depending on the treatment target. HU210, a synthetic derivative of Δ^9-THC, is one of the most potent CB_1 agonists as is the AEA derivative arachidonyl-2'-chloroethylamide, both with very limited activity on CB_2 (Figure 1.5) [60].

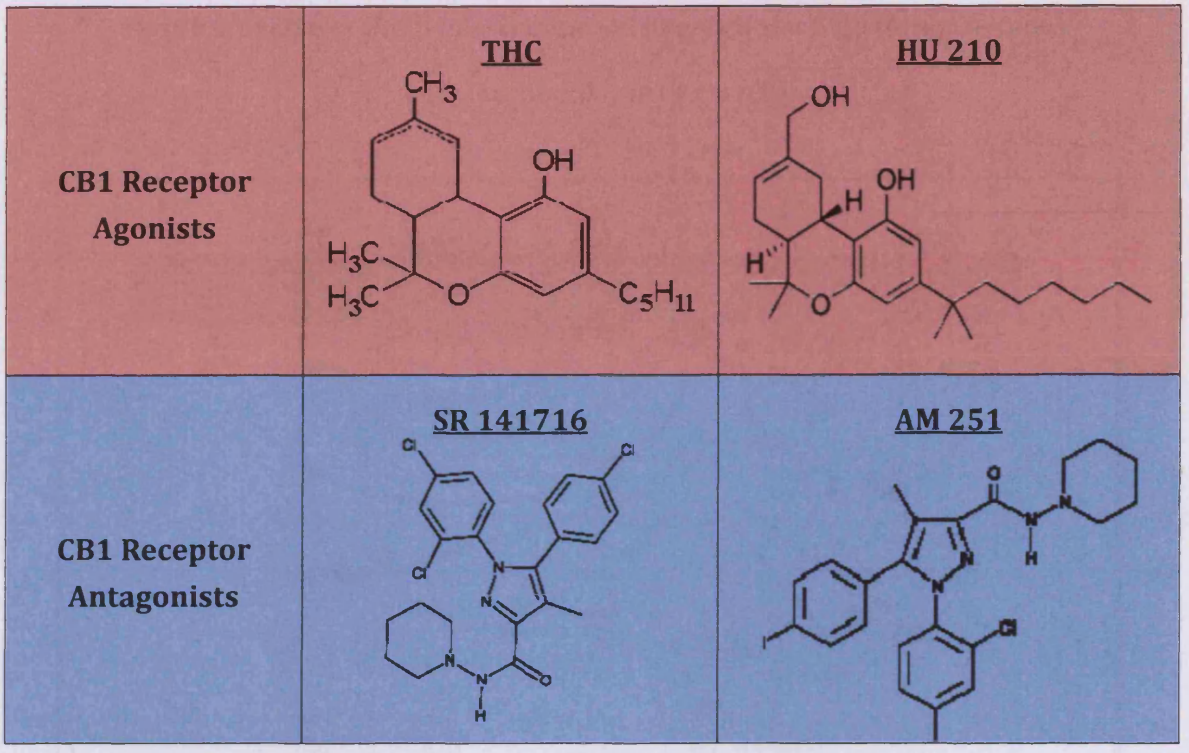
1.8.4.2 Exogenous CB_1 Antagonists

These compounds represent novel therapeutic agents with the potential for treating components of the metabolic syndrome. These ligands act either as competitive antagonists

of CB_1 receptor activation by endocannabinoids, or as inverse agonists that modulate CB_1 receptor activity by shifting it from an active "on" to an inactive "off" state [60]. The first and most extensively used CB_1 receptor antagonist is SR141716, which has also been developed into the pharmacological agent rimonabant for human use, but other compounds that have been characterised include AM251, AM281, and SR14778 (Figure 1.5) [60].

Figure 1.5: Exogenous CB1 Receptor Ligands

Adapted from Di Marzo et al [67]



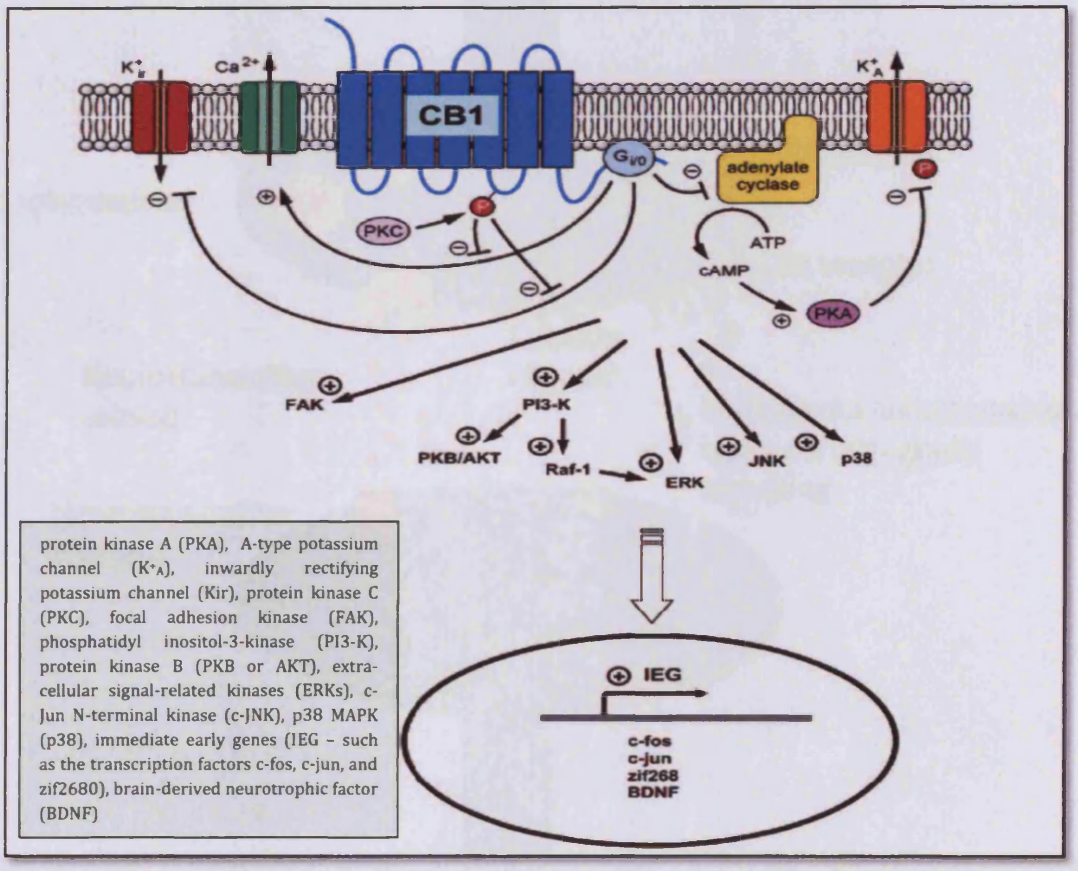
1.8.5 Endocannabinoid-Mediated Intra-Cellular Signalling

Several mechanisms involved in endocannabinoid-mediated signalling have been described over the recent years and some of these signalling cascades have been schematically represented in Figure 1.6. CB_1 receptors are coupled via G-proteins, negatively to adenylate cyclase and positively to mitogen-activated protein kinase, as well as to potassium and

calcium ion channels [68]. G-protein stimulation by CB_1 activation inhibits ATP to cAMP conversion mediated by adenylate cyclase [60]. cAMP is in turn thought to bind to regulatory subunits of protein kinase A (PKA) resulting in the activation of A-type potassium (K^+_A) channel while G-protein mediated effects on protein kinase C (PKC) can result in activation of inwardly-rectifying potassium (K_{ir}) channels and the inhibition of calcium channels [60]. CB_1 activation also stimulates numerous intracellular kinases eventually resulting in gene expression and transcription. It is important to note that these events have been reported in different cell systems and thus may not all occur in the same cell type [60].

Figure 1.6: Effects Of CB1 Activation On Intracellular Signalling Cascades

Adapted from Pagotto et al [60]



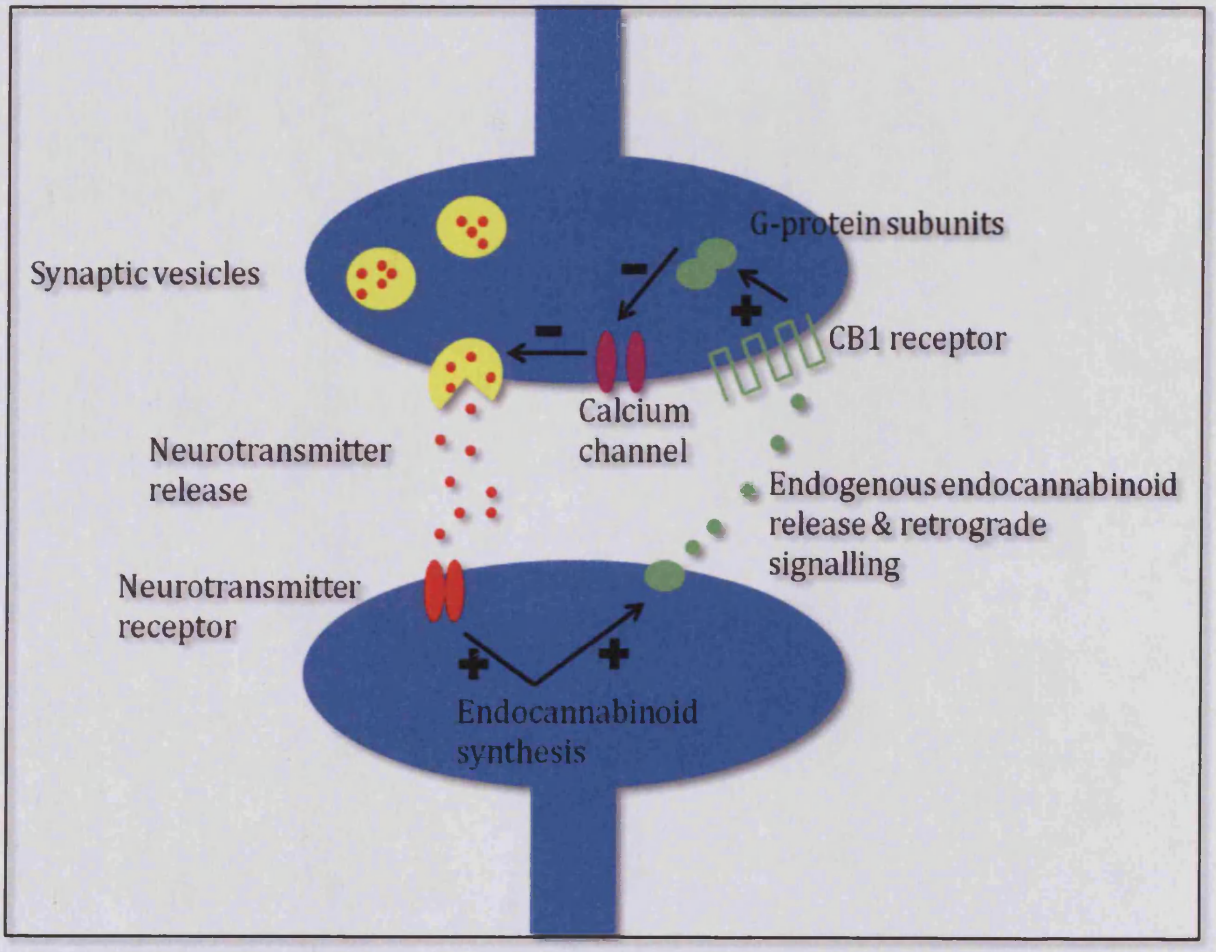
protein kinase A (PKA), A-type potassium channel (K^+_A), inwardly rectifying potassium channel (Kir), protein kinase C (PKC), focal adhesion kinase (FAK), phosphatidyl inositol-3-kinase (PI3-K), protein kinase B (PKB or AKT), extra-cellular signal-related kinases (ERKs), c-Jun N-terminal kinase (c-JNK), p38 MAPK (p38), immediate early genes (IEG – such as the transcription factors c-fos, c-jun, and zif2680), brain-derived neurotrophic factor (BDNF)

1.8.6 Endocannabinoid-Mediated Neuronal Signalling

Neurotransmitters released from vesicles within pre-synaptic neurons activate post-synaptic neuronal synthesis and release of endocannabinoids [69]. The endogenous CB_1 ligands diffuse back and bind to CB_1 receptors located on the pre-synaptic nerve terminals resulting in G-protein activation, calcium channel inhibition and eventual inhibition of neurotransmitter release (Figure 1.7) [69].

Figure 1.7: Endocannabinoid-Mediated Neurotransmitter Signalling

Adapted from Piomelli et al [69]



1.8.7 Role Of The ECS In The Modulation Of Energy Regulation

1.8.7.1 Animal Studies

The vital function of the ECS in normal physiology is proposed by its evolutionary preservation and presence even in lower-level organisms [70]. Its role in the regulation of energy balance has been demonstrated in animal studies where chronic activation of the ECS increases food intake and promotes weight gain [71]. Conversely, CB_1 blockade in the diet-induced obese mouse reduces fat mass while CB_1 receptor knock-out mice maintain a lean phenotype and are resistant to diet-induced obesity [72]. These feeding experiments suggest that endocannabinoids affect the homeostatic control of body weight, not only through central orexigenic mechanisms at known sites in the hypothalamus and limbic forebrain but also through factors independent of food intake by peripheral effects in the regulation of energy metabolism [58]. These peripheral actions of the ECS include mediation of lipogenesis and modulation of adipocytokine profiles in adipose tissue and the liver, and gluconeogenesis in skeletal muscle [60].

1.8.7.2 Human Studies

A potential link between the ECS and obesity has also been suggested by clinical data in human studies. Higher concentrations of circulating endocannabinoids and CB_1 receptor expression have been noted in obese as compared to lean women [73]. Similarly, both AEA and 2-AG levels are significantly enhanced in individuals with binge eating disorders [74]. Genetic studies have also shown an association between a polymorphism in the gene encoding the main endocannabinoid-degrading enzyme, fatty acid amide hydrolase (FAAH), and elevated BMI in white and black patient groups, though not in subjects of Asian origin [75].

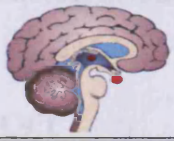
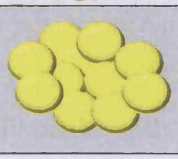
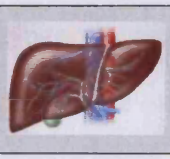
Patients with obesity, or hyperglycaemia associated with type II diabetes, also display greater concentrations of endocannabinoids in visceral fat, or serum, respectively, than corresponding normal-weight, euglycaemic controls [76]. The metabolic effects of the ECS are also borne out in phase III clinical trials of the first selective CB_1 receptor antagonist SR141716, or rimonabant, where treatment promoted significant weight loss and reduction in waist circumference [77]. These have been accompanied by parallel improvements in the metabolic profile, namely reductions in fasting insulin and triglyceride levels, a rise in HDL cholesterol and adiponectin [77-81].

1.8.8 Therapeutic Potential Of CB_1 Receptor Ligands In The Management Of Obesity

Stimulation of the ECS favours several adverse metabolic processes resulting in weight gain, lipogenesis and impaired glucose metabolism, thus making it a novel therapeutic target for managing features of the metabolic syndrome, principally obesity and its associated cardiovascular sequelae. Furthermore, the observations outlined in the above studies raise the possibility that by reducing central adiposity, CB_1 receptors may exert differential effects in visceral as opposed to subcutaneous fat depots, making both important areas for study. The potential sites of action and proposed therapeutic clinical benefits of CB_1 receptor antagonist treatment have been summarised in Table 1.5.

Table 1.5 : Potential Sites And Therapeutic Effects Of CB_1 Antagonists

Adapted from Pagotto et al [82]

Site of Action		Potential Mechanism Of Action	Clinical Implications
	Hypothalamus Nucleus Accumbens	Anorexigenic effect ↓ Food intake	Body weight Abdominal obesity
	Adipose tissue	↑ Adiponectin ↓ Lipogenesis	Dyslipidaemia Insulin resistance
	Muscle	↑ Glucose uptake	Insulin resistance
	Liver	↓Lipogenesis	Dyslipidaemia Insulin resistance
	Gastrointestinal tract	↑ Satiety signals	Body weight Abdominal obesity

1.9 Polycystic Ovary Syndrome (PCOS)

PCOS is the commonest endocrinopathy in women of reproductive age, affecting up to 10% of the premenopausal population [83]. It has become increasingly apparent that in addition to its well-established effects on female reproductive health, PCOS also comprises multiple metabolic abnormalities associated with long-term health risks, including glucose intolerance and insulin resistance, type II diabetes, hypertension, dyslipidaemia, visceral adiposity and endothelial dysfunction [84-91]. Although there is a lack of sufficient epidemiological data in regard to absolute cardiovascular event rates, the increased prevalence of the above cardiovascular risk factors has been clearly documented. Developing therapeutic strategies to

minimise this vascular burden is therefore important. The recognition of insulin resistance as a factor in the pathogenesis of PCOS had resulted in the widespread and accepted use of insulin-sensitising agents in the treatment of this condition. Despite evidence for increased presence of surrogate markers of cardiovascular disease, it is still unclear whether this is associated with accelerated atherosclerosis and endpoint data are lacking, an issue we aim to address in this study.

1.9.1 Diagnosis of PCOS

Patients with PCOS present with a multitude of complaints ranging from hirsutism, acne, menstrual irregularities and infertility, and are therefore seen by a variety of healthcare professionals including primary care physicians, endocrinologists, dermatologists, and gynaecologists, making a clear consensus for diagnosis vital. The first international conference on PCOS was held at the National Institutes of Health in 1990, when three key features were identified by majority opinion as necessary for the diagnosis of PCOS [92]:

1. Clinical and/or biochemical hyperandrogenism
2. Chronic anovulation
3. Exclusion of other endocrine disorders including congenital adrenal hyperplasia, hyperprolactinaemia, Cushing's syndrome, thyroid dysfunction, and androgen-secreting tumours.

The most recent consensus statement from the European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM)-sponsored PCOS workshop group in Rotterdam in 2003 revised the criteria for diagnosis to include two from the following three features in addition to the exclusion of other endocrine diseases [93]:

1. Clinical and/or biochemical hyperandrogenism

2. Oligomenorrhoea or chronic anovulation
3. Radiological evidence of polycystic ovaries

Despite the increasing awareness of insulin resistance and other metabolic abnormalities associated with PCOS, no aspects of these features are included in the current definition.

1.9.2 Pathogenesis Of PCOS

The exact aetiology of PCOS remains unknown though multiple genetic and extrinsic factors contribute towards the phenotypic expression. PCOS was originally thought to arise as a result of abnormal pituitary function and androgen excess; however there is now increasing evidence for insulin resistance as an additional pathogenetic factor.

1.9.2.1 Abnormal Pituitary Function

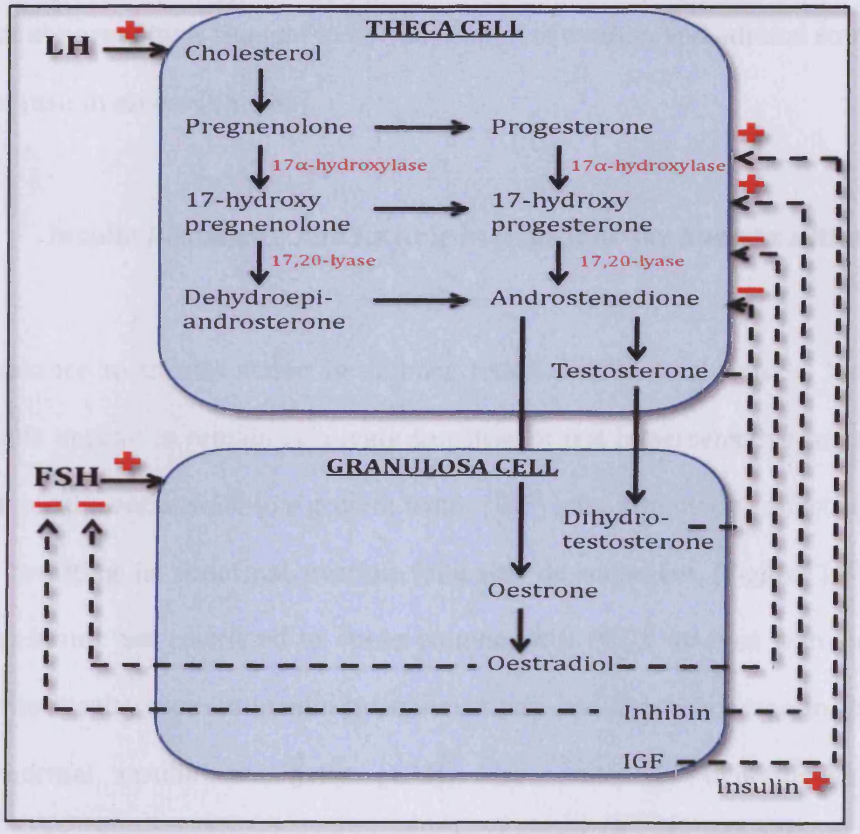
One of the first laboratory abnormalities identified in PCOS is excessive pituitary secretion of luteinising hormone (LH) relative to follicle stimulating hormone (FSH), associated with increased pulses of hypothalamic gonadotrophin releasing hormone (GnRH); this implicated in the pathogenesis of PCOS by increasing ovarian theca cell androgen synthesis [94]. However, abnormal pituitary function alone does not account for all cases as not all women with PCOS exhibit elevated LH concentrations [95].

1.9.2.2 Abnormal Steroidogenesis

Elevated androgen levels in PCOS are thought to result from the dysregulation of steroidogenesis arising from either functional ovarian hyperandrogenism (FOH) or functional adrenal hyperandrogenism (FAH). FOH is found in about 70% of subjects with PCOS characterised by excessive secretion of 17-hydroxyprogesterone (17-OHP) in response to

GnRH agonist stimulation and failure of dexamethasone to suppress free plasma testosterone [95]. FAH is found in approximately 50% of patients with PCOS and can be demonstrated by increased secretion of dehydroepiandrosterone (DHEA) in response to adrenocorticotropic hormone (ACTH) [95]. The most likely cause of the excessive androgen secretion in both glands may be dysregulation of the 17-hydroxylase and 17,20-lyase activities of P-450c17, the rate-limiting steps in androgen biosynthesis (Figure 1.8) [95]. The cause of this abnormal regulation remains unknown, though hyperinsulinaemia may play a role in many cases.

Figure 1.8: Ovarian Steroid Biosynthesis Pathways [95]
(Adapted from Rosenfield et al)



1.9.2.3 Insulin Resistance

A link between abnormal insulin action and reproductive abnormalities was first described in 1921 by Achard and Thiers in their case of "diabète des femmes à barbe" (diabetes in a

bearded woman) [96], and later described as an association between amennorhoea, cystic ovaries and masculinisation by Stein and Leventhal in the 1930s leading to the term Stein-Leventhal syndrome [97]. This association with hyperandrogenism and PCOS was eventually confirmed in the early 1980s [98, 99]. There is evidence for insulin resistance prevalent from an early age in PCOS and at a level disproportionate to that expected from BMI [100]. Up to 10% of women with PCOS will have type II diabetes by the age of 40, and approximately one-third will have an abnormal glucose tolerance test – both rates significantly higher than in matched controls [84]. Also, PCOS is often accompanied by acanthosis nigricans, a dermatological marker of insulin resistance [101]. Although excess androgen levels can cause modest reductions in glucose tolerance as seen in anabolic steroid users, in PCOS the predominant abnormality is thought to be stimulation of ovarian and adrenal steroidogenesis as a result of insulin excess [95, 102].

1.9.3 Insulin Resistance And Its Role In Reproductive Abnormalities In PCOS

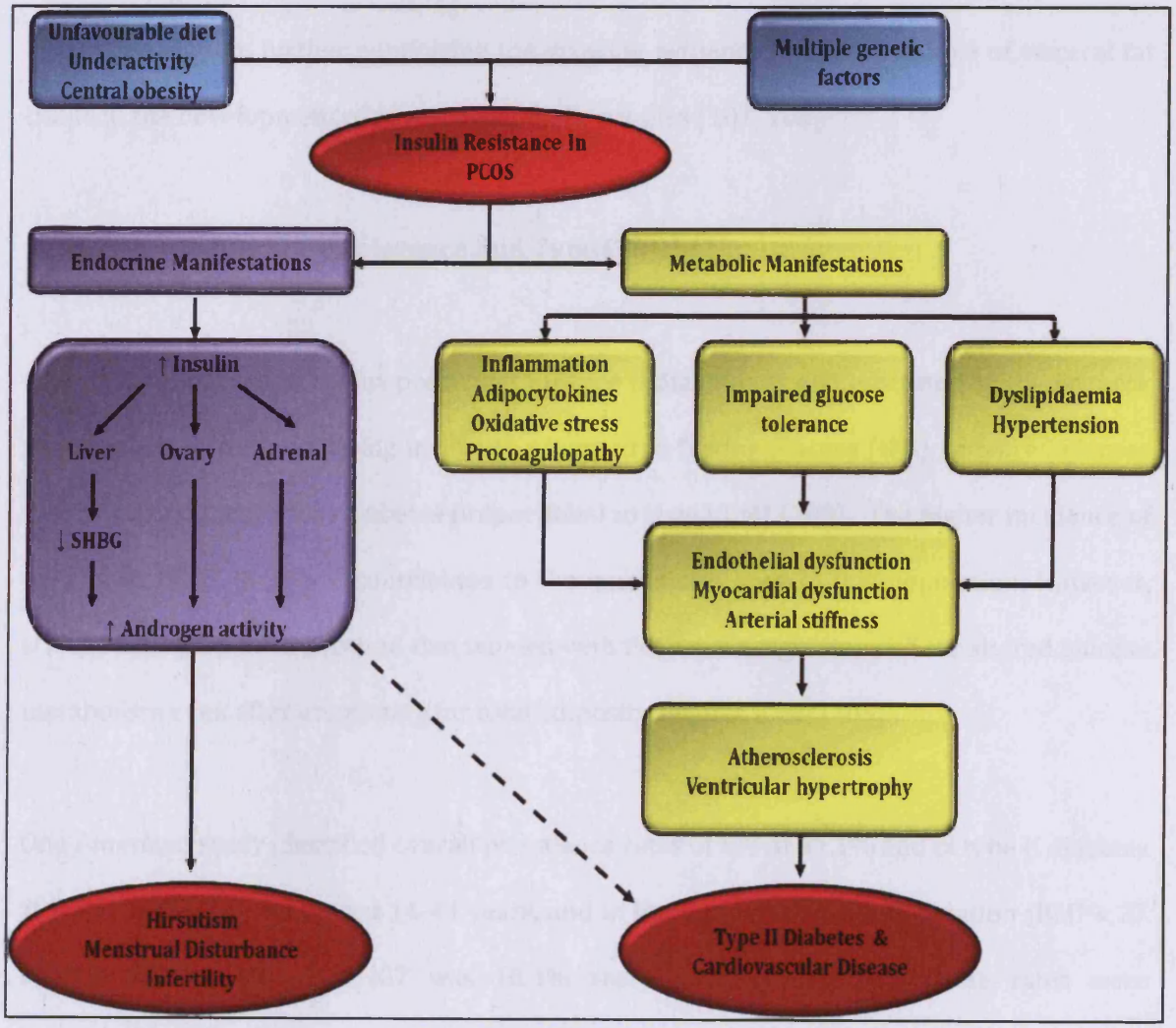
Despite resistance to insulin action in adipose tissue and skeletal muscle, the ovary and adrenal glands appear to remain relatively sensitive, if not hypersensitive to the effects of insulin. Both insulin and insulin-like growth factor (IGF) have stimulating effects on androgen production resulting in abnormal ovarian follicular development (Figure 1.8) [95]. This phenomenon is not just restricted to obese women with PCOS, as even lean subjects have been shown to display ovarian insulin hypersensitivity and hypersecretion in the setting of otherwise normal insulin sensitivity [103]. Also, states of hyperinsulinaemia and hyperandrogenism are associated with reduced circulating levels of sex-hormone binding globulin (SHBG), resulting in further elevations in free androgen concentrations [104]. These processes contribute to the clinical manifestations of increased androgen activity in the form of hirsutism, acne, alopecia, as well as ovarian dysfunction and menstrual irregularity [105].

1.9.4 Insulin Resistance And Its Role In Metabolic Abnormalities In PCOS

Insulin resistance in PCOS is not only linked with the reproductive abnormalities discussed above, but also with a wide spectrum of metabolic abnormalities that are known to predate type II diabetes and cardiovascular disease (Figure 1.9).

Figure 1.9: Consequences Of Insulin Resistance In PCOS

Adapted from Sattar et al [105]



1.9.4.1 Obesity And Central Body Fat Redistribution

Obesity is an extremely common clinical feature in PCOS with 50-60% women having a BMI > 25 kg/m^2 [105]. Numerous studies have also illustrated body fat redistribution with a greater tendency towards accumulation of central adiposity. This is evidenced by greater waist circumference measurements and waist:hip ratios in PCOS independent of BMI [106], and two-fold higher visceral and pre-peritoneal fat thickness in women with PCOS compared to BMI-matched controls [107]. This increased central obesity has also been shown to correlate with higher insulin resistance rates as well as elevated fasting insulin and triglyceride levels in this population, further supporting the growing evidence for a pivotal role of visceral fat tissue in the development of adverse metabolic profiles [107, 108].

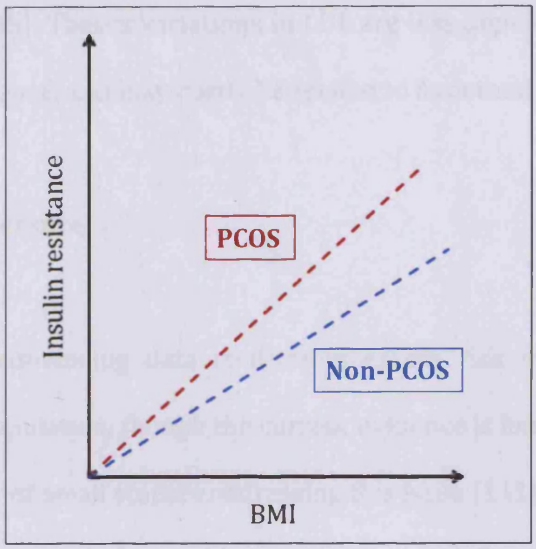
1.9.4.2 Glucose Intolerance And Type II Diabetes

Obesity is one of the strongest predictors for type II diabetes as demonstrated by the Bruneck study which identified a rising incidence of impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and frank diabetes proportional to rising BMI [109]. The higher incidence of obesity in PCOS therefore contributes to the excess risk seen in this population. However, studies have also demonstrated that women with PCOS have a greater risk for altered glucose metabolism even after accounting for total adiposity (Figure 1.10) [105].

One American study identified overall prevalence rates of IGT at 31.1% and of type II diabetes at 7.5% in PCOS women aged 14-44 years, and in the non-obese PCOS population (BMI < 27 kg/m^2) the prevalence of IGT was 10.3% and diabetes 1.5% [85]. These rates were significantly higher than in controls matched for age, weight and ethnicity even at this young age. Overall, current clinical evidence suggests an overall increased risk of IGT/type II diabetes of two to four-fold in women with PCOS compared to age and weight matched controls [105].

Figure 1.10: Increased Insulin Resistance In PCOS

Adapted from Sattar et al [105]



1.9.4.3 Lipids And Lipoprotein Profiles

Dyslipidaemia is the most common metabolic abnormality observed in women with PCOS affecting up to 70% of this population [110]. The atherogenic lipid profile associated with PCOS mirrors that seen in type II diabetes and is generally characterised by elevated serum triglycerides and VLDL, a reduction in HDL concentration and a modest rise in LDL [89, 111]. These findings are present in both obese and lean PCOS subjects and remain significant when compared to matched controls. These studies have also demonstrated an association of the adverse lipid profile with both elevated insulin levels in non-diabetic women as well as an unsurprising correlation with the presence of diabetes.

Insulin is a major positive regulator of lipoprotein lipase, suppresses lipolysis and increases free fatty acid mobilisation from adipose tissue stores, therefore the elevations in triglycerides and reduction in HDL may be more closely related to insulin resistance [112]. 24-40% of women with PCOS also display qualitative changes in LDL which may contribute to an increase in cardiovascular risk in PCOS [113, 114]. The LDL lipoprotein phenotype

reported is an increase in LDL density with a common finding being type III or type IV LDL subclasses (LDL III, density range = 1·033–1·038 g/ml; LDL IV and LDL V in the density range = 1·038–1·050 g/ml) [115]. Theses variations in LDL are less dependent on changes in body weight and insulin resistance, and may partly be related to hyperandrogenism [112, 116].

1.9.4.4 Blood Pressure

There is currently no convincing data confirming excess risk of hypertension in PCOS compared to a control population, though the current evidence is limited by the availability of only a restricted number of small studies addressing this issue [111]. Zimmerman et al found no differences in 24-hour ambulatory blood pressure or left ventricular mass between subjects with PCOS and normal controls, despite significant differences in insulin resistance [117]. However, Fridstrom and colleagues further evaluated the effect of pregnancy on blood pressure in PCOS by means of a case-control trial and demonstrated an increased risk of hypertension in the third trimester and during labour [118]. Whether PCOS therefore represents a high risk state for hypertension still requires further clarification. A large long-term follow-up study by Wild and colleagues, suggested an increased prevalence of hypertension in patients with PCOS, but with no absolute evidence of increased risk of mortality and morbidity from coronary heart disease [119].

1.9.4.5 Haemostatic Factors

There is now increasing evidence linking raised plasma levels of haemostatic factors (including PAI-1, tissue plasminogen activator inhibitor (t-PA) and fibrinogen) with risk for coronary heart disease (CHD). Furthermore PAI-1 is positively associated with insulin resistance and predictive of T2DM in the general population [120, 121]. PAI-1 has been shown to be elevated in PCOS correlating directly with obesity and inversely with insulin sensitivity [87].

1.9.4.6 Inflammation

Chronic, low grade inflammation, as reflected by an increase in surrogate markers such as CRP, independently predicts those at risk for CHD and type 2 diabetes [122]. Women with PCOS have higher circulating CRP concentrations in comparison to controls [91, 123]. These elevations are independent of BMI and are also associated with greater insulin resistance. Preliminary genetic studies have also suggested a potential link between polymorphisms in the genes encoding TNF-α and IL-6 with hyperandrogenism and PCOS [124, 125].

1.9.4.7 Adipocytokine Profiles

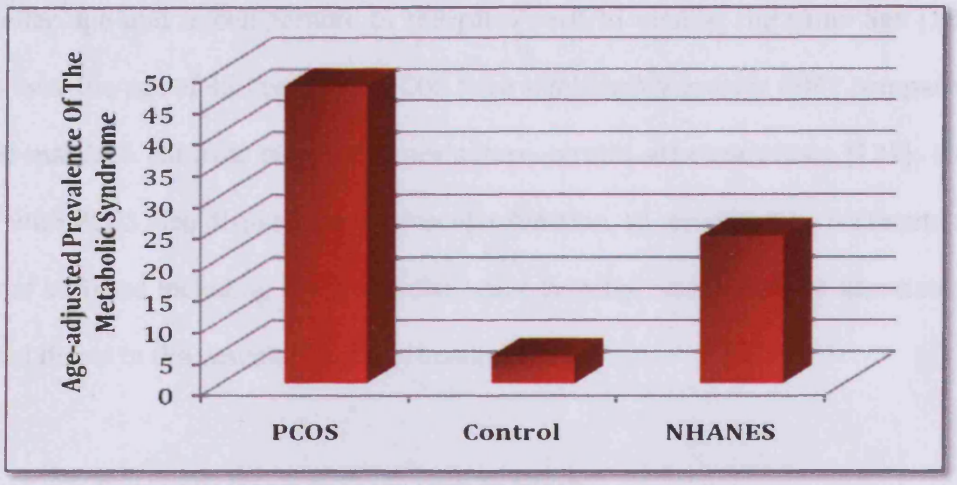
Studies evaluating serum concentrations of adiponectin, an insulin sensitising cytokine, have shown a clear correlation with BMI and central obesity. Obese women with PCOS demonstrate reduced levels of adiponectin though no difference has been seen in comparison to weight-matched controls [126].

1.9.4.8 Prevalence Of The Metabolic Syndrome In PCOS

As discussed previously, the metabolic syndrome represents a potential means of identifying individuals at elevated risk of future CVD. Women with PCOS have a greater prevalence of the metabolic syndrome of 15-50% depending on the criteria used to define the syndrome [127-129]. Dokras et al found a 47.3% prevalence rate of the metabolic syndrome, according to the WHO criteria, in PCOS in contrast to 4.3% in an age-adjusted control population and 23.4% in the NHANES study group (Figure 1.11) [127]. These studies also highlight an increased prevalence of the individual components of the syndrome compared to control populations and this has also been demonstrated in the preceding sections.

Figure 1.11: Prevalence Of The Metabolic Syndrome In PCOS

Adapted from Dokras et al [127]



1.9.5 Vascular Abnormalities In PCOS

1.9.5.1 Endothelial Dysfunction

Endothelial dysfunction, a marker of vascular damage, has recently been linked to the development of atherosclerosis and is now considered to be an independent predictor of future CVD events [130]. Numerous studies have shown early impairment of endothelial structure and function in PCOS, affecting even the young, normal-weight, non-dyslipidaemic, non-hypertensive women [91, 131]. Whether therapeutic interventions to improve endothelial function translate into any clinical benefit with reduced CVD events however, remains unknown.

1.9.5.2 Subclinical Atherosclerosis

There are a growing number of studies highlighting an increased vascular risk in PCOS based on surrogate end-points of CVD, including carotid intima media thickness (CIMT), and coronary artery calcification. In women undergoing coronary angiography, those with more extensive coronary artery disease (CAD) were more likely to have polycystic ovaries on

ultrasound associated with androgenic and metabolic abnormalities [132]. Also, coronary artery and aortic calcification is more prevalent in PCOS than in obese and non-obese women of a similar age and is comparable to the rates seen in men at the same age [128, 133]. Women over the age of 45 years with PCOS have significantly greater CIMT compared to age and BMI-matched controls suggesting premature carotid atherosclerosis [134]. Moreover, women with PCOS also display altered vascular function, as measured by surrogate markers of arterial stiffness including brachial pulse wave velocity, which may be associated with a functional defect in the vascular action of insulin [135].

1.9.5.3 Coronary Heart Disease Risk In PCOS

Despite a clear association between PCOS and an increased prevalence of adverse metabolic features and elevated markers of vascular risk, there is limited evidence confirming an absolute

increase in CVD events in this group and larger studies are still required to elucidate the actual risk. A long-term retrospective cohort study comparing women with PCOS to age-matched controls demonstrated that a history of nonfatal cerebrovascular disease and cardiovascular risk factors including diabetes are more prevalent among women with PCOS [136]. However, this study has been criticised for identifying cases of PCOS based only upon histopathology records from women who had undergone ovarian surgery and thus not truly representing the PCOS population. The Nurses' Health Study is one of the largest studies assessing the risk for CHD and stroke associated with a history of irregular menstrual cycles [137]. 82 439 female nurses were followed prospectively for 14 years and women reporting usually irregular or very irregular cycles had an increased risk for both nonfatal and fatal CVD as well as a non-significant increase in overall stroke risk [137]. Since 80-90% of women reporting menstrual irregularity have PCOS, these data suggested a 50% excess CHD risk in PCOS compared to age and BMI matched controls, in keeping with the greater prevalence of the metabolic syndrome in this group [105].

1.9.6 The Role Of Metformin In The Treatment of PCOS

The mainstay of treatment for PCOS is lifestyle modification. However, if cardiovascular risk remains a concern despite measures to address diet, exercise and smoking cessation, then the insulin sensitiser, metformin, is increasingly being used in the therapeutic adjunct in the management of PCOS. While metformin exerts its predominant metabolic and glucoregulatory action in the liver, considerable interest in its use has arisen by the recognition of its pleiotropic actions on several tissues affected by insulin resistance, including adipose tissue and the endothelium [138]. In subjects with PCOS, metformin not only improves insulin sensitivity [139], but also reduces circulating concentrations of endothelin-1, a marker of endothelial dysfunction [140]. This potential action on the vascular endothelium will be evaluated further in this thesis.

1.10 Thesis Aims And Objectives

The metabolic syndrome comprises a cluster of disturbances, including central obesity, dyslipidaemia and hypertension, centred around insulin resistance. Affected individuals are at increased risk of type II diabetes and CVD, therefore developing novel therapeutic strategies to minimise this is a priority public health challenge.

The adipocyte represents a major molecular target for novel therapeutic agents. CB_1 receptors are expressed in adipocytes, and early trials of a CB_1 antagonist (SR141716) confirm the potential value of this compound in the metabolic syndrome. Some of its effects may be mediated via inhibition of preadipocyte proliferation and differentiation as well as a change in adipocytokine production.

PCOS is the commonest endocrinopathy in young women with well-established effects on reproductive and metabolic health. It is also apparent that PCOS is a vascular disorder

associated with insulin resistance. Metformin therapy improves insulin sensitivity in PCOS but there is limited evidence to support whether these benefits improve vascular risk or indeed whether they represent a direct effect of the drug on the vasculature, or an indirect action mediated through adipokine production or other factors relating to adipose mass.

When the present study was originally designed, the aim had been to investigate the pharmacological effects of a single agent, a CB_1 receptor antagonist, on aspects of the metabolic syndrome using *in vitro* and *in vivo* models. This was however not feasible due to number of factors. Being a novel therapeutic drug, it was not possible to obtain this for independent study in a clinical trial setting at the time this project was commenced. Furthermore the agent was subsequently withdrawn from clinical use shortly afterwards for reasons that will be detailed in the final chapter. Therefore, whilst CB_1 receptor modulators were utilised in the *in vitro* experiments, metformin was chosen as an alternative investigational medicinal product for the *in vivo* studies.

The overall hypothesis for this thesis is that existing (metformin) and novel (CB_1 antagonist) pharmacological agents that target components of the metabolic syndrome may exert their benefits not only by improving insulin sensitivity and obesity, but also through indirect mechanisms involving a switch in the adipokine profile. This hypothesis will be tested using appropriate models of the metabolic syndrome *in vitro* (adipogenesis) and *in vivo* (PCOS) as follows:

1. To evaluate the actions of cannabinoid receptors on proliferation, differentiation and adipokine production in models of *in vitro* adipogenesis.

2. To determine the *in vivo* effects of metformin therapy on arterial stiffness, endothelial function, adipokine profile and insulin sensitivity in young women with PCOS.

CHAPTER 2: ESTABLISHMENT OF PLASMID STANDARD CURVES FOR MEASUREMENT OF TRANSCRIPT EXPRESSION

2.1 INTRODUCTION

Analysis of the activation or inactivation of gene expression has long been used to provide insight into the understanding of the regulation of cellular function and thus the pathophysiology of disease processes. As DNA is transcribed to mRNA and the latter translated to produce functional proteins, quantification of mRNA levels can be used to study gene expression [141]. Numerous techniques have been developed to allow such study including Northern blot analysis, ribonuclease protection assay, microarrays, serial analysis of gene expression as well as conventional and quantitative real time polymerase chain reaction. An overview of some of these methods, including their advantages and limitations, is discussed below.

2.1.1 Northern Blot Analysis

The technique of Northern blotting was developed by James Alwine et al at Stanford University in 1977, and refers to a process by which electrophoretically separated bands of RNA are transferred to a nylon or nitrocellulose membrane, and specific transcripts subsequently detected by hybridisation to radio-labelled DNA probes followed by autoradiography. ^{32}P-labelled probes have been conventionally used but non-radioactive systems have now also become available [142, 143].

Northern blotting allows mRNA size determination and can thus be useful to demonstrate alternatively spliced transcripts or mutations that result in modified mRNA sizes [144]. While this method allows detection of highly specific RNA bands, it supplies information about

tissue distribution, but not cellular location of target RNA, and is further limited by the requirement for large quantities of total RNA from whole tissue. This technique has been superseded, in recent years, by the development of more modern techniques that have increased sensitivity, are less time-consuming and provide more quantitative results.

2.1.2 Ribonuclease Protection Assay

The ribonuclease protection assay (RPA) is a highly sensitive and specific method for detecting RNA and its subsequent quantification. RNA extracted from cells is hybridised with RNA probes, synthesised with the aid of bacteriophage-derived RNA polymerases, containing sequences complementary to those of the gene of interest allowing the formation of double-stranded RNA. Single stranded RNA is then cleaved by RNAse resulting in residual fragments specific to the gene of interest [144, 145]. Based on the quantity of target RNA present in the study sample, information can be derived concerning levels of gene expression. The simultaneous use of multi-probe RNA systems has the advantage of allowing the absolute quantification of multiple species of RNA in a single specimen enabling their comparison, but the method is limited by the large volume of source material required.

2.1.3 cDNA microarrays

In a cDNA array, multiple gene-specific oligonucleotides derived from RNA transcripts are individually arranged on a single matrix which is then probed with fluorescently tagged cDNA representations from total RNA pools [144, 146]. The relative amount of transcript present can therefore be determined by the type of fluorescent signal generated. A single array can contain tens of thousands of probes, therefore this high-throughput method enables the simultaneous study of multiple genes and also allows comparison of gene expression profiles in different settings, for example, in experiments studying the effects of the presence or absence of a specific drug [144]. The main disadvantages include cost, as well as the

challenge of accurate statistical analysis and interpretation of the vast quantities of data generated.

2.1.4 Polymerase Chain Reaction

2.1.4.1 Conventional Polymerase Chain Reaction [147]

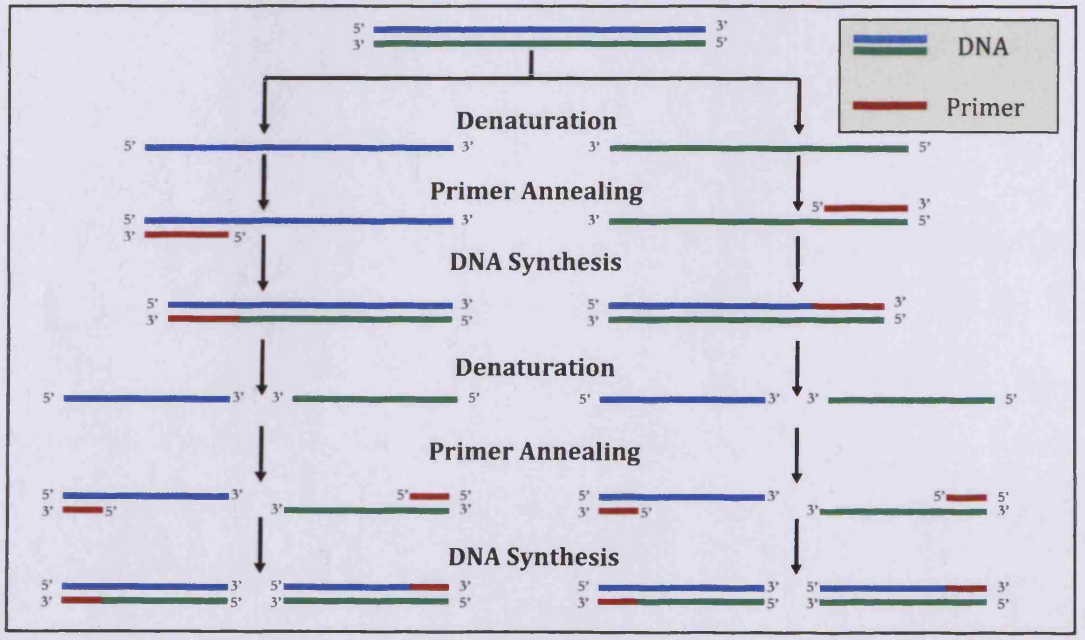
Polymerase Chain Reaction (PCR) allows highly sensitive detection and amplification of short (approximately 100-500 base pairs), target sequences of a larger DNA molecule in a relatively short period of time in an *in vitro* setting. A typical PCR reaction will require a sample of target (genomic or plasmid) DNA, a thermostable DNA polymerase, forward and reverse oligonucleotide primers to recognise the target DNA sequence, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. The reaction mix is then placed in an automated thermal cycler that proceeds through stages at different temperature settings for different periods of time, each series of which is referred to as a single cycle of amplification. Each individual PCR cycle will theoretically double the quantity of the target DNA sequence. Therefore ten cycles will multiply the sequence by a factor of about one thousand, and twenty cycles by a factor of more than a million in just a few hours.

The PCR process occurs in three main stages. The first stage is the initial DNA denaturation step which separates the two intertwined strands of DNA from each other producing single-stranded DNA templates necessary for the DNA polymerase. The complete denaturation of the DNA template at the start of the PCR reaction is of key importance as incomplete denaturation of DNA results in inefficient utilisation of the DNA template in the first amplification cycle and thus poor yield of PCR product (amplicon). This initial denaturation should be performed over an interval of 1 to 5 minutes at 95°C.

The next stage includes the actual amplification cycles and occurs in three steps. The first step denatures the DNA at the start of each cycle. This also occurs at 95°C but lasts for 30 seconds to 2 minutes. Since the PCR product synthesized in the first amplification cycle is significantly shorter than the template DNA, it is completely denatured under these conditions. This is followed by the primer annealing step for which the temperature is reduced to 40-60°C for 30-60 seconds. At this temperature, the oligonucleotide primers can form stable associations (annealing) with the separated target DNA strands and enable the third step of the cycle, i.e. DNA synthesis by a thermostable DNA polymerase performed at 70-75°C lasting 1-2 minutes. The number of times this cycle of denaturation, annealing and DNA synthesis is repeated depends upon the amount of template DNA and expected yield of the PCR product. Usually 25-40 cycles are performed.

After the last cycle, the samples are incubated at 72°C for 5-15min for the final extension stage to fill-in the protruding ends of newly synthesized amplicons. The reactions are then held at 4°C until required for further analysis of e.g. size, quantity and sequence, or for use in further experimental procedures such as cloning in plasmid vectors. A schematic representation of the PCR cycle is given in Figure 2.1.

Figure 2.1: Schematic Diagram Of The PCR Cycle



PCR can be used for semi-quantitative measurement of transcript expression if the number of cycles is kept within the logarithmic phase of amplification. The PCR product amount is then determined by densitometric analysis of ethidium bromide fluorescence upon gel electrophoresis and co-amplification of the transcript of interest with internal controls can allow comparison between different samples.

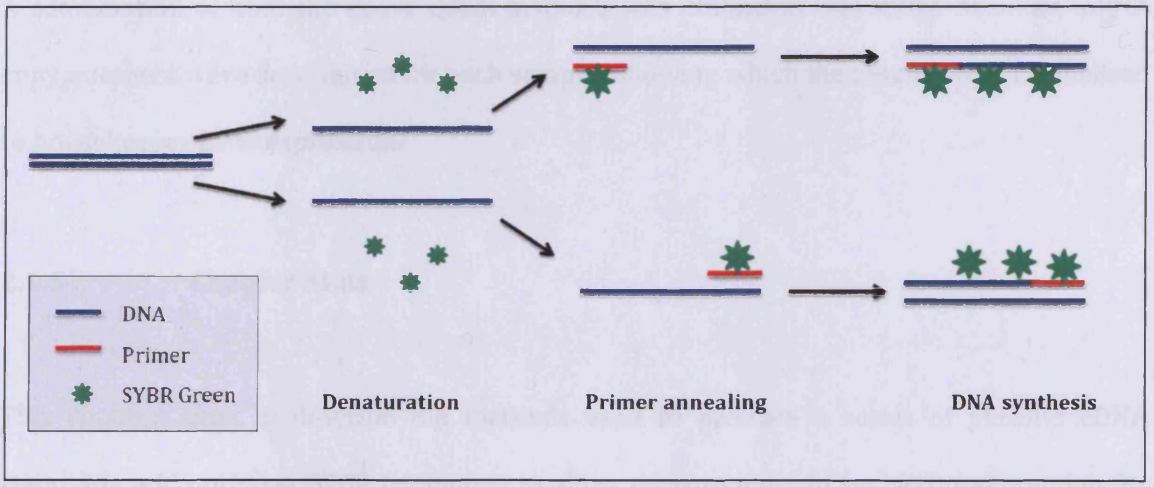
2.1.4.2 Quantitative Polymerase Chain Reaction (QPCR)

QPCR is a highly sensitive technique for studying gene expression by amplification of defined sequences of target RNA. Extracted RNA is initially reverse transcribed to complementary DNA (cDNA), followed by PCR amplification. Quantification of the PCR product occurs during the exponential phase allowing both absolute and relative measurement of the gene of interest. QPCR has the advantage of allowing the simultaneous analysis of multiple samples by standardising data to a known calibrator (housekeeper gene), thus enabling the direct comparison of samples collected from separate populations and different points in time [144]. The most sensitive QPCR machines use glass capillaries and are able to detect transcript copy numbers as low as multiples of ten, whereas the higher throughput machines utilise 96 well plates with sensitivity for transcript numbers in the hundreds. It is, therefore, also a useful technique in circumstances where source material is limited. However, the validity of this procedure is dependent upon careful optimisation of all reaction components as well as accurate quantification of the amplification product.

QPCR is identical to conventional PCR but also allows real time monitoring of the reaction process by including a fluorescent reporter molecule either in the form of a double-stranded DNA-binding dye e.g. SYBR® Green (Figure 2.2), or dye-labelled probes e.g. Taqman®. The SYBR® based assays are relatively easier to design and optimise compared to the probe based techniques and have been used to study gene expression for the purposes of the present study. SYBR® Green has a strong affinity for dsDNA and upon binding there is a

1000-fold increase in its fluorescence (Figure 2.2). As the PCR proceeds the quantity of dsDNA synthesised rises which further augments SYBR® binding and thus fluorescence. The first cycle at which the ambient background fluorescence can be distinguished from that generated by PCR amplification is termed the threshold cycle (Ct). This Ct value is directly proportional to the quantity of DNA present in the initial template.

Figure 2.2: Schematic Representation Of SYBR® Green QPCR



2.1.4.3 Methods Of Quantification

QPCR enables both absolute and relative measurement of mRNA.

Absolute quantification measures the total quantity of the target sequence and is usually expressed as the number of copies of mRNA present in a given sample. The gene of interest can be cloned in a plasmid vector allowing measurement of plasmid DNA concentration. A QPCR standard curve can then be generated by amplification of serial dilutions of the plasmid DNA and plotting the log of the initial known template copy number against the Ct value obtained for each corresponding dilution. Therefore copy numbers in experimental samples can be determined by comparison of their Ct values to this standard curve.

Relative quantification allows gene expression to be reported relative to that of an endogenous control or housekeeper gene whose expression is constant in all samples [144]. This process helps control for differences between samples that may have arisen during RNA extraction and reverse transcription. Gene expression can also be expressed as fold-changes relative to a calibrator, such as Day 0 in time-course experiments or by comparison to an untreated, negative control.

A combination of both the above QPCR methods was utilised in this study. Absolute mRNA copy numbers were determined for each sample following which the results were normalised to housekeeper gene expression.

2.1.5 Chapter Aims

This chapter aims to describe the methods used to produce a series of plasmid cDNA standards and optimise QPCR conditions to allow measurement of transcript expression for human and murine CB_1 and CB_2 receptors, and markers of adipogenesis. The transcriptional control of adipogenesis and details of these individual genes is discussed in further depth in Chapter 3.

2.2 MATERIALS AND METHODS

All centrifugation steps were performed in a Rotina 46R Hettich Zentrifugen (Patterson Scientific, Luton, UK), unless otherwise stated.

2.2.1 Genomic DNA Extraction

Ethical approval was obtained to extract genomic DNA from human and mouse whole blood samples which was undertaken with the use of the QIAamp DNA Blood Mini Kit (QIAGEN Ltd, Crawley, UK) as per the manufacturer's instructions. 200 µl of whole blood was combined with 20 µl 20 mg/ml RNAse A (Promega UK Ltd, Hampshire, UK), 25 µl Proteinase K and 200 µl Buffer AL (lysis buffer), incubated at 70°C for 10 minutes then mixed with 210 µl of absolute ethanol by vortexing. The sample was transferred to a QIAamp spin column placed within a 2 ml collection tube, and centrifuged at 8000 rpm for 1 minute. The column was then transferred to a clean 2 ml collection tube and the previous sample discarded. 500 µl of Buffer AW (wash buffer) was added and the column centrifuged at 8000 rpm for 1 minute. The column was again placed in a fresh 2 ml collection tube, combined with another 500 µl of Buffer AW and centrifuged at 13000 rpm for a further 3 minutes. The column was then transferred to a clean 1.5ml Eppendorf tube and DNA eluted with 200 µl of ultra pure water preheated to 70°C, by centrifugation at 8000 rpm for 1 minute.

2.2.2 DNA Quantification

DNA concentration was quantified by measuring absorbance at 260nm (A_{260}) and 280nm (A_{280}) using a Gene Quant Pro spectrophotometer (Gene Quant, GE Healthcare), and purity of the sample assessed by the A_{260}/A_{280} ratio.

2.2.3 Primer Design

Primers are short nucleic acid chains usually 15-30bp in length, specific to the gene of interest, and serve as a starting point for DNA replication. Longer primers provide higher specificity. The primer should not be complementary to itself or to any other primer in the reaction mixture in order to avoid the formation of primer-dimers and hairpins. All possible sites of binding between primers and the template DNA should be noted to ensure correct identification of the target DNA sequence.

Nucleotide mRNA sequences were obtained for the genes encoding human and murine CB_1 and CB_2 receptors, murine markers of adipogenesis - preadipocyte factor 1 (Pref-1), CCAAT-enhancer-binding protein β (C/EBPβ), peroxisome proliferator-activated receptor-γ (PPARγ), and glycerol-3-phosphate dehydrogenase (GPDH); and house keeper gene acidic ribosomal phosphoprotein (ARP), from the online database GenBank available at the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Based on these published sequences, primers were designed using the online PRIMER3 software available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Ideally primers should be designed to hybridise to different exons to avoid amplification from contaminating genomic DNA, however this was not always possible as the genes encoding CB receptors were all noted to contain a single exon.

Once designed, specificity of the primer sequences was analysed with a BLAST (Basic Local Alignment Search Tool) search (http://www.ncbi.nlm.nih.gov/blast) to ensure no cross-reactivity with other genes. Primer sequences were manufactured by Invitrogen (Invitrogen Ltd, Paisley, UK). Plasmid cDNA standards for the human markers of adipogenesis - C/EBPβ, PPARγ, lipoprotein lipase (LPL) and uncoupling protein-1 (UCP-1); and the housekeeper gene adenosine phosphoribosyltransferase (APRT), were kindly provided by Dr Lei Zhang (Centre for Endocrine & Diabetes Sciences, Cardiff University).

2.2.4 PCR Amplification

PCR was performed in compatible 500 µl polypropylene tubes using a Techne Genius DNA Thermal Cycler (Techne Cambridge Ltd, Cambridge, UK). A reagent master mix was prepared to increase reaction consistency and minimise pipetting error. Each reaction contained approximately 100 ng of genomic DNA (human or mouse), 1 µl forward primer (10 pmol/l), 1 µl reverse primer (10 pmol/l), 1 µl dNTPs, 2.5 µl 10x reaction buffer, 0.4 µl (5 units/µl) Taq DNA polymerase (Promega, Southampton, UK), and ultra pure water to complete a total of 25 µl per reaction. Higher magnesium concentrations were required in the reactions containing mouse DNA and primers. This was achieved by using a magnesium-free buffer supplemented with 5 µl of 25 mM $MgCl_2$. A single reaction containing all reagents except genomic DNA was also performed as the negative control to confirm the absence of contamination. The PCR reagents and thermal cycling settings are shown in Tables 2.1 and 2.2.

Table 2.1: PCR Reagents For Amplification From Genomic DNA

Reagents	Volume per reaction
Sterile H_2O	to complete final volume of 25 µl
10x Taq DNA polymerase buffer (100 mM Tris-HCl, 500 nM KCl, 15 mM $MgCl_2$)	2.5 µl
dNTP mix (10mM each dATP, dCTP, dGTP, dTTP)	1 µl
Forward primer (10 pmol/l)	1 µl
Reverse primer (10 pmol/l)	1 µl
Taq DNA polymerase (5 units/µl)	0.4 µl
Genomic DNA	100 ng
Total volume	25 µl

Table2.2: PCR Thermal Cycling Protocol

	Temperature	Time	№ of cycles
Initial Denaturation	95°C	5 minutes	1
Denaturation	95°C	1 minute	40
Annealing	60°C	30 seconds	
DNA synthesis	72°C	1 minute	
Final extension	72°C	5 minutes	1

2.2.5 Agarose Gel Electrophoresis Of PCR Products

Following PCR the product size was verified using agarose gel electrophoresis. A 2% gel was prepared with 40 ml TAE 1x buffer (Tris-Acetate-EDTA), 0.8 g agarose and 2 µl of 10mg/ml ethidium bromide (Sigma). (To make 50x TAE buffer add 121g Tris base in 250ml H_2O. stir to dissolve. add 28.6mL acetic acid. add 50ml 0.5M EDTA pH 8.0) The same concentration of ethidium bromide i.e. 0.5 µg/ml, was maintained in the TAE running buffer. The solution was heated in a conventional microwave to enable the agarose to dissolve completely and then allowed to set by cooling at room temperature for 20 minutes in the minigel apparatus. 10 µl of each amplicon and 2 µl of 6x loading dye (Promega, UK) were placed in individual wells with 5 µl of 100 bp DNA ladder (Promega) and 1 µl 6x loading dye in the final lane for comparison. The gel apparatus was run at 150 volts for 30 minutes and then visualised under an ultraviolet light source connected to a computer using an Alpha Imager™ gel documentation system (Alpha Innotech, San Leandro, California, USA).

2.2.6 PCR Product Purification

This protocol and reagents provided by QIAGEN (QIAGEN Ltd, Crawley, UK), was followed to purify each PCR product from primers, dNTPs, DNA polymerase and salts present in the PCR reaction, resulting in pure DNA for use in the subsequent experimental procedures.

3 volumes of Buffer QG (solubilisation and binding buffer) was added to 1 volume of the PCR reaction product and mixed thoroughly by vortexing. Before proceeding it was necessary to ensure yellow colouration of the mixture indicating a pH ≤ 7.5, i.e. optimal for DNA adsorption to the QIAquick membrane. The solution was transferred to a QIAquick spin column placed within a 2 ml collection tube and centrifuged at 13000 rpm for 1 minute. 0.75mls of Buffer PE (wash buffer) was applied to the column and centrifuged again for 1 minute. The flow-through was discarded, column centrifuged for an additional minute and then placed in a clean collection tube. DNA was eluted by the addition of 50 µl of Buffer EB (Elution buffer - 10mM Tris·Cl, pH 8.5) to the column and a final centrifugation step at 13000 rpm for 1 minute.

2.2.7 DNA Concentration Calculation By Agarose Gel Analysis

The purified PCR product size was verified by agarose gel electrophoresis as described in section 2.2.5. The quantity of the PCR product was estimated by comparison of the signal intensity against standard quantitative molecular weight markers on a 1 kb and 100 bp DNA ladder (Promega) allowing calculation of DNA concentration within the purified product using the following formula:

$$\frac{\text{Estimated weight (ng)}}{\text{Volume of PCR product loaded in gel apparatus (µl)}} = \text{DNA concentration (ng/µl)}$$

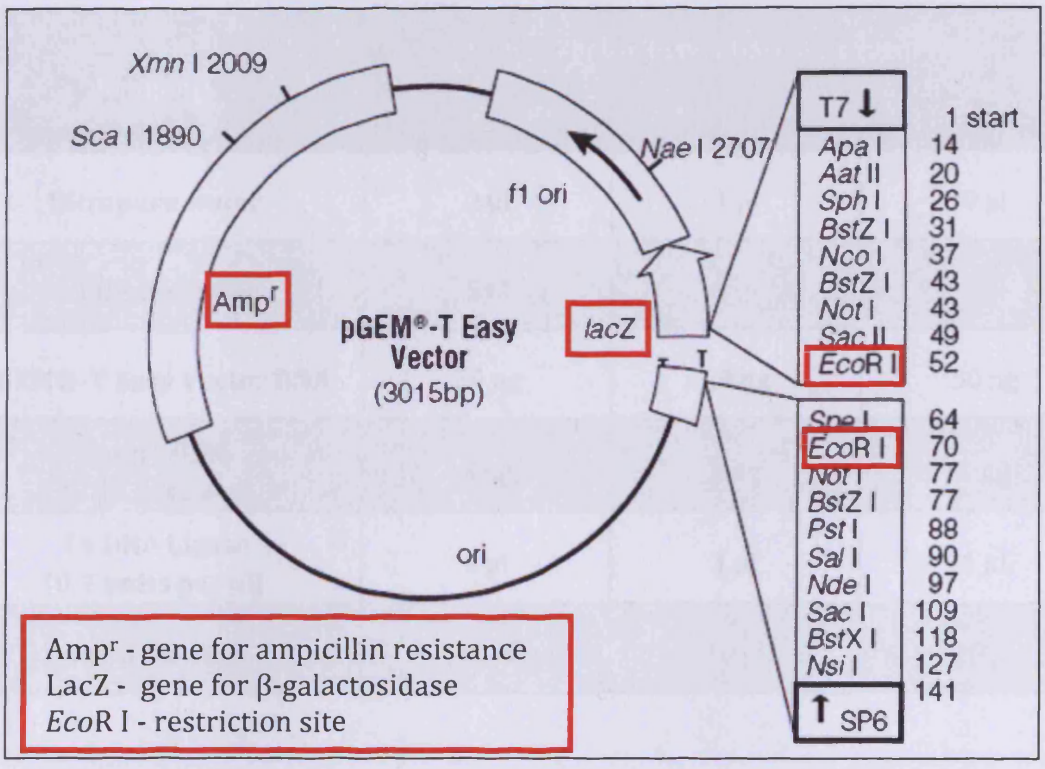
2.2.8 Plasmid Vector Ligation

A plasmid is a DNA molecule capable of autonomous replication. Insertion of the gene to be replicated and then transfection into competent cells allows large scale DNA amplification by bacterial culture. The commercially available pGEM®-T Easy plasmid vector (Promega, Southampton, UK) contains genes encoding ampicillin resistance (Amp^r) as well as the α-

subunit of *Lac*Z (β-galactosidase) allowing later selection of suitably transformed cell colonies. The bacterial host DNA encodes the remaining Ω–subunit thus forming functional β-galactosidase upon successful transformation of competent cells. Key features of the vector are illustrated in Figure 2.3 including the multiple cloning region contained within the *Lac*Z coding region which is flanked by recognition sites for restriction enzymes e.g. *Eco*R1, which can be used to verify the presence of an insert, providing the insert itself has no *Eco*R1 site, in which case 1 or more of the other restriction enzymes would be used for digestion.

Figure 2.3: pGEM®-T Easy Plasmid Vector Map & Sequence Reference Points

(adapted from Promega technical manual available at www.promega.com/tbs)



Purified PCR amplicons were ligated with the pGEM®-T Easy plasmid in vector-insert ratios of 1:1, 1:3, and 3:1 based on molarity. The amount of PCR product (insert) to be included in ach reaction was calculated using the following formula obtained from the Promega website at www.promega.com/tbs:

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{vector : insert molar ratio} = \text{ng of insert}$$

Each reaction contained 50 ng vector, 5 µl of 2x ligase buffer, 1 µl (0.1 units) T4 bacteriophage-derived DNA ligase (Promega), either 1, 3 or 9 ng insert DNA, and ultrapure water to complete a total of 10 µl per reaction, and then incubated at room temperature for 6 hours prior to transfection into competent cells. This protocol has been summarised in table 2.3.

Table 2.3: Protocol For pGEM®-T Easy Vector Ligation With PCR Amplicons

Reagents	Vector:Insert base pair ratio		
	1:1	1:3	3:1
Ultrapure water	2 µl	1 µl	0 µl
2 x ligase buffer	**5 µl**	**5 µl**	**5 µl**
pGEM®-T Easy Vector DNA	50 ng	50 ng	50 ng
Insert DNA (PCR product)	**3 ng**	**9 ng**	**1 ng**
T4 DNA Ligase (0.1 units per µl)	1 µl	1 µl	1 µl
Total volume	**10 µl**	**10 µl**	**10 µl**

2.2.9 Transformation Of Competent Cells

2.2.9.1 Preparation Of Culture Media

Luria Bertani (LB) medium (USB, Cleveland, Ohio) was prepared for use as bacterial growth medium by adding 5 g LB to 200 mls distilled water and autoclaving for 1 hour. The medium

was then cooled to 55°C before being supplemented with 200 µl of 100 mg/ml ampicillin for a final concentration of 100 µg / ml.

10 mls of sterile modified SOC solution (super-optimal broth with catabolic repressor) was prepared by combining 10mls LB with 100 µl of 2M glucose solution (3.6 g glucose dissolved in 10 mls distilled water and filter sterilised) for a final concentration of 20mM glucose.

100 mg X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was dissolved in 2mls dimethylformamide for a stock solution concentration of 50 mg/ml and stored sheltered from light at -20°C.

2.2.9.2 Preparation Of Agar Plates

14.8g LB-agar was added to 400mls distilled water and autoclaved for 1 hour. The solution was then incubated at 55°C for 2 hours and supplemented with 400 µl of 100 mg/ml ampicillin (final concentration 100 µg/ml) and 640 µl of 50 mg/ml X-gal (final concentration 80 µg/ml). 25mls of the final solution was added to each 10 cm petri dish and allowed to set at room temperature. The agar plates were then stored inverted at 4°C until use.

2.2.9.3 Transfection

There are two main methods for preparing competent bacterial cells for transformation – incubation in cold calcium containing buffers (chemical) or by electroporation. Both these techniques render the cell membranes transiently permeable to extracellular DNA in the environment thus allowing transfection with plasmid DNA. The commercially available chemically competent *Escherichia coli* (*E. Coli*) DH5α (Invitrogen) were used in these experiments. 4 µl of plasmid ligation products were combined with 20 µl aliquots of *E. Coli* DH5α and incubated on ice for 30 minutes. The samples were then heat-shocked at 42°C for

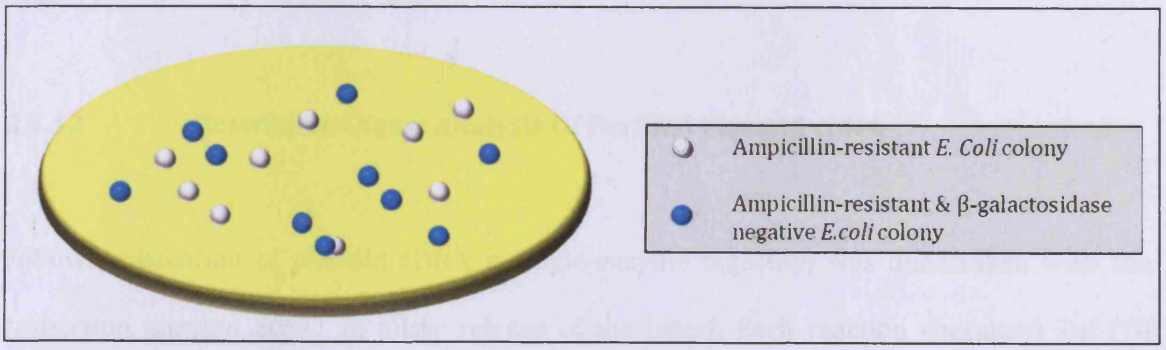
90 seconds and returned to ice for 5 minutes. 1 ml of sterile SOC medium (LB containing 20mM glucose) was added to each sample and incubated at 37°C for 1 hour with gentle shaking at 150 rpm following which the samples were centrifuged at 3000 rpm for 5 minutes. 600 µl of the medium supernatant was discarded and following gentle resuspension, 200 µl of the remaining transformation mix was spread onto LB-agar plates containing 100 µg/ml ampicillin and 80 µg/ml X-gal and incubated, inverted, overnight at 37°C.

2.2.10 Culture Of Selected Colonies

X-gal supplemented in the agar plates is a colourless galactose sugar that is metabolised by β-galactosidase to 5-bromo-4 chloroindole, an insoluble bright blue product. Successful cloning of an insert into the pGEM®-T Easy Vector interrupts the coding sequence of β-galactosidase allowing recombinant clones to be identified by colour screening on indicator plates.

Using this method of blue-white screening, *E.coli* colonies were selected (Figure 2.4) using a micro-inoculator loop and used to inoculate 5 ml of LB medium containing 100 mg/ml ampicillin (Sigma-Aldrich Company Ltd, Dorset, UK). Cultures were grown overnight in an orbital shaking incubator at 200 rpm at 37°C.

Figure 2.4: Selection Of Bacterial Colonies Containing Plasmid Vector



2.2.11 Small-Scale Isolation Of Plasmid cDNA By Alkali Lysis (Mini-prep)

1.5 ml of overnight culture fluid containing transformed cells was transferred to a 1.5 ml Eppendorf tube and centrifuged at 13000 rpm for 1 minute. The cell pellet was then resuspended in 1 ml of SET (20% sucrose, 50mM Tris.HCl, 50mM EDTA, pH 7.5) and centrifuged again at 13000 rpm for 1 minute. The supernatant was removed and pellet resuspended in 150 µl SET. This was then combined with 5 µl of 4mg/ml DNAse-free RNAse A (Promega UK Ltd, Hampshire, UK) and 350 µl of freshly prepared lysis solution (0.2M NaOH, 1% SDS [8.8 ml H_20, 0.2 ml 10M NaOH and 1 ml 10% SDS]). Samples were kept on ice for 10 minutes, combined with 250 µl 3M sodium acetate (pH 5.2) and mixed by inverting the tube several times and kept on ice for a further 30 minutes. This was followed by centrifugation at 15000 rpm for 5 minutes at 4°C. The supernatant was transferred to a clean tube to which an equal volume of isopropanol was added and then centrifuged at 13000 rpm for 10 minutes at room temperature. The supernatant was discarded and DNA pellet washed with 1 ml 70% v/v ethanol 30 % $T_{10}E_1$ (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and centrifuged for 5 minutes. The supernatant was again discarded and DNA pellets air-dried prior to resuspension in 20 µl $T_{10}E_1$ buffer.

DNA concentration was quantified by measuring absorbance at 260nm (A_{260}) and 280nm (A_{280}) using a Gene Quant Pro spectrophotometer, and purity of the sample assessed by the A_{260}/A_{280} ratio.

2.2.12 Restriction Digest Analysis Of Purified Plasmid cDNA

Following isolation of plasmid cDNA, a single-enzyme digestion was undertaken with the restriction enzyme *Eco*R1 to allow release of the insert. Each reaction contained 1µl (10 units/ µl) *Eco*R1 (Promega), 1 µl 10x reaction buffer, approximately 1 µg cDNA and made up to 10 µl with H_20. The samples were then incubated in a 37°C water bath for 2 hours.

2.2.13 Agarose Gel Electrophoresis

Following digestion the product size was verified using agarose gel electrophoresis. A 1.2% gel was prepared with 50 ml Tris-acetate-EDTA (TAE) 1x buffer, 0.6 g agarose and 2.5 µl of 10mg/ml ethidium bromide. The same concentration of ethidium bromide i.e. 0.5 µg/ml, was maintained in the TAE running buffer. The undigested and digested plasmids were compared to 100bp and 1kb DNA ladders. Bands corresponding to the 3 kb plasmid vector were seen in all lanes. Samples which also confirmed the presence of an insert of the expected size, released by restriction digest, were selected for DNA sequencing prior to proceeding to large-scale plasmid DNA preparation.

2.2.14 Automated DNA Sequencing

The plasmid cDNA products resulting from the alkali lysis procedure containing inserts of the expected size were analysed to ensure correct DNA sequence incorporation into the plasmid vectors and successful amplification in bacteria.

The sequencing reactions were performed on a Techne Genius DNA Thermal Cycler (Techne Cambridge Ltd, Cambridge, UK). Two reactions were undertaken for each individual cDNA specimen, one containing the corresponding forward primer, and one with the reverse primer. Each reaction contained 2 µl Big Dye Terminator Cycle Sequencing Ready Reaction (v3.1, ABI Prism, PE Biosystems), 2 µl 5x Big Dye reaction buffer, 1 µl 10 pmol/L primer (Invitrogen), approximately 200 ng cDNA, and made up to 10 µl with H_20. The PCR settings used are shown in Table 2.4.

Table 2.4: PCR Protocol For DNA Sequencing

Temperature	Time	№ of cycles
96°C	3 minutes	1
96°C	30 seconds	30
60°C	4 minutes	

2.2.14.1 Sodium Acetate Precipitation

Following PCR, 1.5 µl of 3M Sodium Acetate, 31.5 µl ice-cold 95% v/v ethanol and 10 µl sterile water was added to each sample. These were incubated at room temperature for 15 minutes and then centrifuged at 15,000 rpm for 20 minutes at 4°C. The supernatant was discarded and the DNA pellet washed with 250 µl ice-cold 70% v/v ethanol and centrifuged further at 15,000 rpm for 5 minutes at 4°C. The supernatant was removed and samples incubated for 1 minute at 95°C prior to being resuspended in 10 µl 70% v/v ethanol and analysed on an ABI 310 Genetic Analyser. The resulting DNA sequences were analysed with a BLAST search to ensure correspondence with the genes of interest and alignment with the initial primer sequences.

2.2.15 Maxiprep

100 mls of LB medium containing 100 µg/ml ampicillin was inoculated with 1 ml of bacterial culture preserved prior to mini-prep. Cultures were grown in a 200 ml conical flask overnight in an orbital shaking incubator at 250 rpm at 37°C. Plasmid DNA was then extracted using the Qiafilter Plasmid Maxi Kit (Qiagen) according to the following protocol. The bacterial cells were harvested by centrifuging the overnight culture fluid at 3600 rpm for 30 minutes at 4°C and supernatant discarded. The cell pellet was resuspended in 10 mls Buffer P1

(resuspension buffer with RNase A) by vortexing, combined with 10 mls Buffer P2 (alkaline lysis buffer), incubated at room temperature for 5 minutes and then neutralised with 10 mls chilled Buffer P3 (neutralisation buffer). The lysate was poured into the barrel of a QIAfilter cartridge, and incubated at room temperature for a further 10 minutes prior to filtration through a QIAGEN-tip, allowing binding of plasmid DNA to an anion-exchange resin. Protein and RNA impurities were removed by washing with 30mls Buffer QC (medium salt wash) and DNA eluted with 15 mls Buffer QF. DNA was further concentrated and purified by precipitation with 10.5 mls room-temperature isopropanol added to the eluted DNA and centrifugation at 11000 rpm for 30 minutes at 4°C. The supernatant was discarded, DNA pellet washed with 5 mls room temperature 70% v/v ethanol and centrifuged at 11000 rpm for another 10 minutes at 4°C. The DNA pellet was then air-dried for 10 minutes, resuspended in 100 µl sterile water and final DNA yield calculated by measuring absorbance at 260 nm using a Gene Quant Pro spectrophotometer, and purity of the sample examined by the A_{260}/A_{280} ratio.

The purified plasmid DNA was again analysed by restriction enzyme digest and agarose gel electrophoresis as described in sections 2.2.12 and 2.2.13.

2.2.16 Calculation Of Transcript Copy Numbers From DNA Concentration

Determination of transcript copy numbers from DNA concentration required use of the following formulae obtained from http://www.promega.com/biomath:

1. Conversion of DNA concentration from µg/ml to pmol/ml based on the following formula:

$$\frac{\mu g\ DNA}{ml} \times \frac{pmol}{660pg} \times \frac{10^6\ pg}{1\mu g} \times \frac{1}{N} = pmol\ DNA/ml$$

where: N = # of nucleotides in plasmid DNA (vector + inserted PCR product)

660pg = average molecular weight of a nucleotide pair.

2. Calculation of transcript copy numbers.

Based on the Avogadro constant there are 6×10^{23} copies of a molecule in 1 mole, and 1 pmol = 10^{-12} moles. Therefore:

$$\frac{6 \times 10^{23} \text{ copies} \times \text{pmol DNA/ml}}{10^{12}} \times 10^{-3} = \text{copies/µl}$$

2.2.17 Storage Of Plasmid DNA Stock Solutions

Following calculation of transcript copy numbers, the maxi-prep plasmid DNA samples were diluted in sterile water to create stock solutions of 10^9 copies per µl and stored at -80°C.

2.2.18 QPCR

2.2.18.1 QPCR optimisation

Efficiency of QPCR is dependent upon a number of factors. Primers should be carefully designed and checked for specificity to the gene of interest and where possible primers should span intron-exon boundaries to avoid amplification from contaminating genomic DNA. Amplification efficiency is also affected by differences in the melting temperature (T_m) of the forward and reverse primers which in turn is dependent upon the primer's own

concentration within the reaction. Therefore the optimal Tm for each primer can be achieved by altering the primer concentrations.

The most favourable primer concentrations were determined by performing the primer matrix test (Table 2.5), where QPCR was performed using differing forward and reverse primer concentrations for known copy numbers of plasmid DNA. The optimum primer combination was that which resulted in the lowest Ct values at both high and low copy numbers of the target gene.

Table 2.5: Primer Concentrations Used In The Primer Matrix Test

Forward Primer / Reverse Primer	100 nM	300 nM	500 nM
100 nM	100nM Forward 100nM Reverse	300nM Forward 100nM Reverse	500nM Forward 100nM Reverse
300 nM	100nM Forward 300nM Reverse	300nM Forward 300nM Reverse	500nM Forward 300nM Reverse
500 nM	100nM Forward 500nM Reverse	300nM Forward 500nM Reverse	500nM Forward 500nM Reverse

2.2.18.2 QPCR Amplification Efficiency & Quality Checks

As well as optimising the primer concentrations further quality checks were performed to ensure maximum QPCR efficiency. The Ct value should occur during the exponential phase of cDNA amplification, therefore the standard curve generated by plotting the Ct values against the log of the transcript copy numbers should generate a linear graph with a correlation coefficient close to 1.0.

A second quality check is analysis of the melting point (T_m), the temperature at which 50% of dsDNA dissociates into single strands. This is identified during QPCR by a significant reduction in fluorescence. The correct DNA product for a specific pair of primers will have the same T_m dependent on the nucleotide base composition, with GC-rich DNA requiring a higher T_m than AT-rich DNA. At the end of QPCR amplification, the temperature is therefore increased to the T_m to identify a single peak representing a single amplified product - the presence of other smaller peaks indicates contaminating DNA, primer-dimers, or alternatively spliced transcripts.

QPCR experiments should also include negative controls where mRNA is used as the nucleic acid template without having undergone reverse transcription (should not yield an amplification product). Another precaution to avoid genomic DNA contamination is to treat mRNA samples with DNAse prior to reverse transcription.

2.2.18.3 QPCR Protocol

QPCR was performed using Stratagene's Brilliant® SYBR® Green Master Mix. Each 25 µl reaction contained 12.5 µl Master Mix, forward and reverse primer (as per optimised concentrations) and ultrapure water. Reactions were set up in Stratagene 96-well PCR plates and centrifuged for 1 minute at 3000 rpm prior to being transferred to the Stratagene MX3000P® thermocycler. The thermal cycling protocol is listed in Table 2.6.

2.2.19 QPCR Product Verification

The QPCR products were analysed by agarose gel electrophoresis, to confirm the presence of products of the expected base-pair size, and by DNA sequencing as described previously to ensure correct sequence amplification.

Table 2.6: QPCR Thermal Cycling Protocol

	Temperature	Time	№ of cycles
Enzyme activation	95°C	10 minutes	1
Denaturation	94°C	30 seconds	40
Annealing	60°C	60 seconds	
Extension	72°C	30 seconds	
Dissociation	95°C	60 seconds	1
	55°C	30 seconds	
	95°C	30 seconds	

2.3 RESULTS

2.3.1 Genomic DNA concentrations

The human and genomic DNA concentrations are shown in table 2.7. A_{260} and A_{280} are the optical spectrometer measurements of absorbance at the wavelengths of 260 nm and 280 nm respectively. A_{260} is used to measure nucleic acid concentration and A_{280} is used to measure protein concentration. A ratio of $A_{260}/A_{280} > 1.8$ suggests little protein contamination in a nucleic acid sample.

Table 2.7: Quantification Of Genomic DNA Concentration

	A_{260}	A_{280}	Concentration ng/µl	A_{260}/A_{280}
Human	0.129	0.065	129	1.985
Murine	0.049	0.028	49	1.75

2.3.2 Primer Sequences

Tables 2.8A and 2.8B list details of all the human and murine primers used during these experiments, including the primer nucleotide sequence, exon location, expected PCR product size and T_m. There were difficulties encountered while designing primers and it was not always possible to obtain forward and reverse primers derived from differing exons due to lack of product specificity or failure of PCR amplification.

Table 2.8A: Primer Sequences for Human CB Receptors and Markers Of Adipogenesis

	Gene		5' to 3' Sequence	Exon	Concentration	Product size	Tm (°C)
HUMAN PRIMERS	CB1	Forward	AAG ACC CTG GTC CTG ATC CT	1	100nM	188	60
		Reverse	CGC AGG TCC TTA CTC CTC AG	1	300nM		60
	CB2	Forward	TAG ACA CGG ACC CCT TTT TG	1	100nM	241	60
		Reverse	TTC TCC CAA GTC CCT CAT TG	1	100nM		60
	APRT	Forward	GCT GCG TGC TCA TCC GAA AG	3	100nM	247	60
		Reverse	CTT TAA GCG AGG TCA GCT GC	5	100nM		60
	C/EBPβ	Forward	AAC TTT GGC ACT GGG G	1	100nM	304	60
		Reverse	GGC CCG GCT GAC AGT T	1	100nM		60
	PPARγ	Forward	CAG TGG GGA TGT CTC ATA	3	300nM	390	60
		Reverse	CTT TTG GCA TAC TCT GTG AT	5	500nM		60
	LPL	Forward	GAG ATT TCT CTG TAT GGA CC	7	300nM	275	60
		Reverse	CTG CAA ATG AGA CAC TTT CTC	9	300nM		60
	UCP-1	Forward	GGG GCT TCA GCG GCA AAT CAG	2	500 nM	236	60
		Reverse	TAT AAG TCC CCG TGT AGC GAG TT	3	300 nM		60

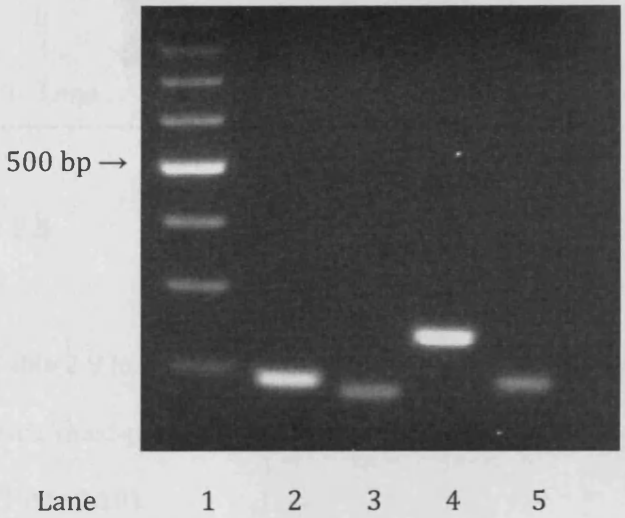
Table 2.8B: Primer Sequences for Murine CB Receptors and Markers Of Adipogenesis

	Gene		5' to 3' Sequence	Exon	Concentration	Product size	Tm (°C)
MURINE PRIMERS	CB1	Forward	ACA GGG CAG TAC CCC TTC TT	1	100nM	175	60
		Reverse	AGC CCC TGG TGG TAT TCT CT	1	100nM		60
	CB2	Forward	TCA TTG CCA TCC TCT TTT CC	1	500nM	188	60
		Reverse	GAA CCA GCA TAT GAG CAG CA	1	100nM		60
	ARP	Forward	GAG GAA TCA GAT GAG GAT ATG GGA	7	100nM	172	60
		Reverse	AAG CAG GCT GAC TTG GTT GC	7	100nM		60
	Pref-1	Forward	CGT GAT CAA TGG TTC TCC CT	5	300nM	148	60
		Reverse	AGG GGT ACA GCT GTT GGT TG	5	500nM		60
	C/EBPβ	Forward	CAA GCT GAG CGA CGA GTA CA	1	500nM	157	60
		Reverse	CAG CTG CTC CAC CTT CTT CT	1	500nM		60
	PPARγ	Forward	TTT TCA AGG GTG CCA GTT TC	6	300nM	220	60
		Reverse	AAT CCT TGG CCC TCT GAG AT	6	300nM		60
	GPDH	Forward	ATG CTC GCC ACA GAA TCC ACA C	8	500nM	124	60
		Reverse	AAC CGG CAG CCC TTG ACT TG	8	500nM		60

2.3.3 PCR Amplification

Figure 2.5 illustrates the human and murine CB_1 and CB_2 PCR products visualised on a 2% agarose gel stained with 0.5 µg/ml ethidium bromide. Comparison was made to a 100 bp DNA ladder to ensure the amplified product bands were of the expected size.

Figure 2.5: Human and Murine CB PCR Products

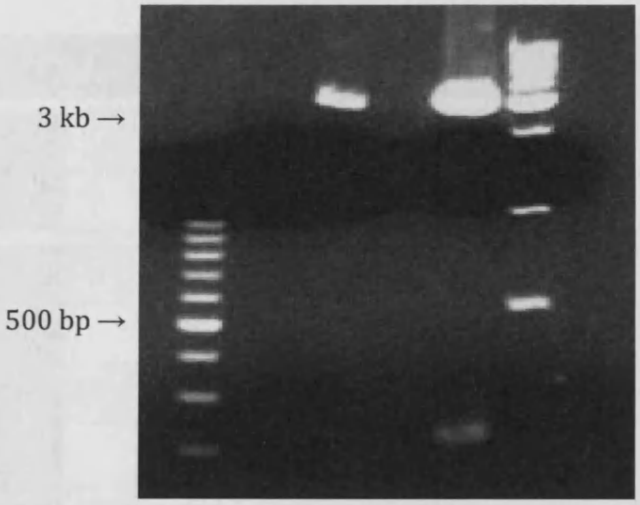


Lane	Gene	PCR Product size
1	100 bp DNA ladder	-
2	Human CB1	188 bp
3	Murine CB1	175 bp
4	Human CB2	241 bp
5	Murine CB2	188 bp

2.3.4 Restriction Digest Analysis Of Mini-Preps

Figure 2.6 provides an example of two Human CB_1 mini-prep products following *Eco*R1 restriction enzyme digest, visualised on a 1.2% agarose gel stained with 0.5 µg/ml ethidium bromide. Comparison was made to a 1 kb and 100 bp DNA ladder. Bands corresponding to the 3 kb plasmid vector size were seen in both samples but only 1 contained an insert of the expected size, released by restriction digest. This sample was selected for DNA sequencing followed by large-scale plasmid DNA preparation.

Figure 2.6: Human CB_1 Mini-Prep Restriction Digest Products



Lane	Contents
1	100 bp DNA ladder
2	Plasmid vector only
3	Plasmid vector and CB_1 insert
4	1 kb DNA ladder

2.3.5 Measurement of Maxi-Prep DNA Concentration

Table 2.9 lists the plasmid DNA concentrations determined by spectrophotometer analysis of each maxi-prep. This information was then used to calculate the transcript copy numbers (Table 2.10).

Table 2.9: Maxi-Prep DNA Concentrations

	Gene	A_{260}	A_{280}	Concentration ng/µl	A_{260}/A_{280}
Human	CB1	0.106	0.055	106	1.927
	CB2	0.331	0.185	331	1.789
Murine	CB1	0.882	0.435	882	2.028
	CB2	1.727	0.879	1727	1.965
	ARP	0.398	0.208	398	1.913
	Pref-1	1.639	0.845	1639	1.94
	C/EBPβ	0.760	0.412	760	1.845
	PPARγ	2.371	1.277	2371	1.857
	GPDH	1.256	0.623	1256	2.016

Table 2.10: Transcript Copy Numbers Determined From Maxi-Prep DNA Concentration

	Gene	DNA µg/ml	DNA pmol/ml	Copies/µl
Human	**CB1**	106	50	3×10^{10}
	CB2	331	156	1×10^{11}
Murine	**CB1**	882	419	2.5×10^{11}
	CB2	1727	817	5×10^{11}
	ARP	398	189	1×10^{11}
	Pref-1	1639	785	5×10^{11}
	C/EBPβ	760	363	2×10^{11}
	PPARγ	2371	1110	6.5×10^{11}
	GPDH	1256	606	4×10^{11}

Plasmid DNA standards for the human markers of adipogenesis (C/EBPβ, PPARγ, LPL) and housekeeper (APRT) were kindly provided by Dr Lei Zhang (Centre for Endocrine & Diabetes Sciences, Cardiff University).

2.3.6 Optimisation Of QPCR Primer Concentrations

Table 2.11 lists the Ct values obtained for the primer matrix test when applied to human CB_1 and the optimum primer combination which resulted in the lowest Ct values at both high (10^6) and low (10^2) copy numbers. Table 2.12 lists the optimised primer concentrations for all the genes of interest for this study.

Table 2.11: Human CB_1 Primer Matrix Test

(The Ct values presented are the mean of a single QPCR reaction performed in triplicate. The lowest values have been highlighted indicating the optimum primer combination)

Ct Values for 10^6 copies			
For / Rev	100nM	300nM	500nM
100nM	14.37	15.01	15.62
300nM	14.03	16.44	15.33
500nM	14.78	14.85	16.81

Ct Values for 10^2 copies			
For / Rev	100nM	300nM	500nM
100nM	27.02	28.63	29.03
300nM	26.94	28.93	30.18
500nM	27.56	27.83	30.25

Table 2.12: Optimised Concentrations For All QPCR Primers

Human gene	Forward primer nM	Reverse primer nM
CB1	100	300
CB2	**100**	**100**
APRT	100	100
C/EBPβ	**100**	**100**
PPARγ	300	500
LPL	**300**	**300**

Murine gene	Forward primer nM	Reverse primer nM
CB1	100	100
CB2	**500**	**100**
ARP	100	100
Pref-1	**300**	**500**
C/EBPβ	500	500
PPARγ	**300**	**300**
GPDH	500	500

2.3.7 QPCR Standard Curves

In order to allow estimation of copy numbers of the genes of interest in unknown samples, standard curves for reference were generated for each gene by QPCR analysis of samples with known copy numbers determined by serial dilutions of plasmid DNA stock solutions. The standard curves were obtained by plotting the Ct values against the log of the transcript copy

numbers to produce a linear graph with a correlation coefficient (RSq) close to 1.0 and amplification efficiency approaching 100%. Representative examples of the QPCR standard curves and amplification plots for human and murine CB_1 and CB_2 are given in Figures 2.7A-D.

Figure 2.7A: SYBR Green® QPCR Standard Curve & Amplification Plots for Human CB_1

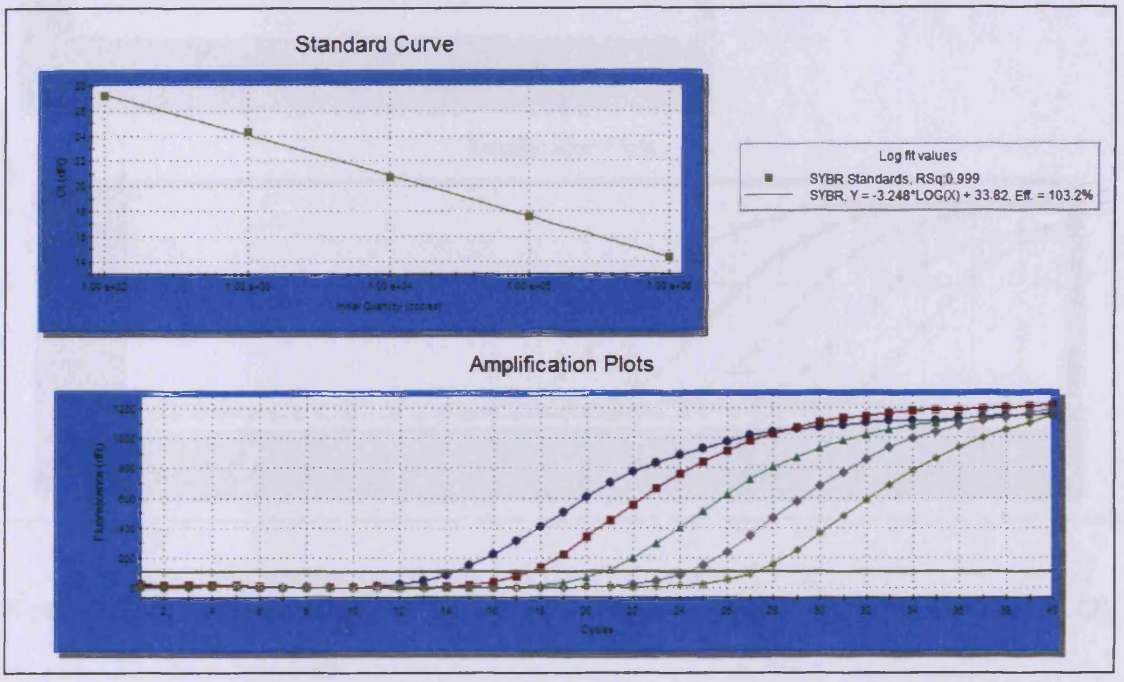


Figure 2.7B: SYBR Green® QPCR Standard Curve & Amplification Plots for Murine CB_1

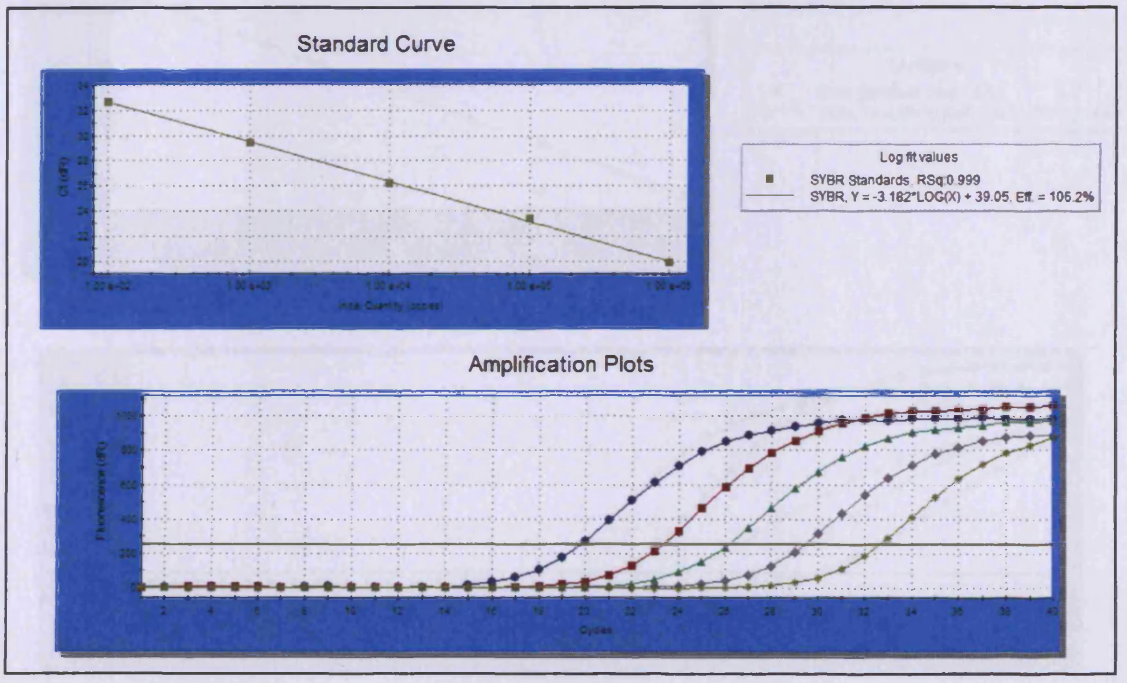


Figure 2.7C: SYBR Green® QPCR Standard Curve & Amplification Plots for Human CB_2

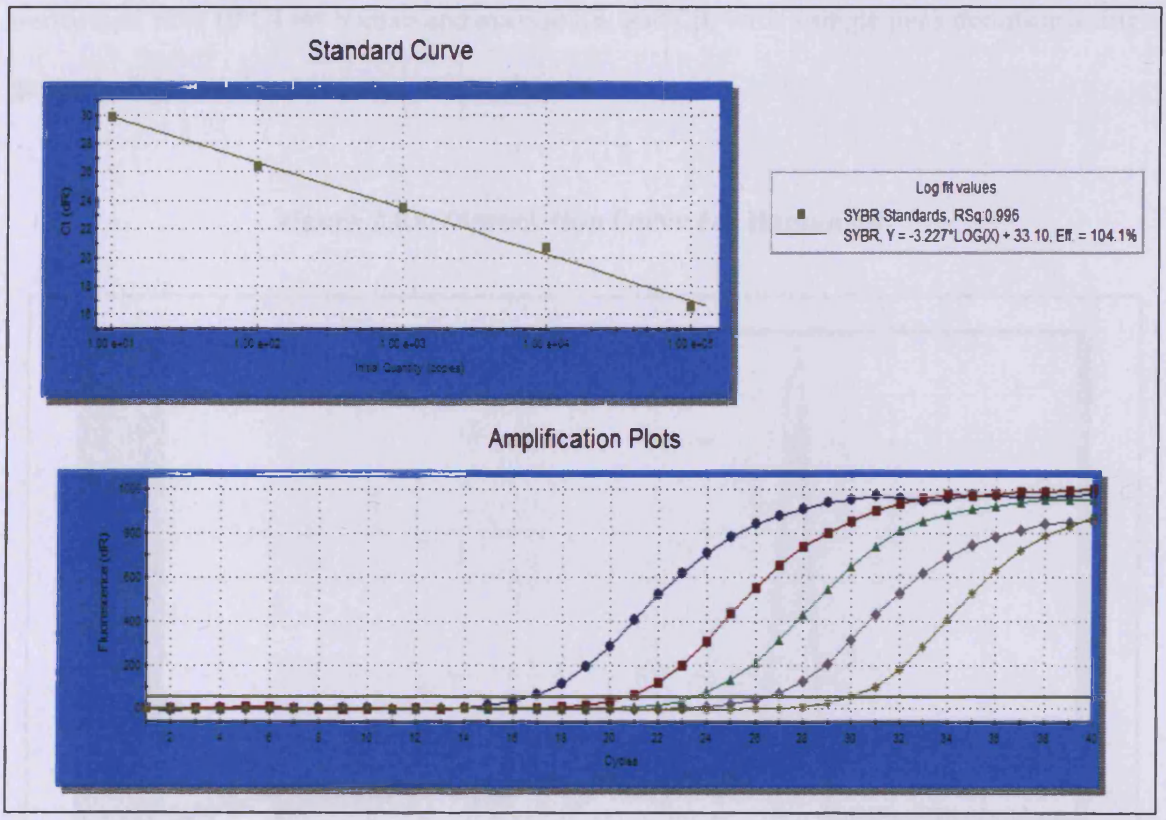
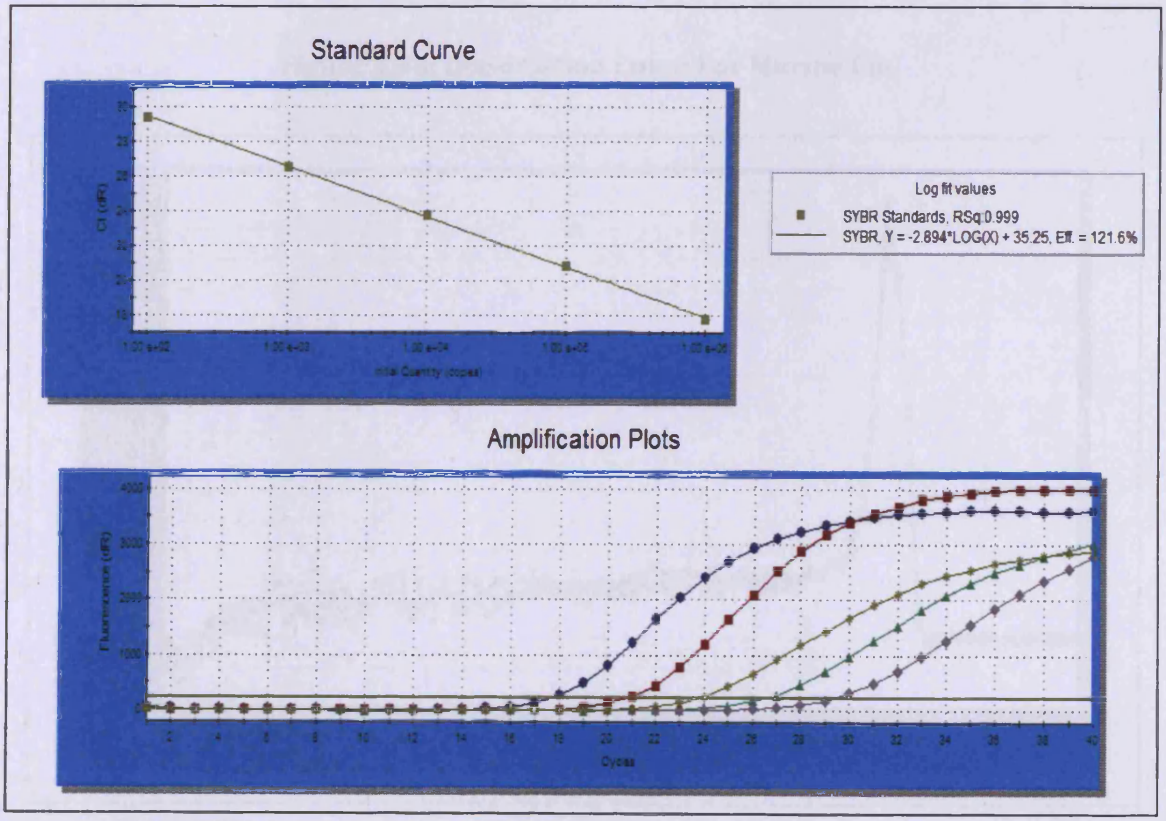


Figure 2.7D: SYBR Green® QPCR Standard Curve & Amplification Plots for Murine CB_2



Figures 2.8A-D illustrate the dissociation curves produced during melting point analysis performed after QPCR for human and murine CB_1 and CB_2 with a single peak denoting a single gene product and the absence of primer dimers.

Figure 2.8A: Dissociation Curve For Human CB_1

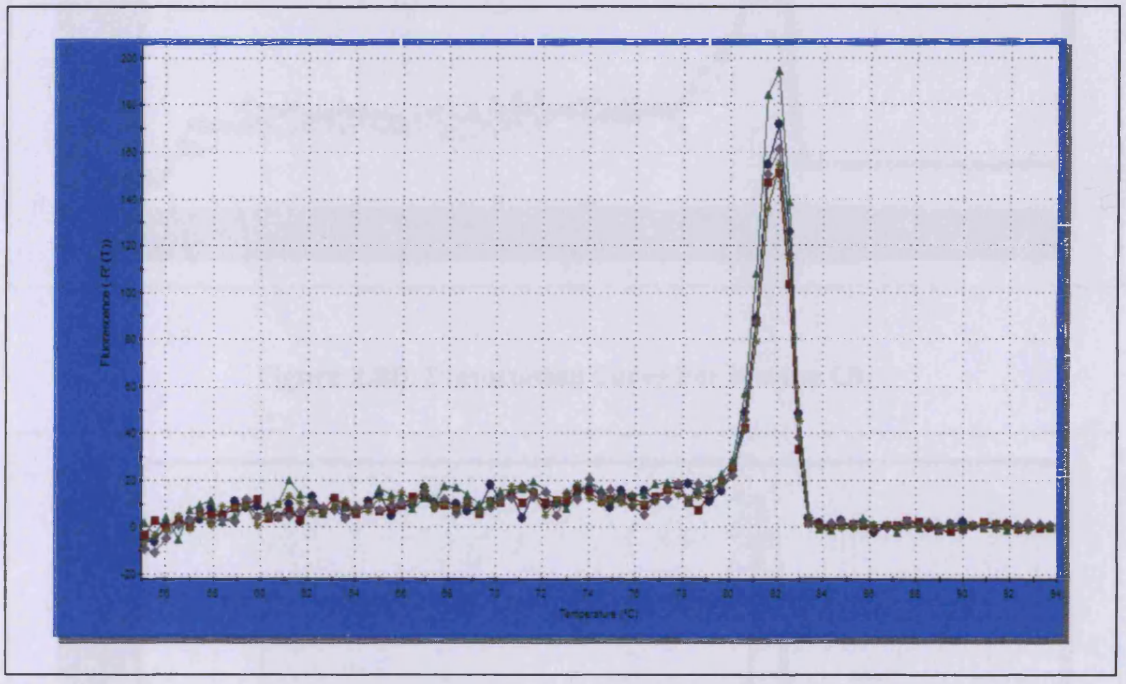


Figure 2.8B: Dissociation Curve For Murine CB_1

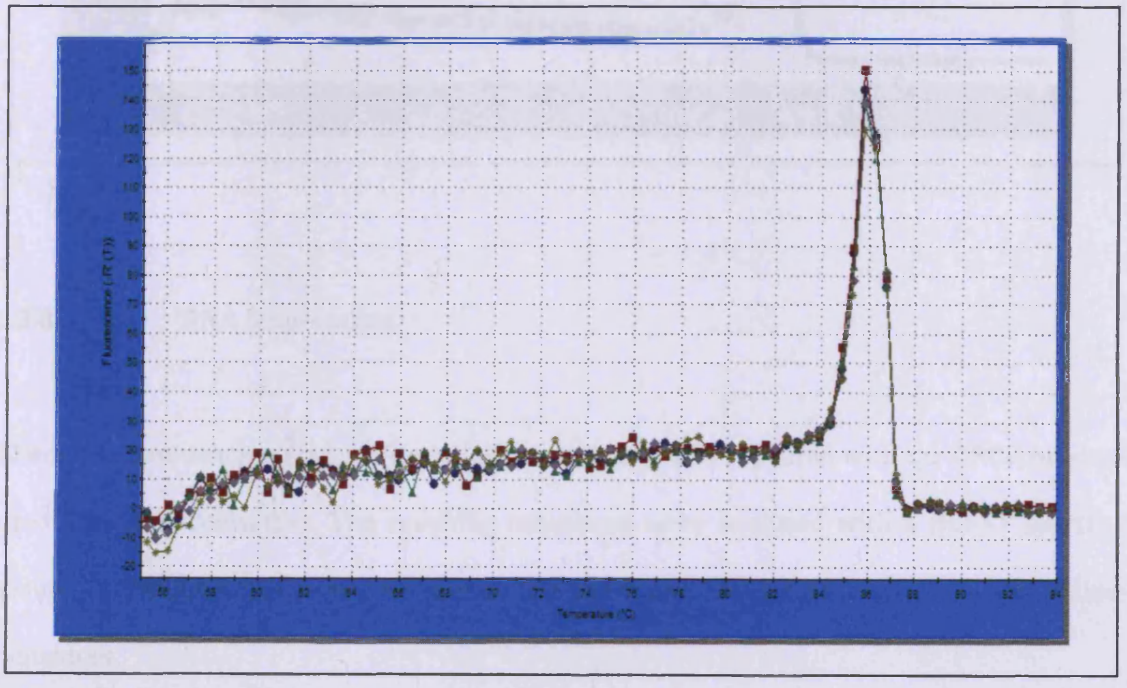


Figure 2.8C: Dissociation Curve For Human CB_2

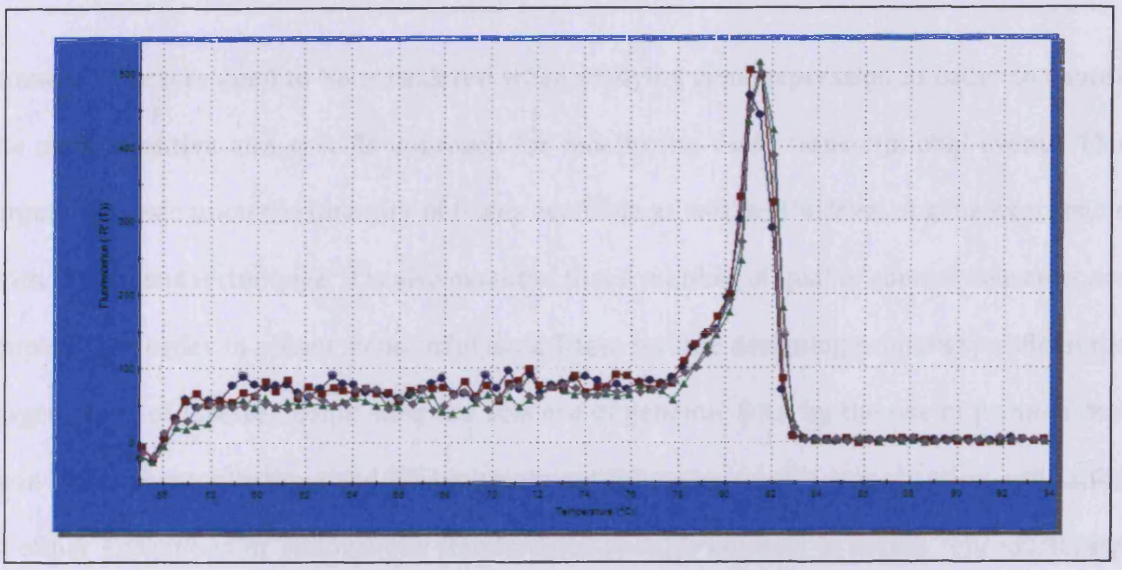
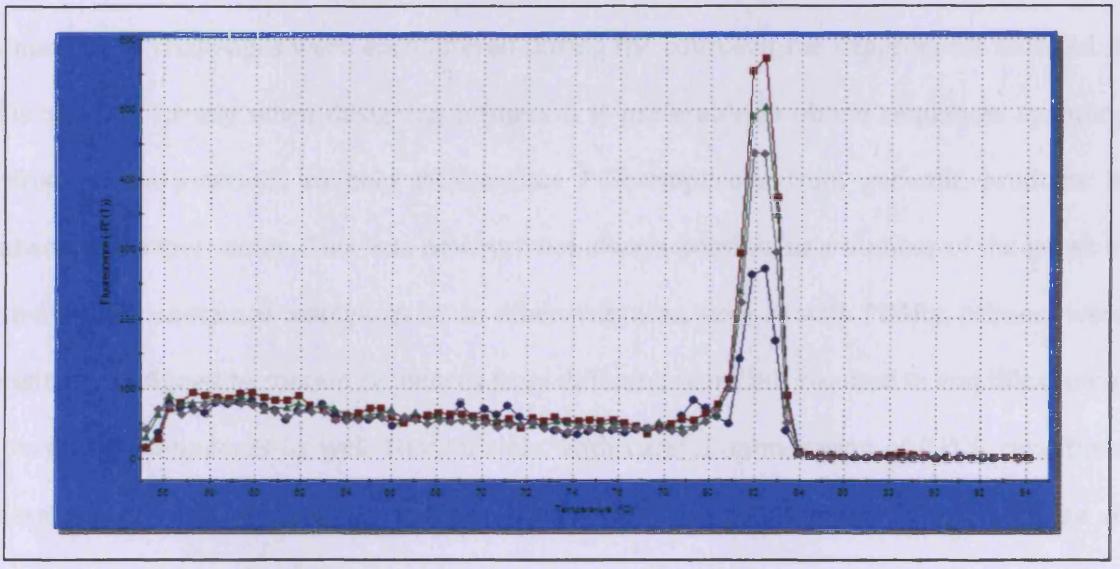


Figure 2.8D: Dissociation Curve For Murine CB_2



2.3.8 DNA Sequencing

Direct DNA sequencing was performed after mini-prep and repeated with the QPCR products after primer optimisation. The resulting sequences were analysed with a BLAST search to ensure correspondence with the genes of interest and alignment with the initial primer sequences.

2.4 DISCUSSION

Numerous factors need to be considered when studying gene expression in order to choose the most sensitive and specific approach for monitoring these transcriptional events. This largely depends upon the quantity of tissue available as well as the level of gene expression. With PCR-based techniques, it is also essential that a number of quality control measures are employed in order to obtain meaningful data. These include designing primers specific to the target genes of interest, confirming the absence of genomic DNA by the use of primers that span intron/exon junctions and DNAse treatment following reverse transcription, utilisation of either exogenous or endogenous standards or positive controls to assess reproducibility, and the use of negative controls to verify the absence of contamination.

A number of challenges were encountered during the course of the experiments outlined in this chapter. Ideally when designing primers it is preferable to obtain sequences spanning intron/extron junctions to help differentiate PCR amplicons from genomic products in subsequent experiments. This was however not always possible as a number of the genes of interest only contained a single exon. In other instances, such as with PPARγ, primers were originally designed to contain sequences from different exons but resulted in amplification of non-specific sequences as well. Nevertheless, with careful optimisation of QPCR conditions there are benefits of using absolute quantification. The results obtained for the gene of interest can be compared to transcript expression of housekeeper genes and can thus take into account efficiency of the reverse transcription step, DNAse treatment and QPCR amplification itself. This does require the use of housekeeper genes whose expression is constant in the samples to be studied, and this can be quite a challenge when inducing differentiation.

QPCR with SYBR Green® is a sensitive and reproducible method for quantification of transcript expression during adipogenesis. There are, however, limitations with this

technique as well. Even higher sensitivity for detection of transcript numbers can be achieved with the use of capillary tubes within a Light Cycler heated with hot air instead of QPCR using 96-well plates heated on thermal blocks but the number of samples which can be run simultaneously is reduced. Another shortcoming with QPCR is that detection of transcripts does not necessarily correlate with the translated functional protein being present in the cell. Therefore alternative ways of verifying these results should also be employed. For example, in the pre-adipocyte differentiation experiments undertaken in the following chapters adipogenesis is confirmed not only by measurement of mature adipocyte-specific transcript expression, but also by morphological appearance on Oil red O staining and ELISA measurement of adiponectin. Alternative methods, such as the use of hydrolysis probes or cDNA arrays can provide simultaneous information about multiple genes in a large number of samples but can be more complex to design and are accompanied by increased expense.

This chapter has described the techniques used to clone purified PCR products from human and murine genomic DNA and optimise QPCR conditions, eventually resulting in a series of standard curves for measurement of CB_1 and CB_2 receptor expression as well as markers of differentiation in both species. This method was subsequently applied to quantify transcript expression in response to CB receptor modulation during *in vitro* adipogenesis using cell lines and human primary cultures as detailed in the following chapters.

CHAPTER 3: EFFECT OF CANNABINOID RECEPTOR MODULATION ON PROLIFERATION AND DIFFERENTIATION IN PRE-ADIPOCYTE CELL LINES.

3.1 INTRODUCTION

As previously discussed in Chapter 1, excess adipose tissue is associated with clinical features of insulin resistance and the metabolic syndrome, therefore the adipocyte represents a major molecular target for novel pharmacotherapies aiming to reduce this burden associated with long-term risk of diabetes and CVD. Whereas adipose tissue was initially considered to be a passive connective tissue store of excess energy in the form of triglycerides, it is now also recognised as an endocrine organ capable of metabolic signalling [148]. This was first highlighted in the mid-1980s when research published by the Spiegelman and Flier laboratories demonstrated that adipocytes are an abundant source of a circulating serine protease-adipsin [149]. Since this time numerous other adipocyte secretory proteins, known as the adipocytokines, have been described including Acrp30 or adiponectin [150, 151], TNFα [152], leptin [153], resistin [154], and visfatin [155]. These have been shown to play key roles in energy homeostasis, regulation of appetite and satiety, reproduction, and insulin sensitivity as well as influencing vascular and immunological functions and have been discussed further in Chapter 1 [148].

3.1.1 Cellular Composition Of Adipose Tissue

The predominant cell-type found in adipose tissue is the adipocyte though numerous additional cells are also present, collectively known as the 'stromal vascular cells' which include pluripotent stem cells and pre-adipocytes both of which possess the ability for

adipogenesis, as well as endothelial cells, pericytes and macrophages all of which help support normal adipose tissue function [156].

3.1.2 Development Of Adipose Tissue

The embryonic development of adipose tissue begins in the second trimester when mesenchymal cells with adipogenic potential form clusters at various sites in close proximity to vascular structures, predominantly at the neck, shoulder and pelvis as well as surrounding essential organs including the heart and kidneys [157, 158]. Adipose tissue has been shown to secrete specific growth factors including transforming growth factor-β (TGF-β) which modulate angiogenesis, suggesting a developmental link with adipogenesis [157]. Adipose tissue is present from birth and retains the potential for growth at all stages of life dependent upon the site of the fat depot, the nature of the diet, environmental and genetic factors. Adipose tissue growth occurs by both ongoing lipid accumulation in existing mature adipocytes resulting in cells varying from 25 to 200 μm in diameter, but also by further proliferation and differentiation of pre-adipocytes present within the adipose store [159].

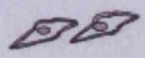
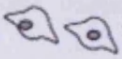
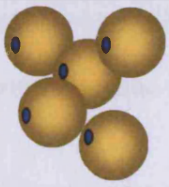
3.1.3 Cellular Development & Transcript Expression During Adipogenesis

The sequence of events occurring during adipogenesis has been studied at length in *in vitro* models and appears to follow a well recognised temporal sequence illustrated in Table 3.1 [160]. Adipocytes are derived from multipotent mesenchymal stem cells which have multiple differentiation capabilities including adipogenesis, osteogenesis, chondrogenesis and myogenesis. Therefore the early stages in adipogenesis involves determination and commitment to the adipocyte lineage by conversion of the multipotent stem cell to a unipotent adipoblast and then a pre-adipocyte [148]. Though morphologically similar to its precursors this cell acquires the machinery that is necessary for lipid transport and synthesis, insulin sensitivity and the secretion of adipocyte-specific proteins [161]. The molecular

regulation of these early events is not yet clearly understood but the latter stages have been more extensively characterised.

Table 3.1: Cellular Development And Transcript Expression In Adipogenesis

(adapted from Schaffler et al [148] & Ailhaud et al [157])

Stage			Cell	Gene expression profile
I	Determination		Mesenchymal stem cell (multipotential)	-
II	Cell commitment		Adipoblast (unipotential)	-
III	Mitosis & Clonal expansion		Pre-adipocyte I (no lipid)	**EARLY** Pref-1 Krox 20
IV			Pre-adipocyte II (lipid-containing)	**INTERMEDIATE** C/EBPs PPARγ
V	Growth arrest		Immature adipocyte (lipid-containing, multilocular)	**LATE** Lipolytic & lipogenic enzymes e.g. LPL, FAS & GPDH
VI	Terminal differentiation		Mature adipocyte (lipid-filled, unilocular)	**VERY LATE** Adiponectin Leptin Resistin Visfatin

During *in vitro* adipogenesis using murine models, pre-adipocytes undergo a proliferative phase and clonal expansion eventually followed by growth arrest at the G_1/S phase of the cell cycle secondary to cell-to-cell contact inhibition, though this step is not essential in primary cultures [162]. This process is associated with the downregulation of early differentiation

markers including Pref-1, a known repressor of adipogenesis [163]. The subsequent stages of adipocyte maturation are associated with a cascade of expression of intermediate adipogenic factors including CCAAT-enhancer-binding proteins (C/EBP α, β & δ) and peroxisome proliferator-activated receptor-γ (PPARγ) [161]. C/EBPβ & C/EBPδ are amongst the earliest seen and are implicated in the induction of adipogenesis by inducing PPARγ expression through C/EBP binding sites in the PPARγ promoter [158, 164]. PPARγ has been described as the 'master regulator' of adipogenesis and is essential for both adipogenesis itself and maintenance of the differentiated state [165]. PPARγ is the most conserved member of the PPAR family and controls gene expression through heterodimerisation with the retinoic X receptor (RXR). There are 3 PPARγ isoforms derived from the same gene through the use of alternate promoters [166, 167]. PPARγ-1 and PPARγ-3 encode for the same protein and are found in numerous cell types including adipose tissue, macrophages and colonic epithelium whereas PPARγ-2 is adipose tissue-specific though the precise roles of these differing isoforms of PPARγ are yet to be established [166, 167]. Numerous studies have demonstrated the critical role for PPARγ in pre-adipocyte differentiation. For instance *in vitro* experiments by Tontonoz et al have shown that PPARγ overexpression in isolation is sufficient to induce adipogenesis [168]. Conversely PPARγ knockout mice exhibit reduced adipose tissue mass while dominant-negative mutations in the human PPARγ gene have been described in subjects with partial lipodystrophy [169-172]. *In vivo* studies in murine models have demonstrated inducible knockout of PPARγ to be associated with adipocyte death [160, 173], whereas PPARγ over-expression alone can induce adipogenesis [168].

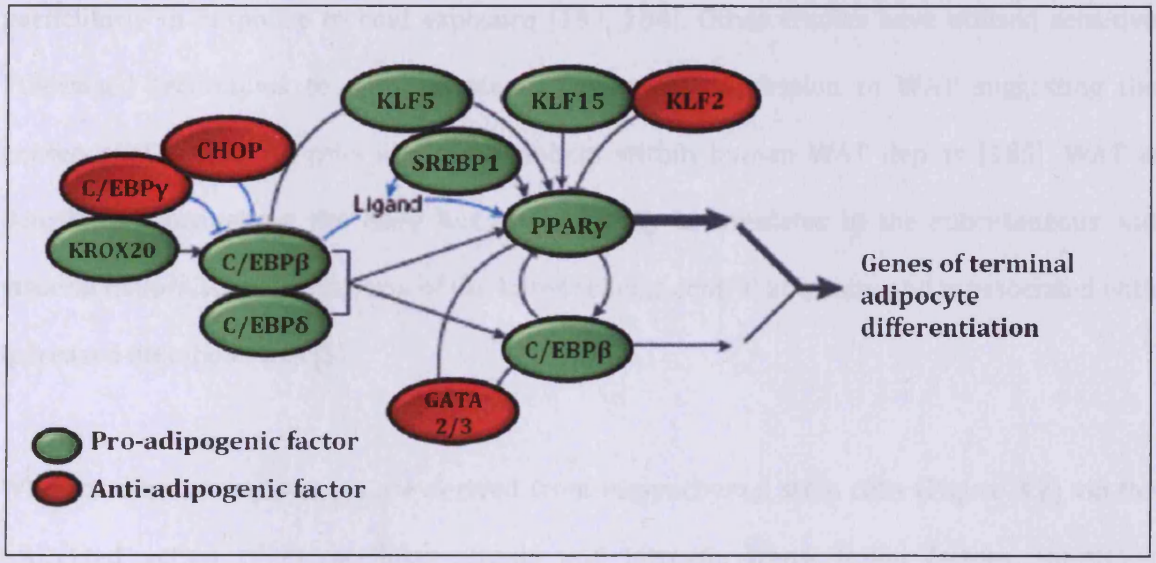
The transcriptional cascade regulating adipogenesis is centred around PPARγ itself whose expression is mediated by several pro-adipogenic and anti-adipogenic factors as illustrated in Figure 3.1 [161]. These include the Krupple-like factors (KLF) which can both promote adipocyte differentiation (KLF 5 & 15) and enhance glucose transport mediated through increased expression, and translocation to the cell membrane, of glucose transporter 4 (GLUT4) [174, 175], as well as inhibit the process by inhibiting PPARγ expression (KLF2)

[176]. PPARγ expression, and thus adipogenesis, is also enhanced indirectly by Krox20, a C/EBPβ promoter [177], as well as directly by sterol response element binding protein 1c (SREBP1c) [178]. Conversely, adipogenesis can be inhibited by reduced C/EBP expression by the transcription factor homologous to C/EBP (CHOP) [161], as well as PPARγ promoter inhibition by the glutamyl amino transferidase subunit A transcription factors (GATA) [179] .

The terminal stages of adipocyte maturation are associated with expression of enzymes required for adipocyte function including lipoprotein lipase (LPL), fatty acid synthase (FAS) and glycerol-3-phosphate dehydrogenase (GPDH) and eventual adipocytokine production [148, 157].

Figure 3.1: Transcriptional Regulation Of Adipogenesis

(adapted from Rosen et al [161])



3.1.4 White & Brown Adipose Tissue

Two types of adipocytes are found in mammals: the white and brown adipocyte. Whereas white adipose tissue (WAT) serves as an energy reservoir, the function of brown adipose tissue (BAT) is to dissipate energy in the form of heat by non-shivering thermogenesis [180].

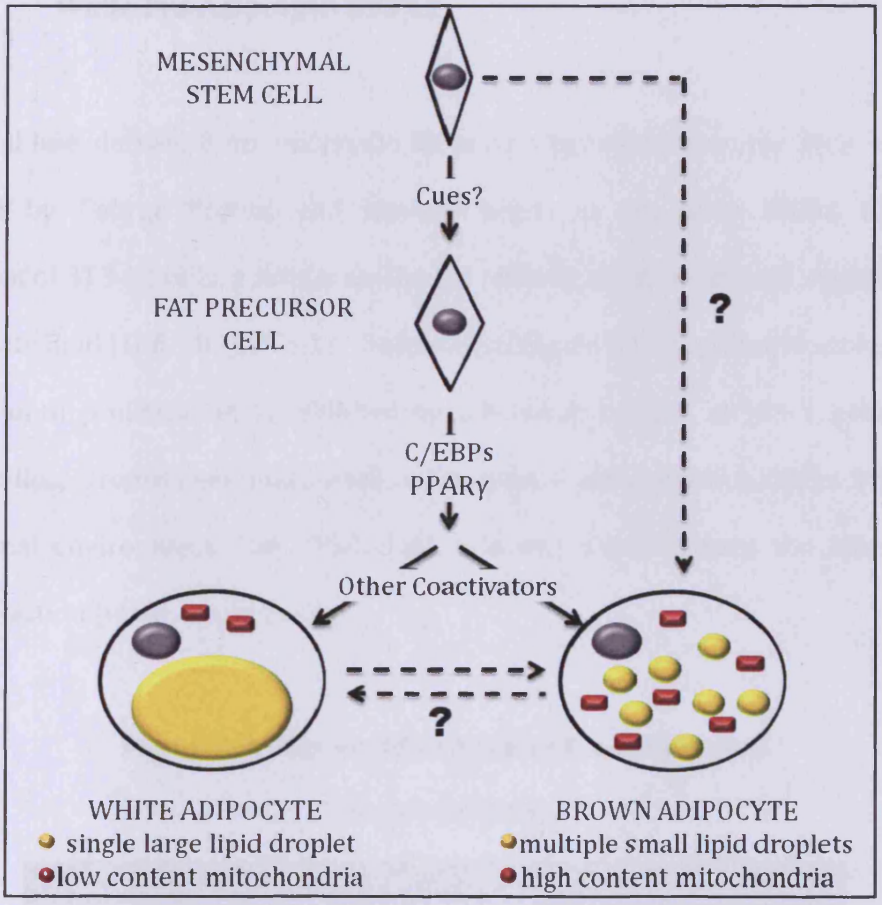
Brown adipocytes store less lipid and contain more mitochondria than white adipocytes, and although brown adipocytes have virtually the same enzymatic machinery as white fat cells, the accumulated lipid in BAT is predominantly used as a source of fatty acids for oxidisation in these mitochondria, as opposed to acting as a systemic energy storage depot [158] . Brown adipocytes have a similar gene expression profile to white adipocytes but also express some distinct genes, including the uncoupling protein-1 (UCP-1) which allows energy conversion to heat [161]. Whereas WAT is present in humans throughout life, until recently BAT was not thought to be found beyond the neonatal period where its location is limited to surrounding the great vessels in the thoracic cavity and interscapular regions [158]. Recent studies using 2-[^{18}F]fluoro-2-deoxy-glucose (taken up by members of the glucose transporter family e.g. GLUT4) positron emission tomography (FDG PET), initially to trace tumour metastases, have identified BAT deposits in adult humans between the anterior neck and thorax [181, 182]. Further studies have also demonstrated that these areas of BAT are metabolically active, particularly in response to cold exposure [183, 184]. Other studies have utilised sensitive PCR-based techniques to demonstrate UCP-1 mRNA expression in WAT suggesting the presence of brown fat cells in small numbers within human WAT depots [185]. WAT is distributed throughout the body but preferentially accumulates in the subcutaneous and visceral depots. An accumulation of the latter reflects central adiposity and is associated with increased metabolic risk [3].

White and brown adipocytes are derived from mesenchymal stem cells (Figure 3.2) via the concerted action of extracellular signals and intrinsic transcription factors. Questions surround several issues in BAT development, including the identity of specific factors that promote brown fat adipogenesis, and whether BAT and WAT share common fat precursor cells or derive separately from stem cells via brown- and white-specific pre-adipocytes [158]. The mechanism by which these stem cells become committed to either white or brown adipocyte differentiation, and the extent to which white and brown adipocytes can be

transdifferentiated to one another, has not yet been fully elucidated, but represents a potential physiological and pharmological target for anti-obesity interventions.

Figure 3.2: White & Brown Fat Development From The Mesenchymal Stem Cell

(adapted from Rosen et al [158])



3.1.5 Cell Lines

The diffuse nature of adipose tissue differentiation limits the potential to use *in vivo* and *ex vivo* analyses to study adipogenesis, therefore a series of adipoblast cell lines have been developed that differentiate into adipocytes when treated with appropriate hormonal inducers, and thus allow *in vitro* studies to take place [160]. One of the most commonly used cell lines that readily differentiates into WAT is 3T3-L1 which has been used in the present

study [186], but others have also been developed including 3T3-F442A and Ob 17 [187]. A BAT model was also studied using a cell line derived from simian virus transformation of human brown pre-adipocytes known as PAZ6 [188], though the physiological significance of BAT in adult humans remains controversial.

3.1.5.1 White Pre-Adipocyte: 3T3-L1

The 3T3 cell line, derived from embryonic fibroblasts harvested from the Swiss mouse, was established by George Todaro and Howard Green in the early 1960s, followed by development of 3T3-L1 cells, a similar and highly resilient cell line with an exaggerated ability to accumulate lipid [186, 189]. 3T3-L1, illustrated in Figure 3.3, are grown in standard culture conditions until proliferation is inhibited by cell-to-cell contact, at which point the cells accumulate lipid droplets and mature into adipocytes, a process which can be influenced by the hormonal environment. The 3T3-L1 cell line was obtained from the American Type Culture Collection (www.atcc.org).

Figure 3.3: Murine 3T3-L1 White Pre-Adipocytes

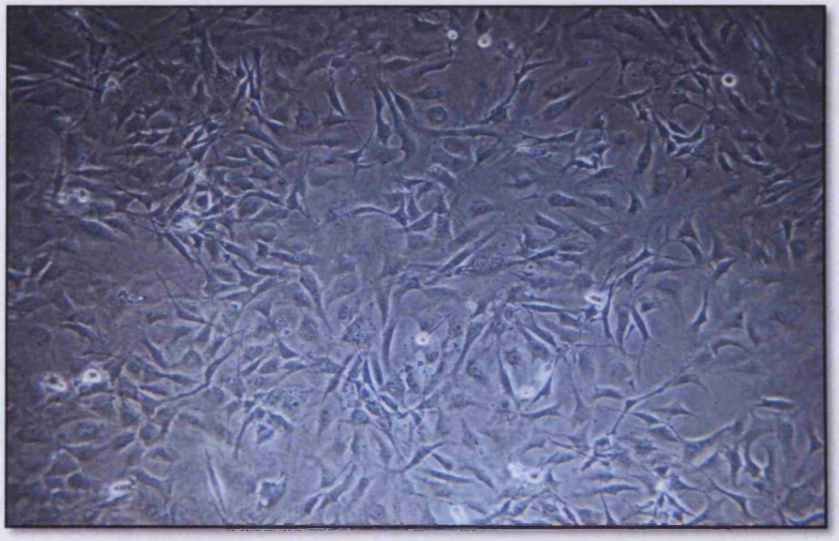
(magnification 200x)

3.1.5.2 Brown Pre-Adipocyte: PAZ6

The PAZ6 cell line was kindly provided by Vladimir Zilberfarb (Université Paris Descartes) and is illustrated in Figure 3.4.

Figure 3.4: Human PAZ6 Brown Pre-Adipocytes

(magnification 200x)



The PAZ6 cell line was established by transfection of stromal cells derived from human infant BAT with simian virus-40 antigen under control of the vimentin promoter and, in the appropriate hormonal environment, differentiates into mature BAT as confirmed by morphological changes of lipid droplet accumulation and QPCR detection of LPL (mature adipocyte marker) and UCP-1 (mature brown adipocyte marker) expression [188].

3.1.6 Hormonal Induction Of Adipogenesis

Over the years a number of agents have been empirically selected to induce *in vitro* adipogenesis and this evidence has been used to establish a differentiation protocol within our lab at the Centre for Endocrine and Diabetes Sciences at Cardiff University. One of the

earliest observations for efficient adipocyte differentiation has been the requirement for insulin to increase both the number of cells recruited for differentiation as well as enhance lipid accumulation [190]. However pre-adipocytes express few insulin receptors and the insulin effect has been shown to be mediated by cross-activation of the insulin-like growth factor-1 (IGF-1) receptor [191]. Further supplementation of the differentiation medium with fetal calf serum rich in IGF-1 therefore also promotes adipogenesis [192]. The glucocorticoid dexamethasone has also been used to inhibit pre-adipocyte proliferation *in vitro* and induces differentiation by inhibiting expression of Pref-1 and inducing C/EBPδ [164, 193, 194]. Given the crucial role of PPARγ in the regulation of adipogenesis, it is unsurprising that PPARγ agonists such as the thiazolidinedione pioglitazone, have also been used to stimulate differentiation [195].

Another compound known to induce adipogenesis is 3-isobutyl-1-methylxanthine (IBMX), a compound thought to act by increasing intracellular cAMP as well as C/EBPβ expression [196]. Numerous other hormones have also been used as components of *in vitro* differentiation protocols, though their roles *in vivo* may not be as well established. These include the addition of growth hormone [197], thyroid hormone [198], retinoic acid [199], and prostacyclin [200].

3.1.7 *In Vitro* Evaluation Of Pre-Adipocyte Proliferation

As previously discussed, adipose tissue accumulates by both pre-adipocyte proliferation and differentiation, as well as mature adipocyte hyperplasia. Therefore a study of both these processes is required when investigating modulators of adipose tissue. The first of these, cell proliferation, represents a change in cell numbers arising from cell division or mitosis. Stimulators of cell proliferation will increase the rate at which cells divide, whereas inhibitors will cause either growth arrest or a reduction in the rate of cell division. Therefore the effect

any compound may exert on cellular proliferation can be investigated by determining absolute cell numbers before and after a treatment period.

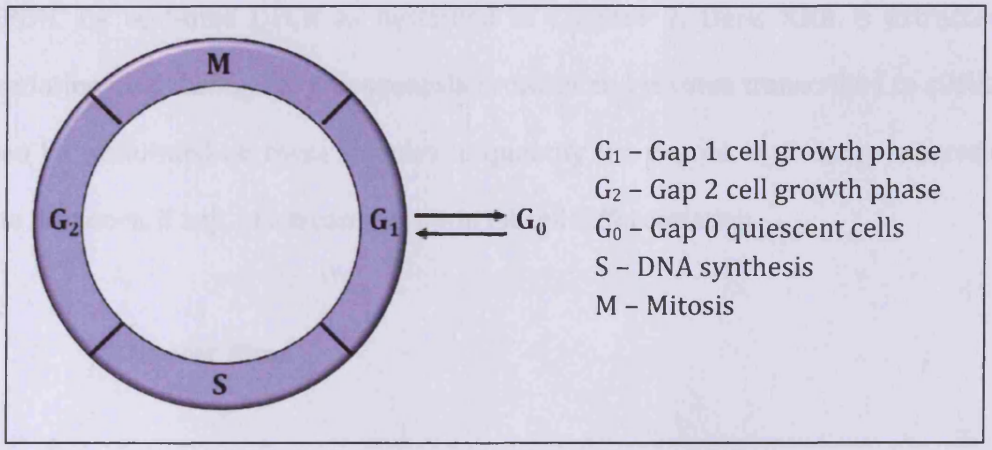
Several techniques are recognised as validated methods of assessing proliferation. The first and simplest method is direct visual counting with the use of instruments such as a haemocytometer to estimate the number of cells per volume of culture suspension. However such methods are limited by both the laborious nature and potential for observer bias, and have thus been superseded by the development of electric particle counters [201]. Other techniques include radioactive isotope labelling of DNA in dividing cells such as the ^{3}H-thymidine incorporation assay, where radioisotope accumulation in samples undergoing increased proliferation can be measured by scintillography [202].

Where a reduction in cell proliferation is observed in response to treatment exposure, it is important to exclude cell toxicity as a cause of the decrease in cell numbers. This was performed in the present studies by staining with trypan blue, a dye taken up by cells where membrane integrity has been lost due to cell necrosis or apoptosis, and by analysis of the cell cycle.

3.1.7.1 Cell Cycle Analysis

The cell-division cycle refers to the series of events leading to cell division and thus proliferation. The cell cycle can be divided into four phases as illustrated in Figure 3.5, the first three of which are collectively known as the interphase: Gap 1 (G_1) phase for cell growth, DNA synthesis phase (S), Gap 2 phase for further cell growth (G_2), and mitosis (M) [203]. Cells can either permanently or temporarily exit the cycle at G1 and become quiescent in a Gap 0 (G_0) phase, and it is at this point that adipogenesis can occur in pre-adipocytes. Checkpoints also exist at G_1/S and G_2/M to block cell division if adverse environmental factors are present or if cell repair is required [204].

Figure 3.5: Stages Of The Cell Cycle



The amount of DNA present in a cell varies according to the stage of the cell cycle. Therefore, if cells undergoing investigation are fixed in ethanol and stained with a DNA stain such as propidium bromide, the amount of DNA present in any cellular sample can be quantified. Plotting this value against the known cell number can provide an indication of the distribution of cells throughout the cell cycle in a given sample, and thus enable the effect of compounds on cell proliferation and the cell cycle to be studied.

3.1.8 *In Vitro* Assessment Of Adipogenesis

Numerous techniques have been established for assessment of adipogenesis. This can be performed by simple direct inspection under light microscopy to observe for morphological features of adipocyte maturation, predominantly a rounding up of cell shape and the acquisition of lipid droplets, coupled with a count of the number of foci of differentiation per high powered microscopic field. This method is again limited by observer bias thus the more robust and objective methods outlined below have been employed in this study.

Oil red O staining can be used to quantify the amount of lipid accumulation within a sample by firstly applying the stain, then extracting the absorbed stain in 100% isopropanol and measurement using colourimetry [205]. Another more specific technique is the measurement

of the expression of transcript specific to the target cell, e.g. terminal adipocyte markers LPL and GPDH, by real-time QPCR as described in Chapter 2. Here, RNA is extracted from differentiating cells during the adipogenesis protocol and reverse transcribed to cDNA. QPCR can then be performed on these samples to quantify the degree of receptor expression and thus the influence, if any, of a treatment upon fat cell differentiation.

3.1.9 Chapter Aims

The ECS plays an important role in regulating weight and energy balance in humans and this system is activated to a greater extent in obese individuals. In animal studies, the administration of exogenous and endogenous cannabinoids is associated with robust increases in food intake and can promote weight gain, an effect believed to be mediated through activation of the CB_1 receptor. Conversely, animal studies of CB_1 blockade have demonstrated reductions in food intake and weight loss with repeated compound administration. These reductions in body weight appear to be greater in obese animals and may be the result of a dual effect on both food intake and metabolic processes. Clinical trials of SR141716 treatment in humans have also demonstrated significant reductions in body weight, waist circumference and improvement of lipid and glucose metabolism. Accordingly, the ECS represents an important pharmacological target for modulation of adipose tissue biology.

This chapter aims to study an *in vitro* model of pharmacological modulation of a key component of the metabolic syndrome – adipose tissue. To this end the actions of CB_1 and CB_2 receptors on proliferation and adipogenesis will be studied using *in vitro* models of both WAT and BAT.

3.2 MATERIALS AND METHODS

3.2.1 Cell Populations

The murine white preadipocyte cell line, 3T3-L1, was readily available in the department and the human brown preadipocyte cell line, PAZ6, was kindly provided by Vladimir Zilberfarb (Université Paris Descartes).

3.2.2 Reagents

CB receptor modulators (Table 3.2) were purchased from Tocris Bioscience (Bristol, UK). All reagents were initially reconstituted in ethanol, and further dissolved in culture medium to produce stock solutions of 10^{-2} molar. The maximum concentration of ethanol in any experiment was 0.1% and this concentration was maintained in all control samples. All other culture medium constituents were obtained from BioWhittaker (Belgium) unless otherwise stated.

Table 3.2: Summary Of CB Receptor Modulators [206]

Compound	Description	Binding affinity (Ki)
Anandamide	Endogenous CB_1 and CB_2 agonist	CB_1 – 89 nM CB_2 – 371 nM
ACEA	CB_1 agonist (>1400-fold CB_1 selectivity over CB_2)	1.4 nM
AM 251	CB_1 antagonist (306-fold CB_1 selectivity over CB_2)	7.49 nM
JWH 133	CB_2 agonist (200-fold CB2 selectivity over CB_1)	3.4 nM

3.2.3 Culture Media

Both the 3T3-L1 and PAZ6 cell lines were routinely cultured in complete medium (CM), the contents of which are listed in Table 3.3.

Table 3.3: Constituents Of Cell Culture Media

Reagent	3T3-L1	PAZ6
Dulbecco's modified eagle medium (DMEM)	80 mls	82 mls
Ham's F12	80 mls	82 mls
Fetal calf serum (FCS)	10%	8%
Sodium pyruvate	2 mls	2 mls
1.5% Sodium bicarbonate	3 mls	n/a
Hepes buffer	n/a	3mls
Penicillin (Cambrex, Berkshire, UK)	100 u/ml	100 u/ml
Streptomycin (Cambrex, Berkshire, UK)	100 µg/ml	100 µg/ml

3.2.4 Routine Maintenance Of Cell Lines

All cell culture experiments were performed in sterile conditions at 37°C in an atmosphere containing humidified 5% CO_2 in air. Both 3T3-L1 and PAZ6 preadipocytes were grown in 75 cm^2 Nunclon™ delta surface flasks (Nunc, Roskilde, Denmark). Culture medium was changed every 48 hours and upon reaching approximately 80% confluence, the cells underwent passage. This was done by aspirating the culture medium and washing the cellular monolayer with 2 mls of 1% w/v trypsin (Cambrex, Berkshire, UK) pre-heated to 37°C. The trypsin was immediately aspirated and the cells were subsequently incubated with a further 2 mls of 1% w/v trypsin until the cells had detached from the flask and were free in suspension, as

confirmed by microscopy. A further 8 mls of culture medium was added to the solution to inactivate the trypsin and the total 10 mls solution was transferred to a universal container and centrifuged at 1000 rpm for 5 minutes. The resultant cell pellet was resuspended in culture medium and transferred either to fresh flasks or plated for experiments.

3.2.5 Cryopreservation Of Cells

At every passage a proportion of cells were used to restock the cell bank. Cells were trypsinised as described above and the cell pellet obtained after centrifugation was resuspended in 0.5 ml of culture medium combined with 0.5 ml of freezing mix (FCS containing 20% dimethyl sulfoxide (DMSO), Sigma-Aldrich Company Ltd, Dorset, UK), and transferred to a 2 ml sterile cryotube. The cryotubes were placed within an insulated polystyrene freezing box containing isopropanol (Fisher Scientific UK Ltd, Leicestershire, UK) to enable a slow cooling rate of 1°C / minute and kept at -80°C for 24 hours prior to long-term storage in liquid nitrogen at -190°C.

3.2.6 Proliferation Studies

3.2.6.1 Cell Culture Protocol

Both 3T3-L1 and PAZ6 preadipocytes were grown to confluence and trypsinised as described previously. The approximate number of cells per ml solution was calculated with the aid of a haemocytometer (Camber counter, Weber Scientific International Ltd, Middlesex, UK). Cells were subsequently diluted and seeded at $1x10^3$ per well in 24 well plates (Nunclon™ delta surface, Nunc, Roskilde, Denmark) and incubated at 37°C overnight to enable optimal attachment. Cells were then incubated in culture medium alone, or supplemented with increasing concentrations of Anandamide (10^{-9} to 10^{-5} molar), ACEA (10^{-9} to 10^{-5} molar),

AM251 (10^{-9} to 10^{-5} molar), or JWH133 (10^{-9} to 10^{-5} molar). Each experiment was completed in quadruplicate and performed at least twice.

3.2.6.2 Direct Cell Counting

The pre-adipocytes were collected for direct cell counting after 24 and 72 hours of treatment. Each well was washed with 0.5 mls of 1% w/v trypsin and then incubated with a further 0.5 mls of 1% w/v trypsin until cells were free in suspension. The contents of individual wells were transferred to corresponding cell counting cuvettes containing 10 mls each of Coulter Isoton II Diluent (Beckman Coulter, Gmbh, Germany). Cell densities were measured using a Coulter particle counter (Z2, Beckman Coulter Gmbh, Germany) with particle size set between 3.8 nm and 9.0nm. Each sample was counted twice and the average value used for statistical analysis.

3.2.6.3 Trypan Blue Toxicity Assay

3T3-L1 and PAZ6 pre-adipocytes were seeded at $1x10^3$ per well in 24 well plates as described previously and incubated for 72 hours in culture medium (CM) alone, or supplemented with increasing concentrations of AM251 (10^{-9} to 10^{-5} molar). Cells were then trypsinised and the resultant cell suspensions were centrifuged at 1000 rpm for 5 minutes and resuspended in 50 µl CM combined with 50 µl 0.1% Trypan blue (Sigma-Aldrich Company Ltd, Dorset, UK). The cells were examined for positive staining, indicating toxic injury, under a light microscope with the aid of a haemocytometer.

3.2.6.4 Cell Cycle Analysis / Flow Cytometry

3T3-L1 and PAZ6 pre-adipocytes were seeded at $1x10^4$ per well in 6 well plates as described previously and incubated for 24 hours in CM alone, followed by a further 48 hour incubation

in CM alone or supplemented with increasing concentrations of AM251 (10^{-8} to 10^{-6} molar). The cell contents of individual wells were trypsinised as described above, centrifuged at 1000 rpm for 5 minutes, fixed in 1 ml cold (4°C) 70% v/v ethanol and stored overnight at -20°C. The samples were again centrifuged at 1000 rpm for 10 minutes, ethanol aspirated and the cell pellet washed twice with PBS. Following this the samples were incubated for a further 20 minutes at 37°C with propidium iodide (50μg/mL in PBS) and RNAse A (50μg/mL) and subsequently analysed with a FACSCalibur flow cytometer. The FACSCalibur flow cytometer operation was kindly undertaken by Dr E. Wang, Department of Immunology, School of Medicine, Cardiff University.

3.2.7 Adipogenesis Studies

3.2.7.1 Cell Culture Protocol

3T3-L1 and PAZ6 cells were plated in 6 well plates and adipogenesis induced in confluent populations of pre-adipocytes by replacing CM with differentiation medium (DM) containing 5% FCS, 33 μM biotin (Sigma Aldrich), 17 μM panthothenate (Sigma Aldrich), 1 nM T3 (Sigma Aldrich), 100 nM dexamethasone (Sigma Aldrich), 1 μM thiazolidinedione (Takeda) and 500 nM insulin (Sigma Aldrich). Cells were maintained in DM alone, or supplemented with 100 nM ACEA or 100 nM AM251, for 12 days with DM changes occurring on days 3, 5, 7, 9 and 12. Morphological changes of differentiation were monitored by microscopic examination.

3.2.7.2 Isolation Of Total RNA From 3T3-L1 & PAZ6

Samples for RNA extraction were collected on days 0, 3, 5, 7, 9 and 12. Culture medium was removed from individual wells and cells washed 3 times with 1 ml PBS. Total RNA was isolated using Invitrogen's TRIzol reagent according to the manufacturer's instructions. Cells were lysed by adding 1 ml TRIzol to each well pipetting the lysate several times and complete

cell lysis confirmed by inspection under a light microscope. The contents of individual wells were transferred to 1.5 ml tubes and stored at -80°C until further use.

To continue RNA isolation, 200 µl chloroform (Fisher) was added to each thawed 1.5 ml tube to separate RNA from DNA and protein. The samples were mixed vigorously and incubated at room temperature for 2 minutes prior to centrifugation at 12000 rpm (Mikro 22R, Heittich Zentrifugen, Germany) for 15 minutes at 4°C. The uppermost RNA-containing aqueous phase was carefully transferred to clean 1.5 ml tubes and the RNA precipitated by adding 0.5 ml isopropanol. The samples were again incubated at room temperature for 10 minutes and centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was removed, the remaining RNA pellet washed with 1 ml 75% v/v ethanol (Fisher) and centrifuged for a further 5 minutes at 4°C at 7500 rpm. The RNA pellet was air dried and redissolved in 20 µl nuclease-free water (Promega) by incubating at 60°C for 10 minutes.

The RNA concentration and quality was assessed by spectrophotometry (GeneQuant, GE Healthcare) and analysis on a 1.2% agarose gel. Samples were stored at -80°C until further use.

3.2.7.3 DNase Treatment Of RNA

To avoid contamination of subsequent experiments with residual traces of genomic DNA, the isolated RNA was treated with Promega's RNase-free DNase according to the manufacturer's instructions. 1 µg RNA, 1 µl DNase 10x buffer, 1 µl DNase were combined in a 200 µl microtube and made up to 10 µl with nuclease-free water. The mixture was briefly centrifuged and incubated for 30 minutes in a 37°C water-bath prior to terminating the reaction by adding 1 µl Stop Solution and transferring the microtubes to a heating block for 10 minutes at 65°C.

3.2.7.4 Reverse Transcription

RNA was reverse transcribed to complementary DNA (cDNA) for use in subsequent QPCR to measure transcript expression using the Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). Each reaction contained 4 µl 5x First-Strand buffer (Promega), 1 µl 40 U/µl RNase inhibitor (Promega), 4 µl 500 ng/µl oligo $(dT)_{15}$ (Promega), 4 µl 10 mM dNTPs mix (Sigma-Aldrich), 1 µg DNase-treated RNA pre-heated at 60°C for 10 minutes, 1 µl 20 U/ µl MMLV reverse transcriptase and made up to 20 µl with sterile water. The reaction mixture was centrifuged briefly and transferred to a Techne Genius DNA Thermal Cycler (Techne Cambridge Ltd, Cambridge, UK). The thermal cycling settings are listed in Table 3.4.

Table 3.4: Reverse Transcription Thermal Cycling Protocol

	Temperature	Time	№ of cycles
Reverse transcription	37°C	60 minutes	1
Enzyme inactivation	95°C	5 minutes	1

The resulting cDNA samples were stored at -20°C until further use.

3.2.7.5 Conventional PCR

Standard PCR was performed in a Techne Genius DNA Thermal Cycler (Techne Cambridge Ltd, Cambridge, UK) using primers for the housekeeping gene phosphoglycerate-1-kinase (PGK1). The forward and reverse primers are located on separate exons and can therefore be used to differentiate between PCR products amplified from reverse-transcribed cDNA (250 bp product) and contaminating genomic (650 bp product) when analysed on a 2% agarose gel. The PCR reaction constituents and thermocycling protocol are listed in Tables 3.5 and 3.6.

Table 3.5: PCR Reagents For Amplification From Genomic DNA

Reagents	Volume per reaction
Sterile H_2O	to complete final volume of 25 µl
10x Taq DNA polymerase buffer (100 mM Tris-HCl, 500 nM KCl, 15 mM $MgCl_2$)	2.5 µl
dNTP mix (10mM each dATP, dCTP, dGTP, dTTP)	1 µl
Forward primer (10 pmol/l)	1 µl
Reverse primer (10 pmol/l)	1 µl
Taq DNA polymerase (5 units/µl)	0.4 µl
Genomic DNA	100 ng
Total volume	25 µl

Table 3.6: PCR Thermal Cycling Protocol

	Temperature	Time	№ of cycles
Initial Denaturation	95°C	5 minutes	1
Denaturation	95°C	1 minute	40
Annealing	60°C	30 seconds	
DNA synthesis	72°C	1 minute	
Final extension	72°C	5 minutes	1

3.2.7.6 QPCR Measurement Of Markers Of Adipogenesis

Real-time QPCR was performed to measure expression of CB_1 and CB_2 receptors in all samples, murine markers of adipogenesis (Pref-1, C/EBPβ, PPARγ, GPDH) and house keeper genes (ARP) in 3T3-L1, and human markers of adipogenesis (C/EBPβ, PPARγ, LPL) and

housekeeper (APRT) in PAZ6 as well as UCP-1 in both cell lines. QPCR was performed using Stratagene's Brilliant® SYBR® Green Master Mix. Each 25 µl reaction contained 12.5 µl Master Mix, forward and reverse primer (as per optimised concentrations detailed in Chapter 2) and ultrapure water. Reactions were set up in Stratagene 96-well PCR plates and centrifuged for 1 minute at 3000 rpm prior to being transferred to the Stratagene MX3000P® thermocycler. The thermal cycling protocol is listed in Table 3.7.

Table 3.7: QPCR Thermal Cycling Protocol

	Temperature	Time	Nº of cycles
Enzyme activation	95°C	10 minutes	1
Denaturation	94°C	30 seconds	40
Annealing	60°C	60 seconds	
Extension	72°C	30 seconds	
Dissociation	95°C	60 seconds	1
	55°C	30 seconds	
	95°C	30 seconds	

3.2.7.7 Oil Red O Staining

Oil red O stains were performed to confirm the presence of lipid droplet accumulation and enable foci of differentiation to be counted. 3T3-L1 pre-adipocytes were seeded in 24 well plates and the differentiation protocol conducted as described above. On day 12, 0.5 g of oil red O was dissolved in 100 mls of isopropanol. 12 mls of this solution was combined with 8 mls of sterile water and filtered immediately. Each well was washed with 1 ml PBS, cells fixed with 0.5 ml 60% v/v isopropanol and washed again with 1 ml PBS. 300 µl filtered oil red O was then applied to each well for 15 minutes then aspirated. Each well was washed 3 times with 1 ml sterile water, examined under the light microscope and photographed for illustration. 200 µl of isopropanol was then added to each well and each individual well's

contents were then transferred to corresponding wells in a 96 well plate for analysis in an optical density reader (OpsysMR microplate reader, Dynex Technologies, Chantilly, USA) set at OD_{490}.

3.2.8 Statistical Analysis

Data were analysed using the SPSS (version 14.0) statistical software package.

The data generated by the proliferation and adipogenesis studies were not normally distributed (determined by generating Q-Q and P-P plots of data obtained) and therefore analysed according to median and interquartile ranges (IQR) using non-parametric statistical tests. The Friedman's test was performed on each experiment and the Wilcoxon Signed Ranks test was performed on individual treatments relative to control conditions. The QPCR data were analysed as absolute values per input microgram of mRNA and corrected relative to the appropriate housekeeper gene expression.

3.3 RESULTS

3.3.1 Endogenous CB Agonist Anandamide Stimulates Proliferation Of 3T3-L1 But Has No Effect On PAZ6

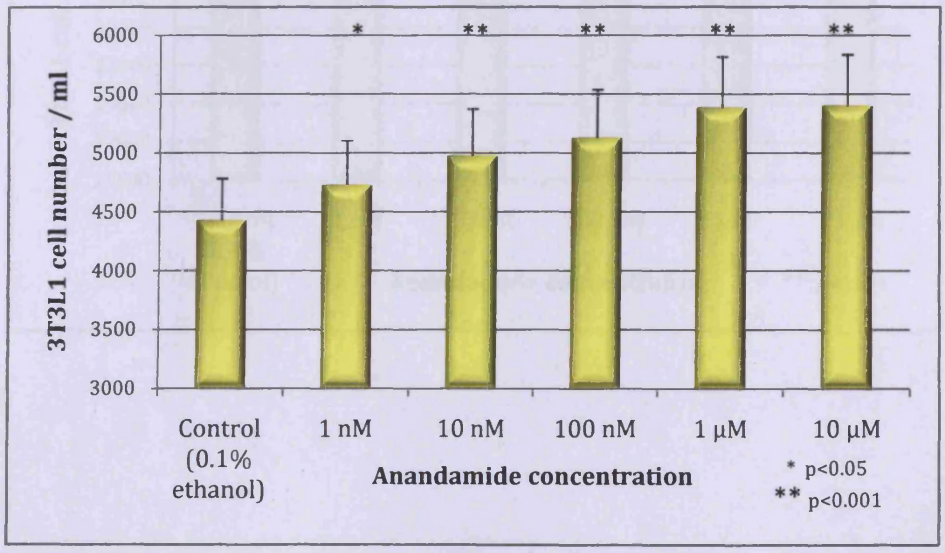
Exposure to the CB agonist anandamide for 24 hours was associated with a concentration-dependent increase in proliferation of 3T3-L1 as illustrated in Table 3.8 and Figure 3.6.

Table 3.8: Mean, Median & IQR For 3T3-L1 Treated With Anandamide For 24 Hours

Anandamide concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	4428	4392	4152-4716	n/a
1 nM	4730	4790	4443-4979	0.009
10 nM	4977	4996	4799-5181	<0.001
100 nM	5128	5143	4988-5252	<0.001
1 µM	5386	5367	5159-5621	<0.001
10 µM	5404	5433	5241-5592	<0.001

Figure 3.6: The Effect Of Anandamide On 3T3-L1 Proliferation At 24 Hours

Cells were counted 24 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



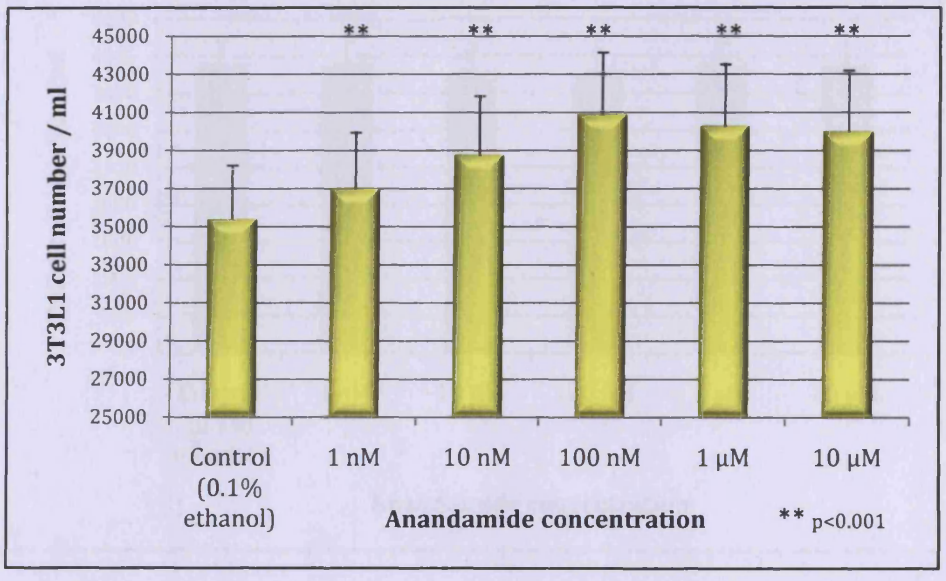
The significant, concentration-dependent increase in proliferation of 3T3-L1 was also observed after 72 hours exposure to anandamide as illustrated in Table 3.9 and Figure 3.7.

Table 3.9: Mean, Median & IQR For 3T3-L1 Treated With Anandamide For 72 Hours

Anandamide concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	35374	35394	35014-35744	n/a
1 nM	36973	36997	36566-37289	**<0.001**
10 nM	38746	38660	38309-39377	**<0.001**
100 nM	40867	40978	40048-41616	**<0.001**
1 µM	40280	40430	39820-40712	**<0.001**
10 µM	39984	40039	39480-40511	**<0.001**

Figure 3.7: The Effect Of Anandamide On 3T3-L1 Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



Treatment with the CB agonist anandamide had no significant effect on proliferation of PAZ6 at 24 hours (Table 3.10 and Figure 3.8) or at 72 hours (Table 3.11 and Figure 3.9).

Table 3.10: Mean, Median & IQR For PAZ6 Treated With Anandamide For 24 Hours

Anandamide concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	3875	3883	3539-4246	n/a
1 nM	3891	4028	3527-4245	0.966
10 nM	3777	3857	3353-4135	0.346
100 nM	3765	3835	3343-4150	0.407
1 µM	3868	3872	3504-4171	0.886
10 µM	3860	3943	3582-4172	0.797

Figure 3.8: The Effect Of Anandamide On PAZ6 Proliferation At 24 Hours

Cells were counted 24 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.

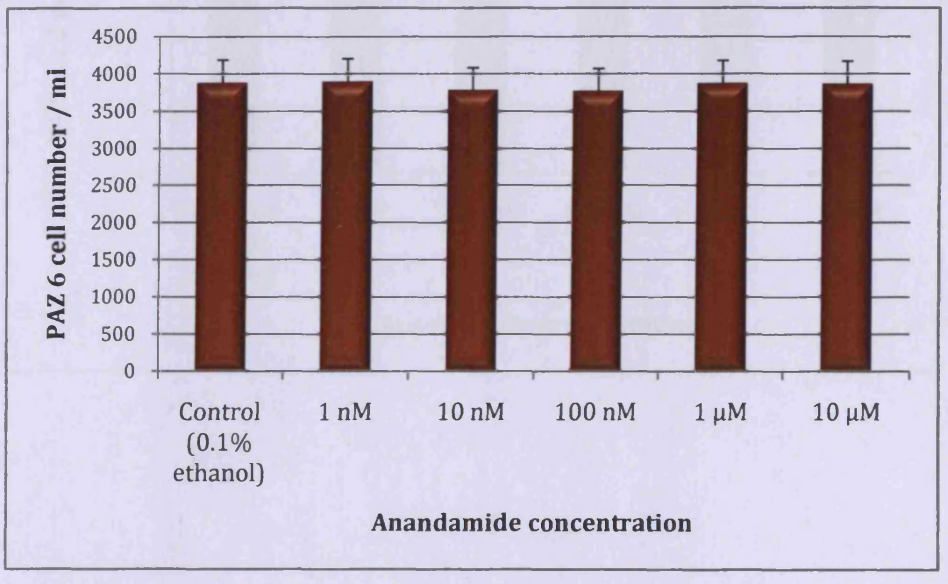
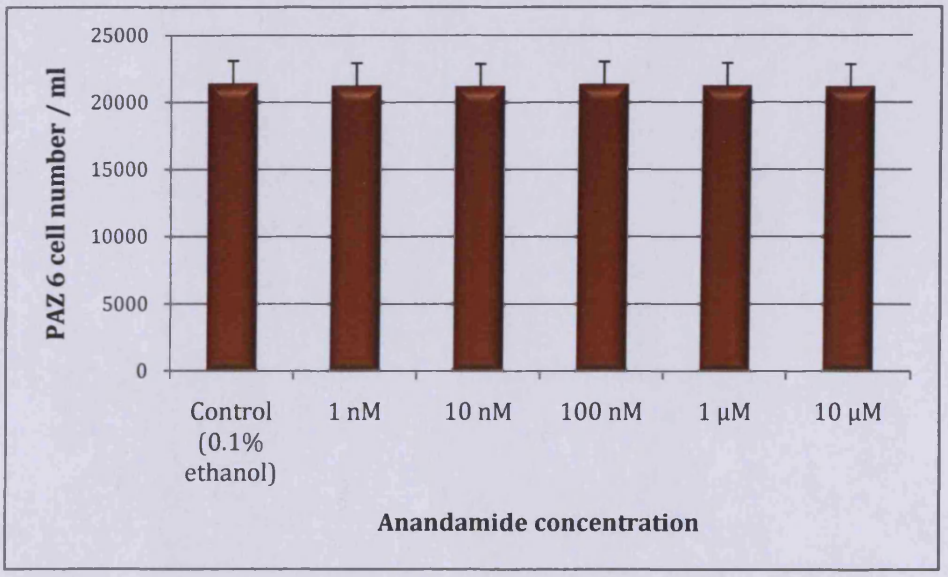


Table 3.11: Mean, Median & IQR For PAZ6 Treated With Anandamide For 72 Hours

Anandamide concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	21405	21535	20896-21841	n/a
1 nM	21239	21255	20893-21659	0.230
10 nM	21193	21270	20937-21482	0.092
100 nM	21345	21429	20950-21633	0.587
1 µM	21239	21195	20864-21721	0.153
10 µM	21157	21010	20709-21720	0.067

Figure 3.9: The Effect Of Anandamide On PAZ6 Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



3.3.2 CB_1 Agonist ACEA Stimulates Proliferation Of 3T3-L1 But Does Not Affect PAZ6

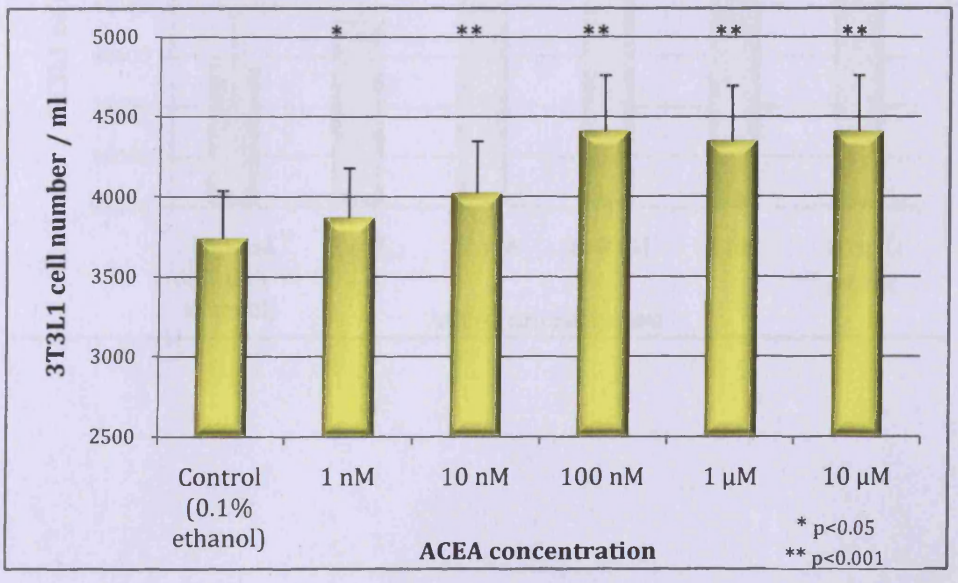
Exposure to the CB_1 agonist ACEA for 24 hours was associated with a significant, concentration -dependent increase in proliferation of 3T3-L1 as illustrated in Table 3.12 and Figure 3.10.

Table 3.12: Mean, Median & IQR For 3T3-L1 Treated With ACEA For 24 Hours

ACEA concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	3736	3736	3560-3900	n/a
1 nM	3867	3811	3752-3961	**0.028**
10 nM	4021	3991	3906-4155	**<0.001**
100 nM	4405	4428	4257-4571	**<0.001**
1 µM	4344	4309	4236-4459	**<0.001**
10 µM	4404	4377	4260-4618	**<0.001**

Figure 3.10: The Effect Of ACEA On 3T3-L1 Proliferation At 24 Hours

Cells were counted 24 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



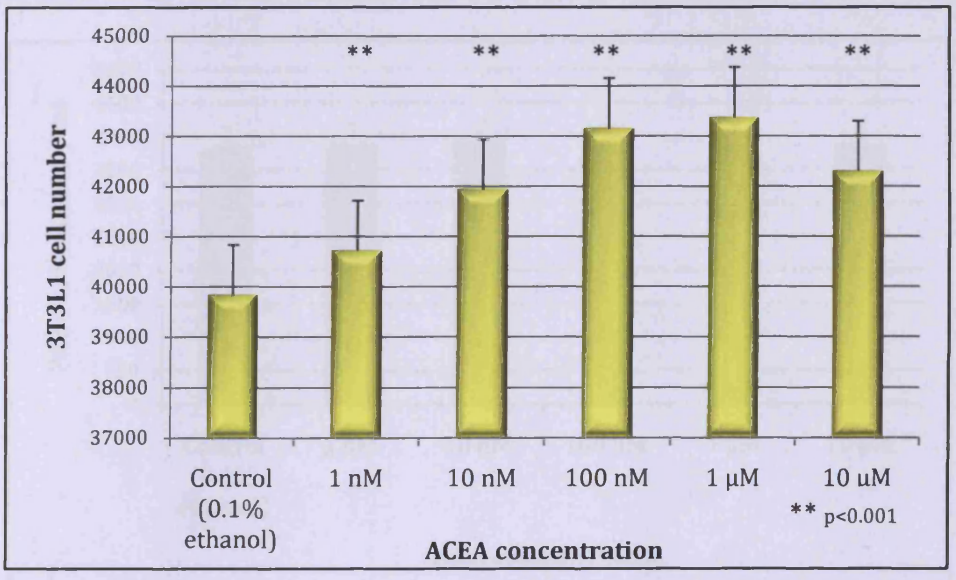
The significant, concentration-dependent increase in proliferation of 3T3-L1 was also observed after 72 hours exposure to ACEA as illustrated in Table 3.13 and Figure 3.11.

Table 3.13: Mean, Median & IQR For 3T3-L1 Treated With ACEA For 72 Hours

ACEA concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	39835	39805	39230-40355	n/a
1 nM	40717	40624	40290-41121	**<0.001**
10 nM	41935	41769	41519-42489	**<0.001**
100 nM	43154	42953	42632-43909	**<0.001**
1 µM	43371	43596	42495-44046	**<0.001**
10 µM	42298	42366	41968-42565	**<0.001**

Figure 3.11: The Effect Of ACEA On 3T3-L1 Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



Treatment with the CB_1 agonist ACEA had no significant effect on proliferation of PAZ6 at 24 hours (Table 3.14 and Figure 3.12) or at 72 hours (Table 3.15 and Figure 3.13).

Table 3.14: Mean, Median & IQR For PAZ6 Treated With ACEA For 24 Hours

ACEA concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	3832	3804	3671-3994	n/a
1 nM	3882	3920	3626-4128	0.627
10 nM	3910	3972	3696-4051	0.201
100 nM	3841	3881	3638-4033	0.710
1 µM	3868	3851	3727-4025	0.689
10 µM	3842	3868	3652-4006	0.886

Figure 3.12: The Effect Of ACEA On PAZ6 Proliferation At 24 Hours

Cells were counted 24 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.

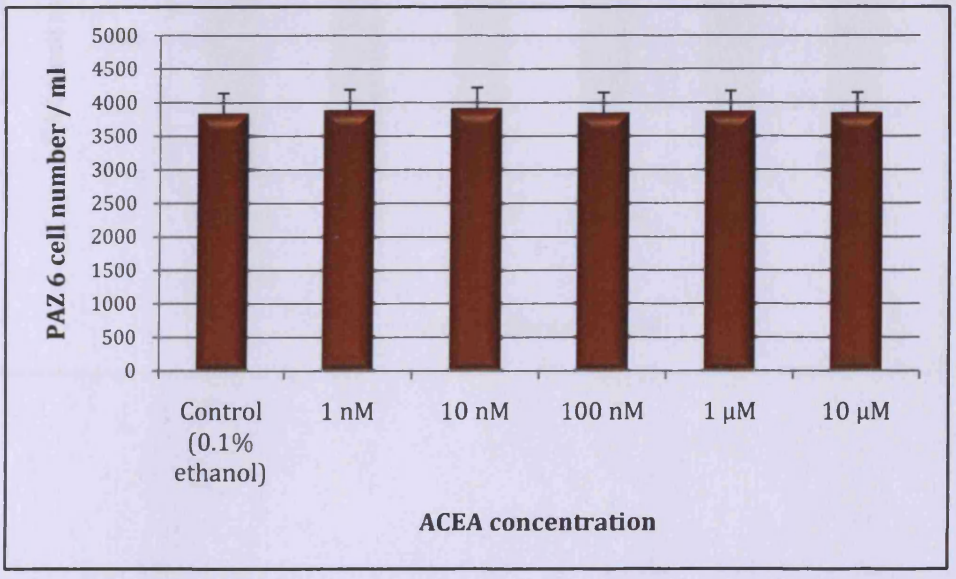
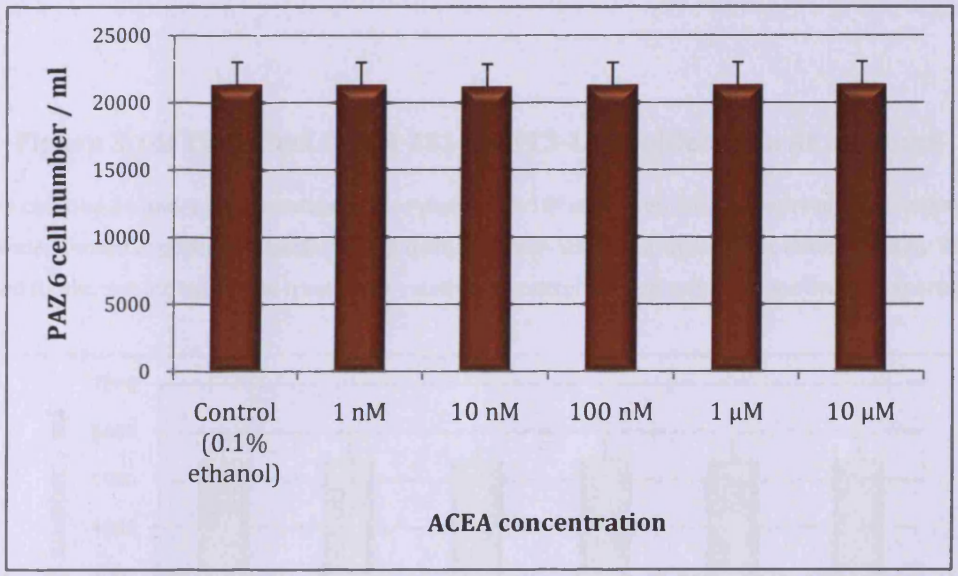


Table 3.15: Mean, Median & IQR For PAZ6 Treated With ACEA For 72 Hours

ACEA concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	21310	21355	21111-21510	n/a
1 nM	21291	21341	21092-21479	0.732
10 nM	21162	21168	20888-21347	0.063
100 nM	21279	21252	21072-21542	0.648
1 µM	21303	21360	21124-21576	0.875
10 µM	21347	21431	21124-21576	0.753

Figure 3.13: The Effect Of ACEA On PAZ6 Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



3.3.3 Effect Of CB_1 Antagonist AM 251 On Proliferation Of 3T3-L1 And PAZ6

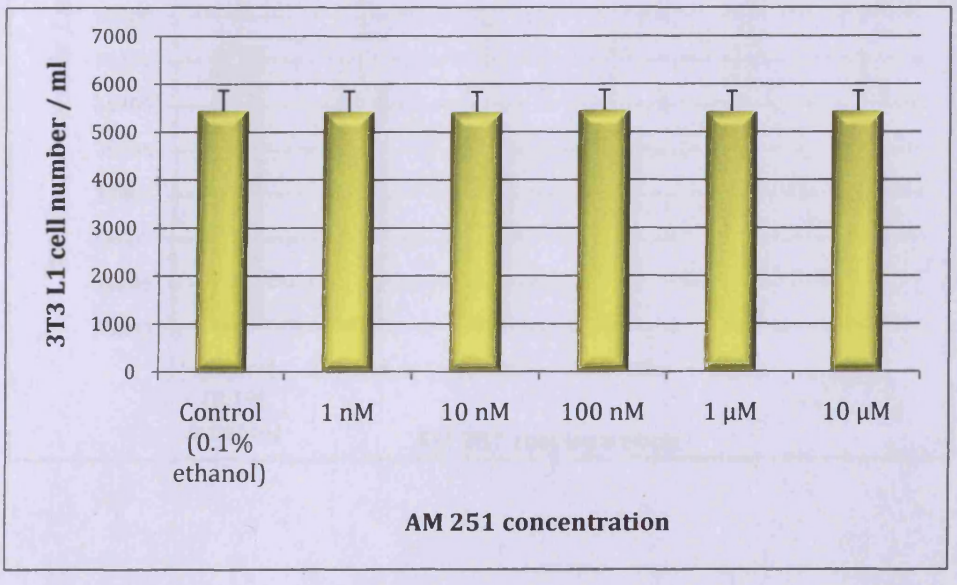
Exposure to the CB_1 antagonist AM 251 for 24 hours did not affect proliferation of 3T3-L1 as illustrated in Table 3.16 and Figure 3.14.

Table 3.16: Mean, Median & IQR For 3T3-L1 Treated With AM 251 For 24 Hours

AM 251 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	5433	5443	5302-5537	n/a
1 nM	5414	5390	5313-5522	0.658
10 nM	5401	5356	5317-5480	0.315
100 nM	5442	5462	5341-5523	0.830
1 µM	5411	5410	5348-5503	0.511
10 µM	5421	5386	5327-5525	0.764

Figure 3.14: The Effect Of AM 251 On 3T3-L1 Proliferation At 24 Hours

Cells were counted 24 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



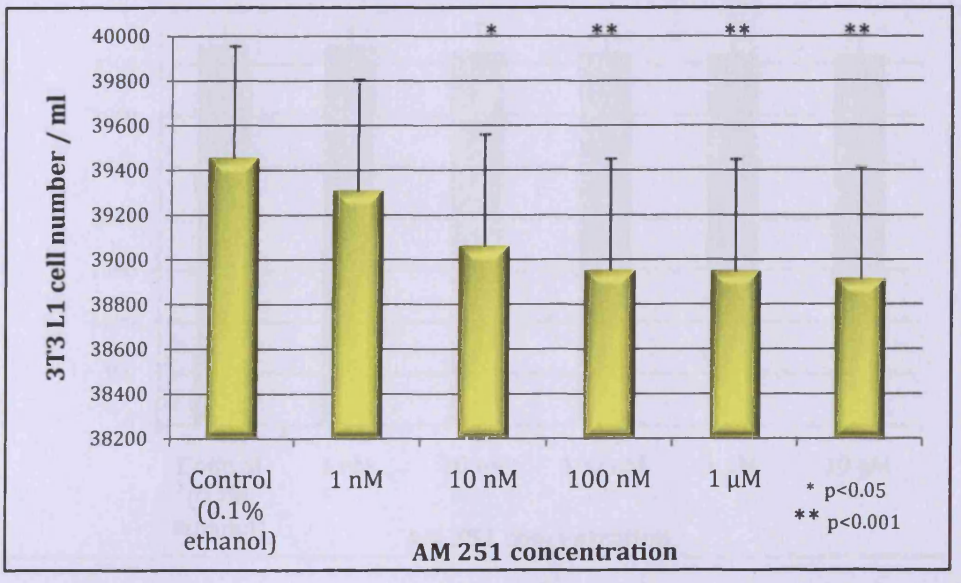
Exposure to AM 251 for 72 hours was associated with a significant, concentration-dependent reduction in proliferation of 3T3-L1 as illustrated in Table 3.17 and Figure 3.15.

Table 3.17: Mean, Median & IQR For 3T3-L1 Treated With AM 251 For 72 Hours

AM 251 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	39454	39577	39018-39812	n/a
1 nM	39307	39368	39097-39457	0.137
10 nM	39059	39077	38972-39151	**0.001**
100 nM	38949	38950	38874-39029	**<0.001**
1 µM	38947	38932	38880-39054	**<0.001**
10 µM	38913	38879	38791-39049	**<0.001**

Figure 3.15: The Effect Of AM 251 On 3T3-L1 Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



Treatment with the CB_1 antagonist AM 251 had no significant effect on proliferation of PAZ6 at 24 hours (Table 3.18 and Figure 3.16) or at 72 hours (Table 3.19 and Figure 3.17).

Table 3.18: Mean, Median & IQR For PAZ6 Treated With AM 251 For 24 Hours

AM 251 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	3678	3704	3539-3830	n/a
1 nM	3663	3717	3517-3764	0.376
10 nM	3607	3582	3495-3725	0.130
100 nM	3610	3620	3556-3682	0.056
1 µM	3594	3605	3450-3662	0.059
10 µM	3579	3619	3479-3673	0.081

Figure 3.16: The Effect Of AM 251 On PAZ6 Proliferation At 24 Hours

Cells were counted 24 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.

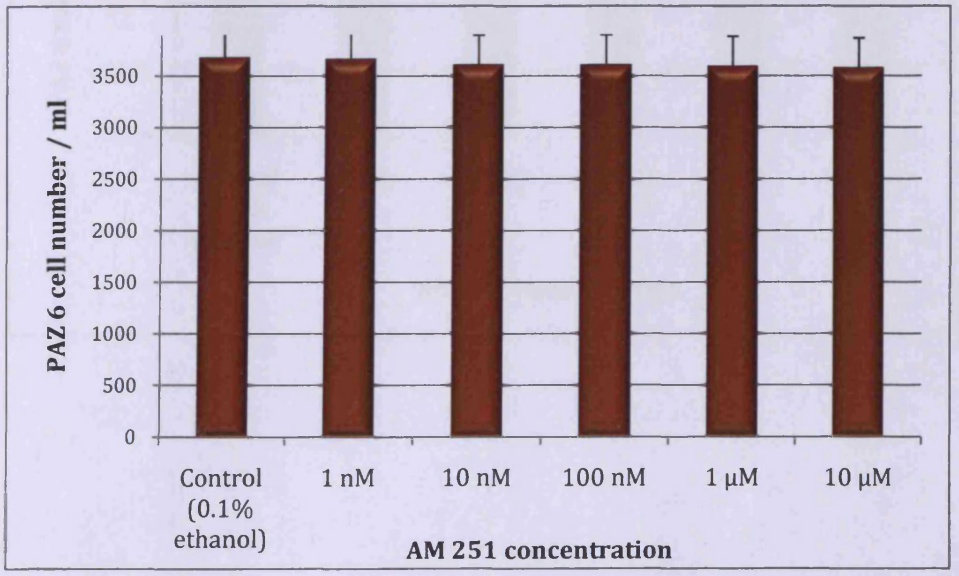
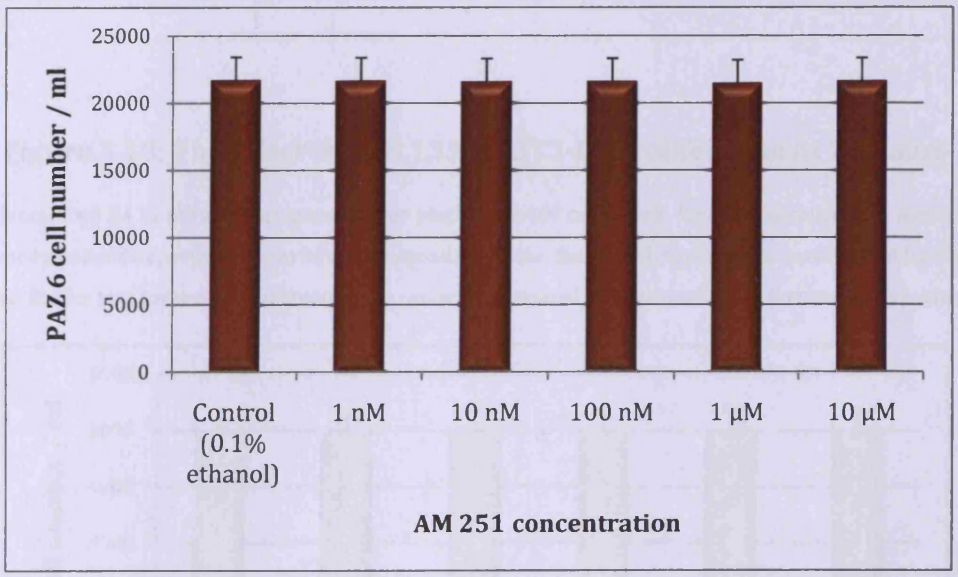


Table 3.19: Mean, Median & IQR For PAZ6 Treated With AM 251 For 72 Hours

AM 251 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	21689	21763	21328-21951	n/a
1 nM	21639	21643	21225-21988	0.587
10 nM	21587	21559	21353-21793	0.136
100 nM	21539	21588	21239-21912	0.368
1 µM	21470	21381	21187-21720	0.058
10 µM	21621	21660	21264-21946	0.346

Figure 3.17: The Effect Of AM 251 On PAZ6 Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



3.3.4 CB_2 Agonist JWH 133 Does Not Affect Proliferation Of 3T3-L1 Or PAZ6

Treatment with the CB_2 agonist JWH 133 had no significant effect on proliferation of 3T3-L1 at 24 hours (Table 3.20 and Figure 3.18) or at 72 hours (Table 3.21 and Figure 3.19).

Table 3.20: Mean, Median & IQR For 3T3-L1 Treated With JWH 133 For 24 Hours

JWH 133 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	4989	4985	4933-5054	n/a
1 nM	4979	4964	4924-5053	0.511
10 nM	4959	4957	4895-5021	0.260
100 nM	4995	5001	4937-5045	0.954
1 µM	4966	4974	4908-5026	0.361
10 µM	4939	4926	4869-5013	0.054

Figure 3.18: The Effect Of JWH 133 On 3T3-L1 Proliferation At 24 Hours

Cells were counted 24 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.

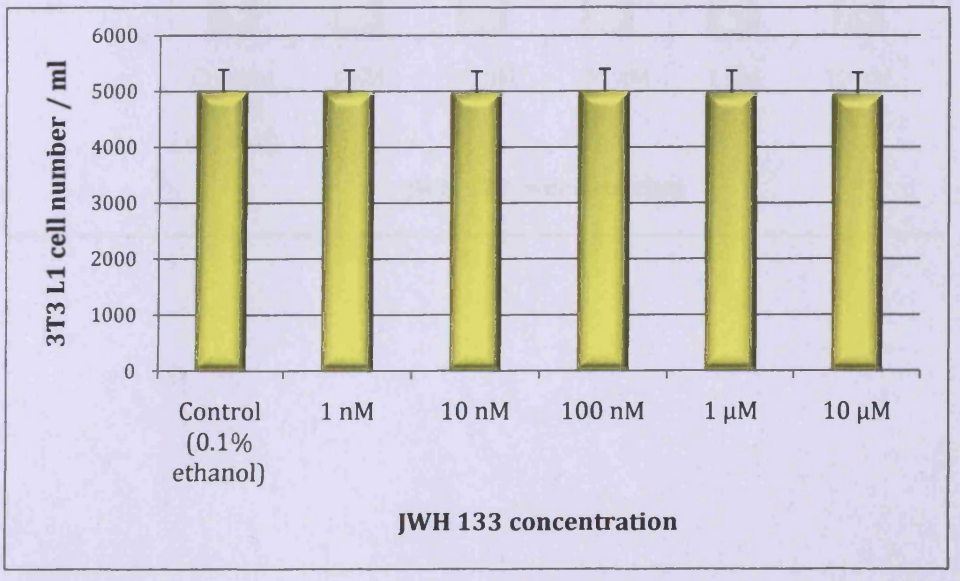
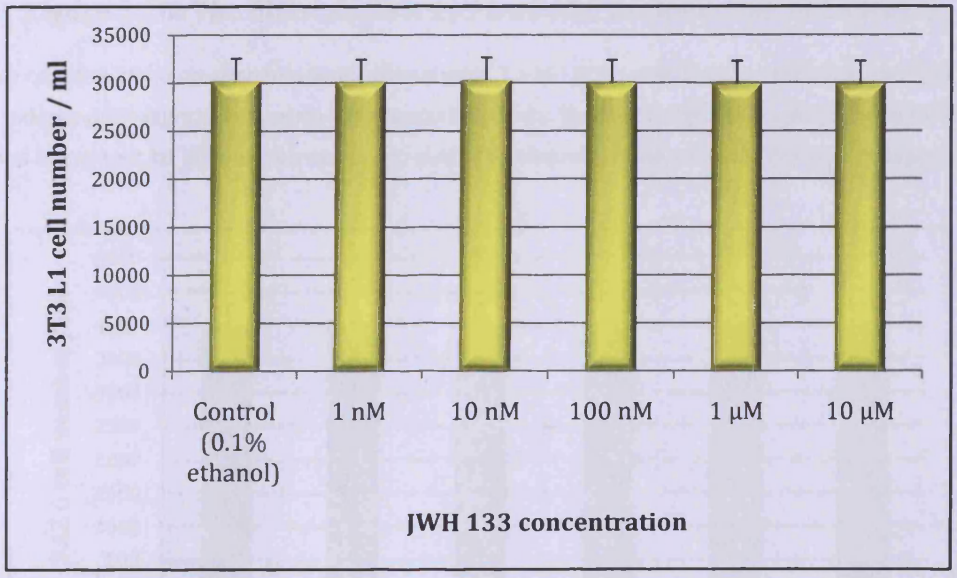


Table 3.21: Mean, Median & IQR For 3T3-L1 Treated With JWH 133 For 72 Hours

JWH 133 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	30167	30120	29572-30778	n/a
1 nM	30049	29866	29273-30869	0.607
10 nM	30249	30385	29451-30860	0.511
100 nM	29995	29840	29198-31045	0.549
1 μM	29879	29823	29102-30709	0.304
10 μM	29897	29802	29245-30634	0.162

Figure 3.19: The Effect Of JWH 133 On 3T3-L1 Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



Treatment with the CB_2 agonist JWH 133 had no significant effect on proliferation of PAZ6 at 24 hours (Table 3.22 and Figure 3.20) or at 72 hours (Table 3.23 and Figure 3.21).

Table 3.22: Mean, Median & IQR For PAZ6 Treated With JWH 133 For 24 Hours

JWH 133 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	3991	3980	3853-4107	n/a
1 nM	4005	4018	3916-4107	0.568
10 nM	4017	3992	3887-4145	0.637
100 nM	3997	3985	3848-4135	0.909
1 µM	4011	4003	3986-4131	0.543
10 µM	3987	3966	3881-4097	0.886

Figure 3.20: The Effect Of JWH 133 On PAZ6 Proliferation At 24 Hours

Cells were counted 24 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.

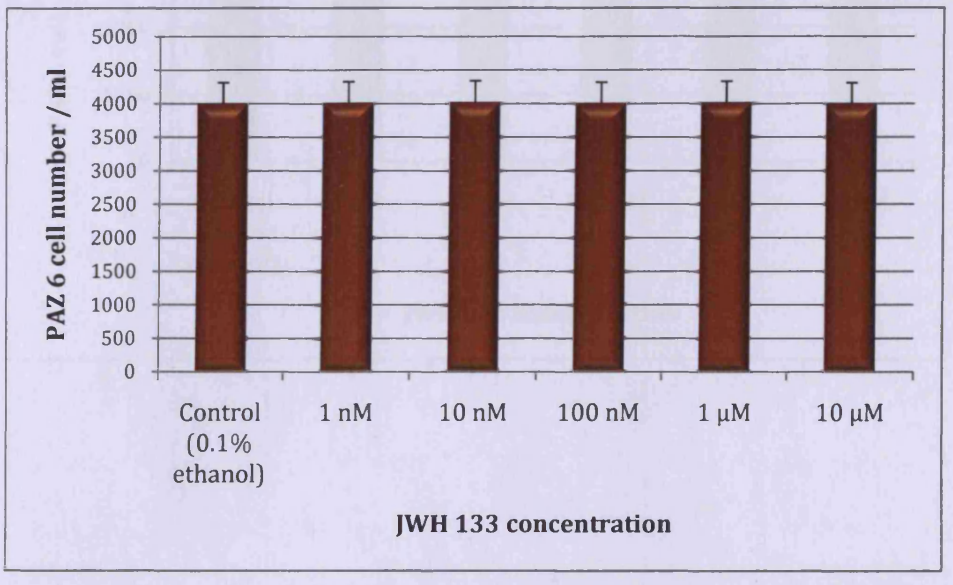
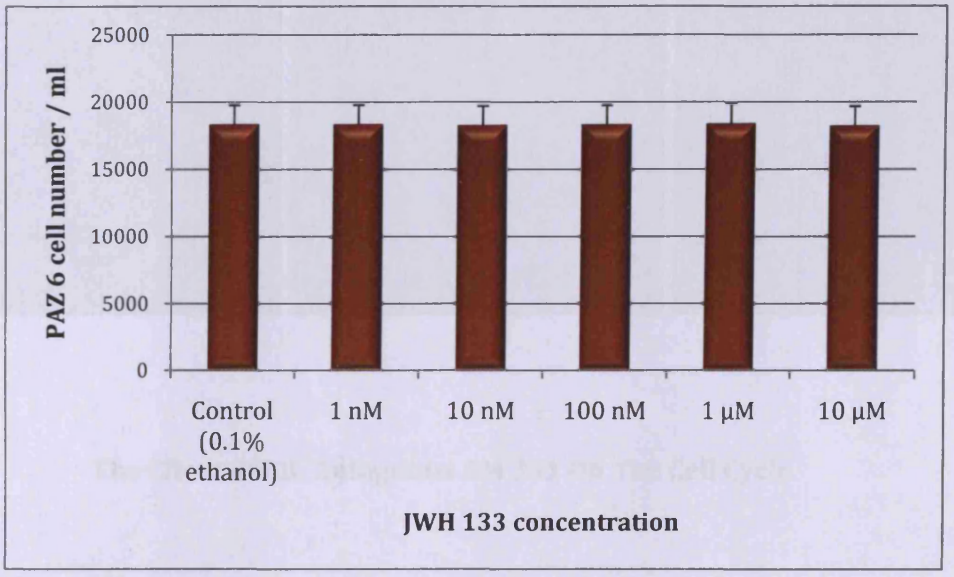


Table 3.23: Mean, Median & IQR For PAZ6 Treated With JWH 133 For 72 Hours

JWH 133 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	18324	18373	18073-18534	n/a
1 nM	18318	18243	18108-18575	0.989
10 nM	18246	18199	18007-18544	0.331
100 nM	18299	18252	18017-18642	0.819
1 µM	18379	18445	18148-18602	0.399
10 µM	18217	18169	17966-18516	0.107

Figure 3.21: The Effect Of JWH 133 On PAZ6 Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SEM from 3 independent experiments performed in quadruplicate. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.

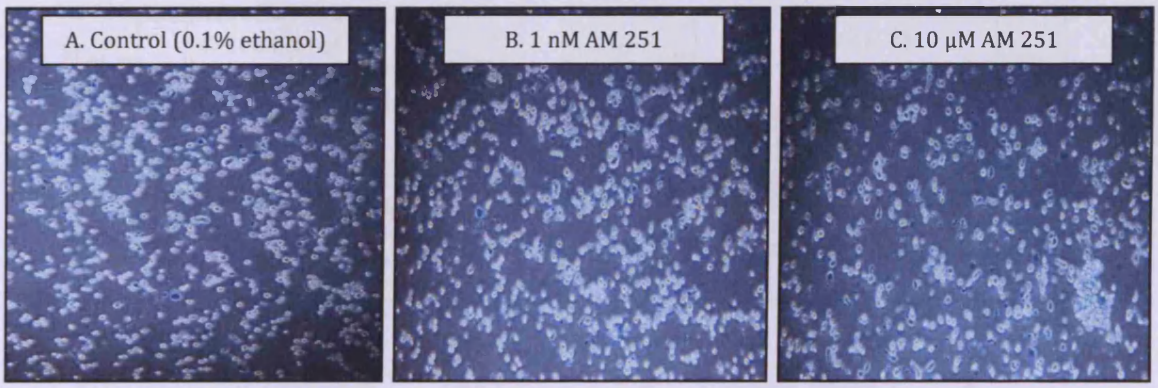


3.3.5 CB_1 Antagonist AM 251 Inhibition Of Proliferation Is Not Due To Cell Toxicity

The previous results indicated that treatment with AM 251 had an inhibitory effect on 3T3-L1 proliferation. Overall cell numbers continued to rise between 24 and 72 hours suggesting cell toxicity arising from exposure to AM 251 to be unlikely. Trypan blue staining was undertaken on control and treated samples to confirm this (Figure 3.22). Some dye uptake was noted in all samples and was likely to be a result of manual cell damage that may have occurred during the experimental procedure. Overall toxicity was estimated to be less than 1% in both control and treated samples.

Figure 3.22: Trypan Blue Staining Of 3T3-L1 After 72 Hours Exposure To AM 251

(Results are from a single experiment performed in quadruplicate. Magnification 200x)



3.3.6 The Effect Of CB_1 Antagonist AM 251 On The Cell Cycle

Cell cycle analysis was undertaken in order to determine whether the inhibition of proliferation observed in 3T3-L1 following treatment with AM 251 was due to interruption of the cell cycle or an increase in cell apoptosis. Figures 3.23 and 3.24 illustrate a significant concentration-related increase in cells in the G0/G1 phase as well as a significant decrease in the number of cells in the S and G2+M phases of the cell cycle suggesting a block at G1/S. Also

the histograms in Figure 3.23 show no pre-G1 peak, indicating that increased apoptosis was unlikely to be the cause of the reduced cell numbers. Statistical significance was determined by the Kruskal-Wallis test to include both control samples and all treatment concentrations.

Figure 3.23: Histograms Illustrating Effect Of AM 251 On 3T3-L1 Cell Cycle Progression

1 x 10^4 cells were plated per well and analysed after 48 hours treatment with control or AM 251 followed by DNA staining with propidium iodide. The x-axis represents the cell cycle and y-axis provides the cell counts. Data are a representative example of 2 independent experiments performed in replicates of six.

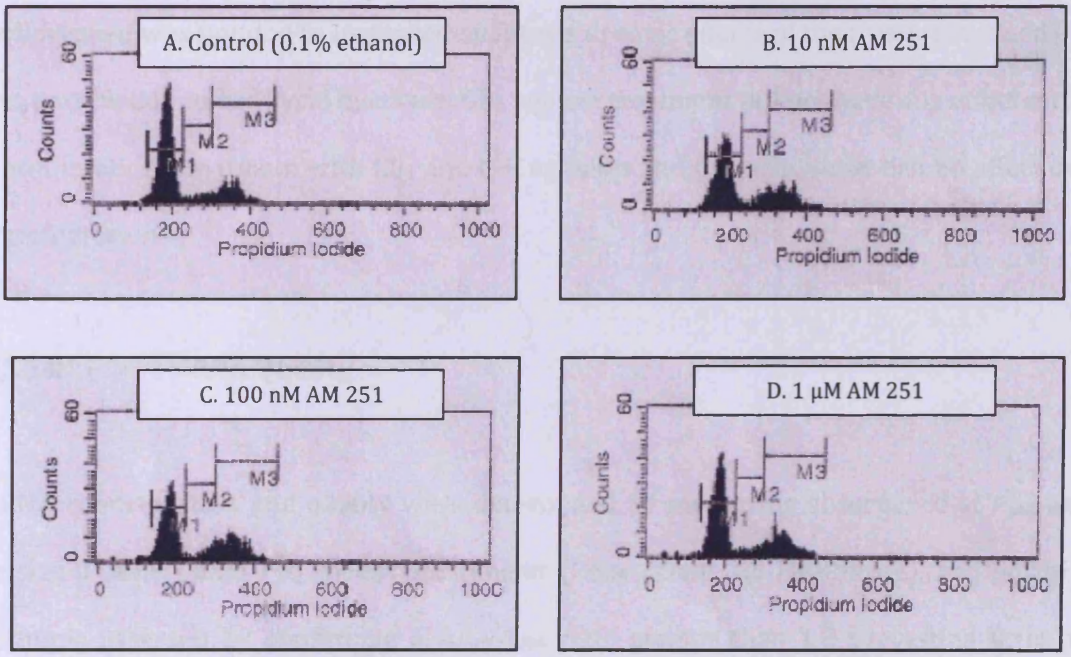
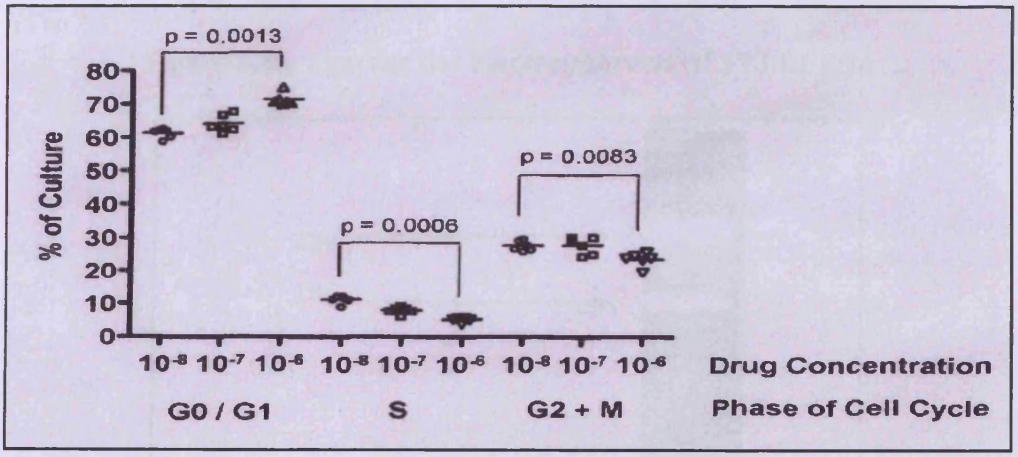


Figure 3.24: The Effect Of AM 251 On 3T3-L1 Cell Cycle

1 x 10^4 cells were plated per well and analysed after 48 hours treatment with control or AM 251. Results are median values of percentages of gated controls for each cell cycle stage



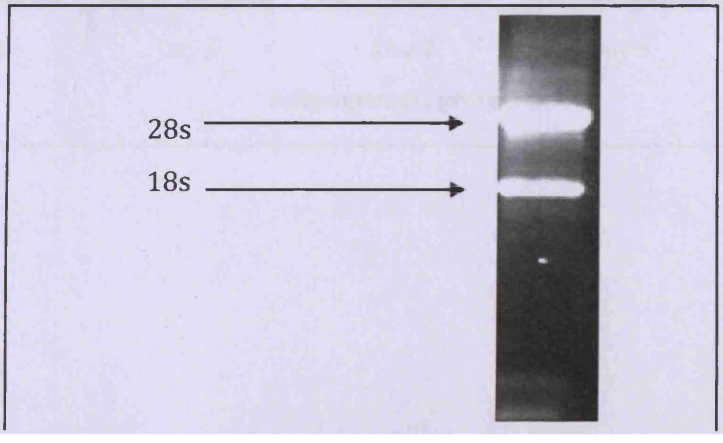
3.3.7 Summary Of The Effects Of Cannabinoid Receptor Modulation On Pre-Adipocyte Cell Line Proliferation

When cultured in ordinary CM, 3T3-L1 were noted to exhibit a population doubling time of approximately 22 hours while this was longer at almost 28 hours for PAZ6 . Treatment with endogenous and exogenous CB_1 agonists resulted in a statistically significant concentration-dependent stimulation of proliferation in 3T3L1 while the CB_1 antagonist had the opposite effect. Further studies with cell cycle analysis and trypan blue staining confirmed that the effect seen was not due to increased apoptosis or toxic effects of the treatments used but may in part be due to cell cycle blockade. CB_2 agonist treatment did not have any effect on 3T3-L1 proliferation Treatment with CB_1 and CB_2 agonists and CB_1 antagonist had no effect on PAZ6 proliferation.

3.3.8 RNA Quality

RNA concentration and quality were determined by measuring absorbance at A_{260} and A_{280} using a Gene Quant Pro spectrophotometer (Gene Quant, GE Healthcare), and purity of the sample assessed by confirming a A_{260}/A_{280} ratio greater than 1.8 suggesting little protein contamination in the nucleic acid sample. A sample of RNA was also analysed on a 1.2% agarose gel with ethidium bromide staining (Figure 3.25). The presence of bands at 28s and 18s confirmed RNA integrity and suitability for reverse transcription.

Figure 3.25: Agarose Gel Electrophoresis Of 3T3-L1 RNA

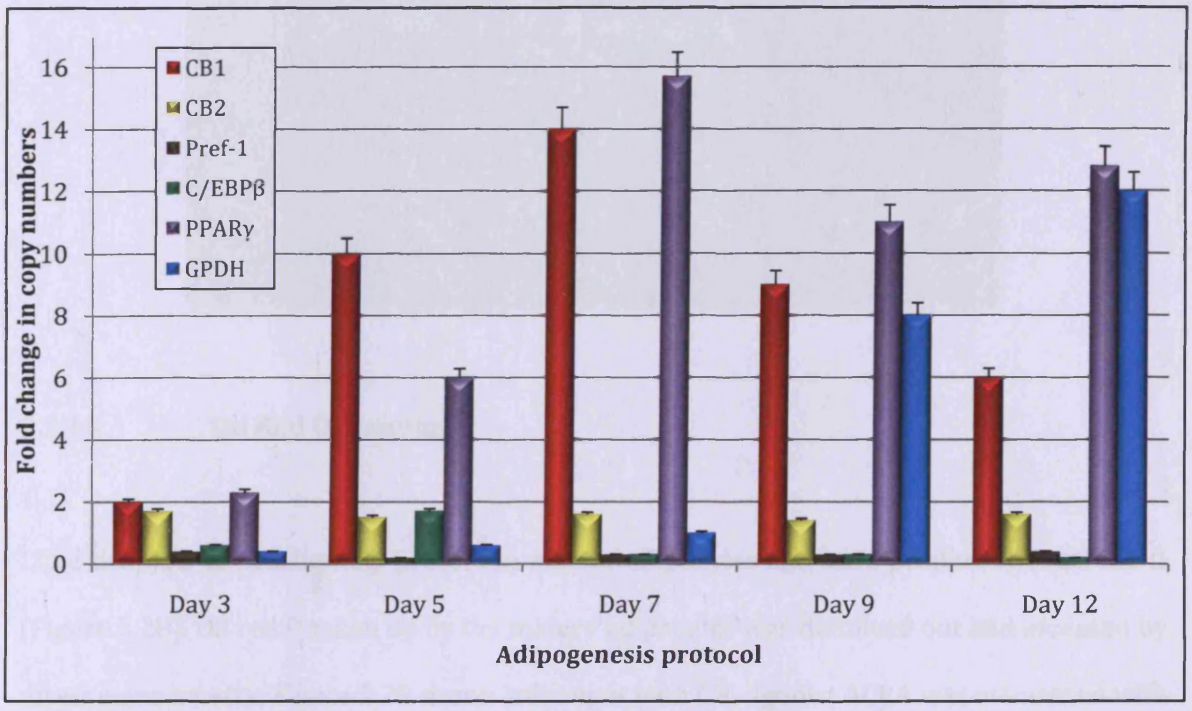


3.3.9 Transcript Expression During 3T3-L1 Pre-adipocyte Differentiation

Figure 3.26 demonstrates transcript expression during adipogenesis in 3T3-L1 cells. While CB_2 expression was largely unaltered, CB_1 receptors were upregulated at the midpoint of the differentiation protocol coincident with PPARγ expression. The increase in GPDH expression, a marker present in mature adipocytes, confirmed successful adipogenesis. Both CB_1 and CB_2 receptor expression was virtually undetectable in PAZ6 with Ct values approaching the limit of detection at the maximum number of programmed PCR cycles and no change was noted during adipogenesis. This may explain the lack of effect of CB modulation on PAZ6 proliferation. Therefore all further differentiation experiments were only performed in 3T3-L1.

Figure 3.26: Transcript Expression During 3T3-L1 Adipogenesis

All transcript copy numbers were normalised to the housekeeper gene ARP. Results are presented as fold-changes in comparison to expression on Day 0 & represent two independent experiments performed in triplicate

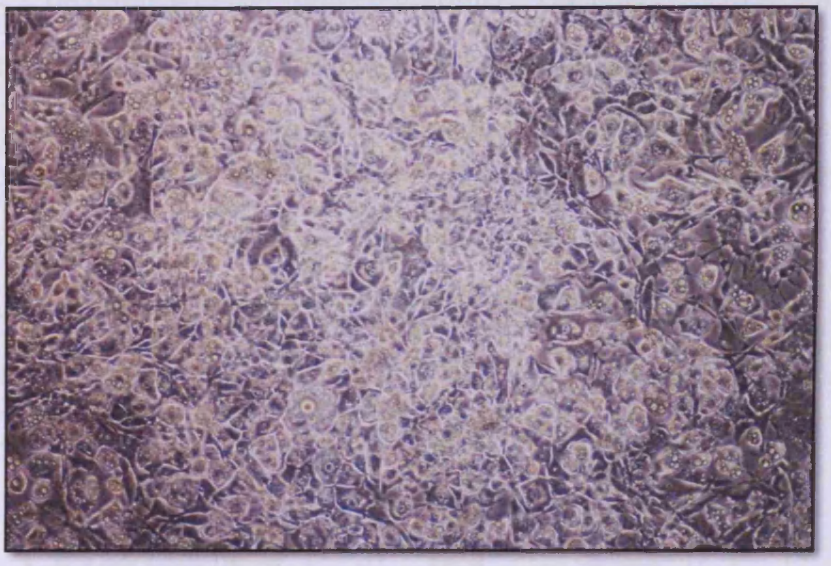


3.3.10 The Effect Of CB_1 Agonist ACEA & CB_1 Antagonist AM 251 On Foci Of Differentiation In 3T3-L1

One method of assessing adipogenesis is by manually counting separate foci of differentiation occurring within a single observed microscopic field. However, 3T3-L1 differentiation was noted to be so widespread that it was not possible to accurately count foci in order to draw comparisons between control and treatment conditions ((Figure 3.27). These effects were therefore studied by means of oil red O staining and QPCR for terminal markers of adipogenesis.

Figure 3.27: Adipogenesis In 3T3-L1

(magnification 200x)



3.3.11 Oil Red O Staining

Lipid droplets are exclusively present in mature adipocytes and stain positive with oil red O (Figure 3.28). Oil red O taken up by the mature adipocytes was dissolved out and assessed by direct colourimetry. Figure 3.29 shows treatment with CB_1 agonist ACEA was associated with an increase in oil red O whereas CB_1 antagonist AM 251 had no significant effect.

Figure 3.28: Oil Red O Staining In Mature 3T3-L1 Adipocytes

(magnification 200x)

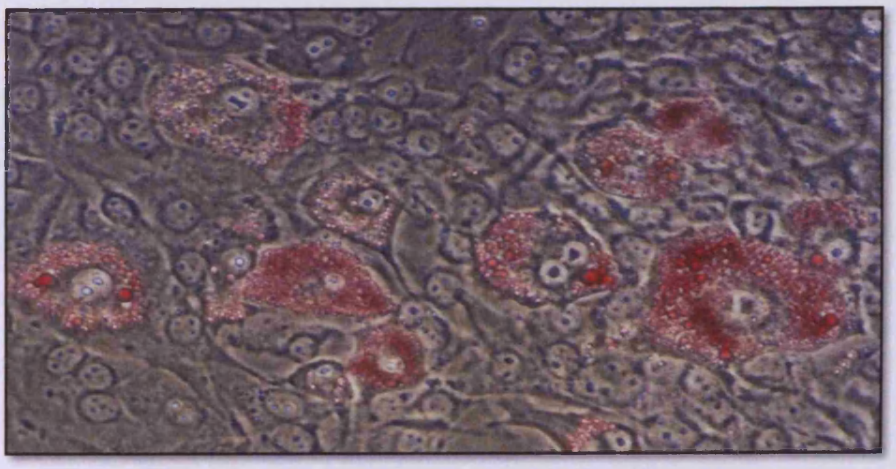
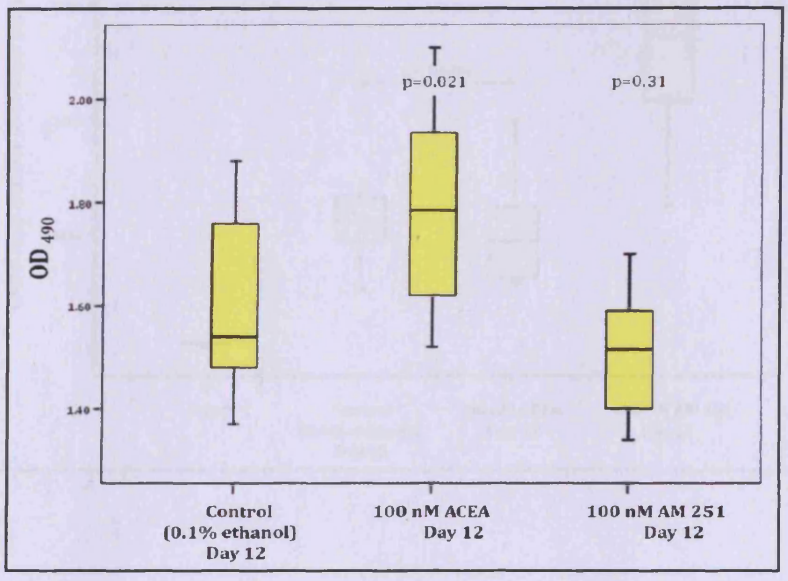


Figure 3.29: Box-and-Whisker Representation Of The Effect Of CB_1 Agonist ACEA & Antagonist AM 251 On Oil Red O Staining in Mature 3T3-L1 Adipocytes

Data represent the median & IQRs expressed as OD490 from 2 independent experiments performed in quadruplicate. Significance was determined by the Wilcoxon Signed Ranks Test for individual treatments relative to control.



3.3.12 The Effect Of CB_1 Agonist ACEA & CB_1 Antagonist AM 251 On Terminal Markers Of Adipogenesis In 3T3-L1

QPCR was performed on cDNA derived from RNA collected on day 0 and day 12 of the differentiation protocol and transcript expression for GPDH was normalised to housekeeper gene (ARP) expression. While exposure to the CB_1 agonist ACEA had no effect on adipogenesis relative to control conditions, supplementation with AM 251 was associated with a significant increase in GPDH expression as illustrated in Figure 3.30 and Table 3.24.

Figure 3.30: The Effect Of AM 251 On GPDH Expression In 3T3-L1 During Adipogenesis

Data represent 2 independent experiments performed in triplicate following 12 days culture in DM, ACEA or AM251. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control.

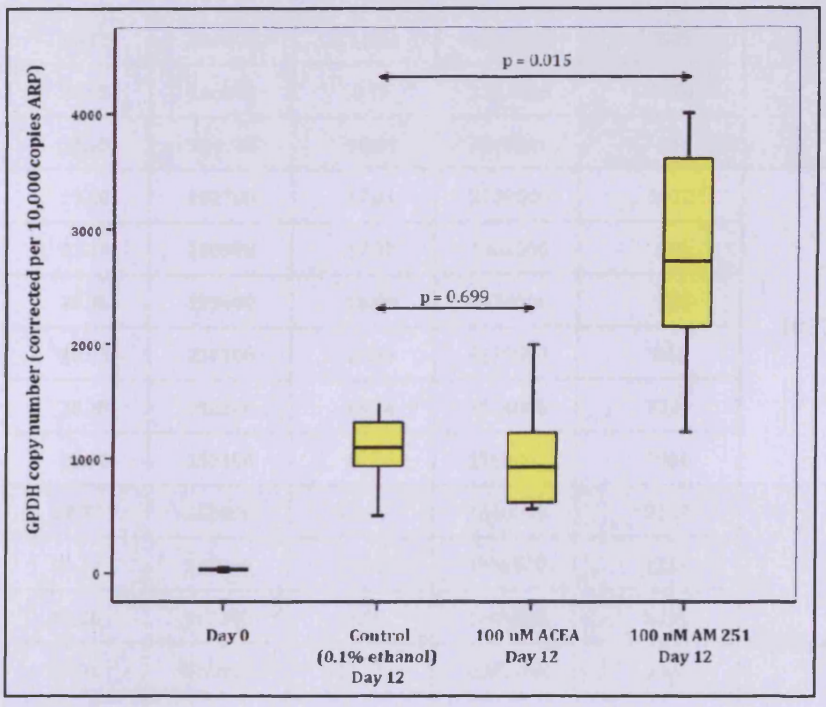


Table 3.24: The Effect Of AM 251 On GPDH Expression In 3T3-L1 During Adipogenesis

Data represent 2 independent experiments performed in triplicate following 12 days culture in DM, ACEA or AM251. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control

Sample	GPDH		ARP		Normalised	Mean	P-value
	Ct	Copy	Ct	Copy			
Day 0	25.44	5736	17.51	2187000	26	31	n/a
	26.14	3392	17.04	3175000	11		
	24.37	7842	18.05	1481000	53		
	25.22	6137	17.55	2158000	28		
	26.13	3416	18.70	908100	38		
	26.75	2974	18.56	1012000	29		
Day 12 Control 0.1% ethanol	21.21	138300	18.51	1053000	1313	1095	n/a
	21.41	118900	18.25	1276000	932		
	20.98	164000	18.35	1000000	1640		
	20.71	200600	16.62	3989000	503		
	20.96	166800	18.19	1357900	1228		
	21.15	144900	18.04	1515000	956		
Day 12 100 nM ACEA	19.80	282700	17.21	2739000	1032	1037	0.699
	21.34	120900	17.87	1962000	616		
	20.75	193800	16.98	3472000	558		
	20.03	257500	17.03	3179000	810		
	20.78	192200	18.34	1574000	1221		
	19.66	350100	18.26	1763000	1986		
Day 12 100 nM AM 251	19.70	362000	17.94	1640000	2207	2734	0.015
	20.43	241900	17.88	1976000	1224		
	18.56	517200	17.96	1605600	3221		
	17.91	836000	17.61	2089000	4002		
	18.03	787400	17.43	2184000	3605		
	19.92	423000	17.90	1973000	2144		

3.3.13 The Effect Of CB_1 Agonist ACEA & CB_1 Antagonist AM 251 On UCP-1 Expression In Differentiated 3T3-L1

QPCR was performed on cDNA derived from RNA collected on day 0 and day 12 of the differentiation protocol and transcript expression for UCP-1 was normalised to housekeeper gene (ARP) expression. Transcript expression was at the limit of QPCR detection (Ct values > 35) and did not alter with adipogenesis in control conditions or when supplemented with ACEA or AM 251.

3.4 DISCUSSION

Adipocyte cell proliferation and differentiation represent two closely related cell processes that control adipose tissue homeostasis [207]. In obesity, adipose tissue accumulates as a result of increased pre-adipocyte proliferation (hyperplasia) and differentiation into mature adipocytes, as well as excess lipid droplet accumulation within adipocytes causing cell hypertrophy. The data from the experiments described in this chapter have demonstrated that CB_1 agonist treatment appears to have a stimulatory effect on pre-adipocyte proliferation in the WAT cell line, 3T3-L1, while CB_1 antagonism may inhibit cell proliferation, thus modifying an important step in adipose tissue accumulation. This inhibitory effect does not appear to be due to increased apoptosis and toxicity, but may partly be secondary to cell cycle blockade with growth arrest in the G1 phase. However, though statistically significant, these effects do appear to be relatively small in absolute terms, therefore further work is required to determine their biological significance.

Similar studies published in the literature have also confirmed an anti-proliferative effect, using the CB_1 antagonist SR141716, where the findings have been associated with inhibition of p42/44 mitogen-activated protein (MAP) kinase activity which has been shown to be involved in the CB_1 signalling pathway [207, 208]. The inhibition of proliferation with CB_1 blockade adds evidence to the importance of the ECS in adipocyte physiology and studies by Matias et al have confirmed the presence of the endogenous endocannabinoids 2-AG and AEA in murine white adipocytes *in vitro* [76]. These findings suggest that adipocytes could contribute directly to dysregulation of the ECS affecting obesity.

In the experiments presented here, CB_1 antagonism induced expression of GPDH, a late marker of adipocyte maturation without an associated increase in lipid droplet accumulation measured by oil red O, thus modifying another process in adipose tissue accumulation. These findings are consistent with those published in previous studies. Gary-Bobo et al have also

demonstrated a CB_1 antagonist-induced increase in expression of terminal markers of adipogenesis, GPDH and adiponectin, that was not accompanied by lipid droplet accumulation [207]. The authors hypothesise that CB_1 antagonism may cause an uncoupling of the association between the inhibition of pre-adipocyte proliferation and lipid accumulation through adipogenesis [207]. Though atypical, this property may also account for some of the anti-obesity effects seen with CB_1 blockade but further work is required to clarify this.

CB_2 agonists and antagonists had no effect on 3T3-L1 proliferation. The results from these experiments suggest that CB_2 does not play a significant role in adipocyte biology as modulation of CB_2 in the present studies had no effect on proliferation, and CB_2 transcript expression remained unchanged throughout adipogenesis.

There do not seem to be any previous studies examining the effects of CB modulation in BAT. Neither CB_1 nor CB_2 modulation had any effect on the BAT human pre-adipocyte cell line PAZ6 proliferation, and transcript expression was virtually undetectable for both receptors throughout adipogenesis. These findings, though negative, are novel as there are no published data of similar studies, though further work would be required to confirm this. It is, however, also important to recognise the limitations of the PAZ-6 cell line, which is not always easy to use and does not differentiate as readily in reponse to environmental stimuli as the 3T3-L1 cell line. These characteristics may also have contributed to the negative findings in the present studies of CB modulation in BAT.

Our experiments did not confirm the presence of the BAT-specific marker, UCP-1, in terminally differentiated 3T3-L1. This is in contrast to a recent study by Perwitz et al who demonstrate that CB_1 blockade with the antagonist SR 141716, or receptor knock down with small interfering RNA (siRNA), induces UCP-1 expression in murine white adipocytes in a time- and concentration-dependent manner at both RNA and the protein level [209]. This was also associated with an augmentation in the number of mitochondria present as

indicated by mitochondria-specific fluorescent staining and electron microscopy. Furthermore, SR 141716 treatment enhanced cytochrome C oxidase activity and increased cellular oxygen consumption [209]. These important findings suggest that CB_1 blockade could promote transdifferentiation of white adipocytes into a BAT phenotype, and may thus contribute to weight loss and improved energy homeostasis. The discrepancy between these studies may be atrributed to the different CB_1 antagonists employed or due to possible differences in the WAT cell lines.

The studies described in this chapter suggest that the inhibition of pre-adipocyte proliferation, along with induction of adipocyte maturation, may in part explain the peripheral anti-obesity effects of CB_1 antagonist treatment by a reduction in total body fat mass. However, these data have been obtained from work performed using *in vitro* models of animal-derived cells, and therefore cannot be conclusively applied to human physiology without further study. The next chapter aims to address this by replicating these experiments in a human model with the use of primary pre-adipocytes.

CHAPTER 4: EFFECT OF CANNABINOID RECEPTOR MODULATION ON PROLIFERATION AND DIFFERENTIATION IN HUMAN PRIMARY PRE-ADIPOCYTES.

4.1 INTRODUCTION

The previous chapter has described the effects of CB receptor modulation on pre-adipocyte proliferation and adipogenesis using *in vitro* models with the 3T3-L1 and PAZ6 cell lines representing WAT and BAT respectively. These studies demonstrated no influence of CB receptor agonism or antagonism on PAZ6 biology, but significant effects, albeit small, were noted in 3T3-L1. However, there are certain limitations associated with conclusions drawn from investigations performed with the use of cell lines. Firstly, 3T3-L1 is a murine cell line therefore any study conclusions have to be applied with caution to human physiology. One difference is that cell lines such as 3T3-L1 undergo one or two cycles of cell division prior to differentiation, whereas human pre-adipocytes can undergo adipogenesis without post-confluence mitosis [161]. Cell lines are specifically developed outside their natural environment with an exaggerated potential for proliferation and lipid accumulation, and are thus not exposed to factors which may influence normal physiology *in vivo*. There is a commercially available immortalised human pre-adipocyte cell line with a preserved differentiating capacity, Chub-S7, derived from the subcutaneous adipose tissue by co-expression of human telomerase reverse transcriptase and papillomavirus E7 oncoprotein genes [210]. The use of this cell line is however, limited by its expense. Furthermore, there is increasing evidence that white pre-adipocytes derived from different locations have distinct molecular and physiological properties. Subcutaneous and visceral adipocytes retain differences in gene expression, adiponectin secretion, and insulin action and signalling, and excess visceral adiposity is associated with an increased risk of DM and CVD [11, 12, 211]. Though 3T3-L1 represents a model of WAT it cannot account for these depot specific

differences that have been observed in humans. In order to address these short-comings it was necessary to repeat some of the previous experiments with human primary cultures derived from both subcutaneous and adipose tissue depots.

4.1.1 Human Primary Cultures

While the use of human primary cultures may be preferred over non-human cell lines for the reasons discussed above, this is not without its own difficulties. Adipose tissue is an internal organ and therefore obtaining samples requires a surgical procedure. Such a project therefore requires approvals from local Research and Development departments as well as the local Research Ethics Committee. These processes are time-consuming and therefore prolong the duration of any proposed study. Care must also be taken to ensure the study is performed in accordance with the 'Declaration of Helsinki' [212]. Individuals participating in these studies must be approached appropriately and informed consent needs to be obtained, as well as co-operation from surgical colleagues and theatre staff not directly involved in the project. Once isolated, primary pre-adipocytes are more susceptible to contamination due to the methods used for extraction and these cells also exhibit considerably slower rates of proliferation compared to cell lines thus similar experiments require longer periods of study. Unlike the immortalised cell lines, primary cells can enter a period of senescence and thus cells of low passage number (in these studies < 2) are required, further limiting the number of experiments possible with any given sample. Despite these drawbacks human primary cultures are still considered the 'gold standard' method for *in vitro* studies and results obtained from such work can be applied to human physiology with greater confidence than cell lines.

4.1.2 Adipose Tissue Depot-Specific Differences

In vivo, WAT is present in a variety of locations, including subcutaneous, omental, pre-orbital, synovial, bone marrow and retroperitoneal depots. Pre-adipocytes isolated from different areas have different adipogenic potential, the basis for which is not fully understood. Individuals with increased visceral adiposity have a greater risk of insulin resistance, dyslipidaemia, and cardiovascular disease than those of equivalent weight but a higher degree of subcutaneous adiposity [3]. Surgical removal of visceral fat by omentectomy improves insulin sensitivity while elimination of subcutaneous fat by liposuction has limited effect on insulin resistance and metabolic abnormalities [213, 214].

Intra-abdominal and subcutaneous adipose tissue exhibit important metabolic differences that may underlie the association of visceral, but not subcutaneous, fat with obesity-related cardiovascular and metabolic problems [215]. Expression of adiponectin and C/EBPα (which regulates adipogenesis and adiponectin gene expression), have been shown to be higher in subcutaneous adipocytes, whereas cellular inhibitor of apoptosis2 (CiAP2) mRNA expression is greater in omental adipocytes [211, 216]. CiAP2 may be involved in the regulation of TNFα signaling, therefore this raises the possibility that depot-specific differences may exist in the regulation of adipocyte apoptosis [216]. Previous studies have also demonstrated a two- to three-fold higher insulin-stimulated glucose uptake in visceral adipocytes as well as exaggerated lipolysis particularly in response to adrenergic stimulation, resulting in higher free fatty acid (FFA) concentrations [211, 217]. Circulating FFAs promote insulin resistance in the liver and muscle, and increase hepatic gluconeogenesis and lipoprotein production.

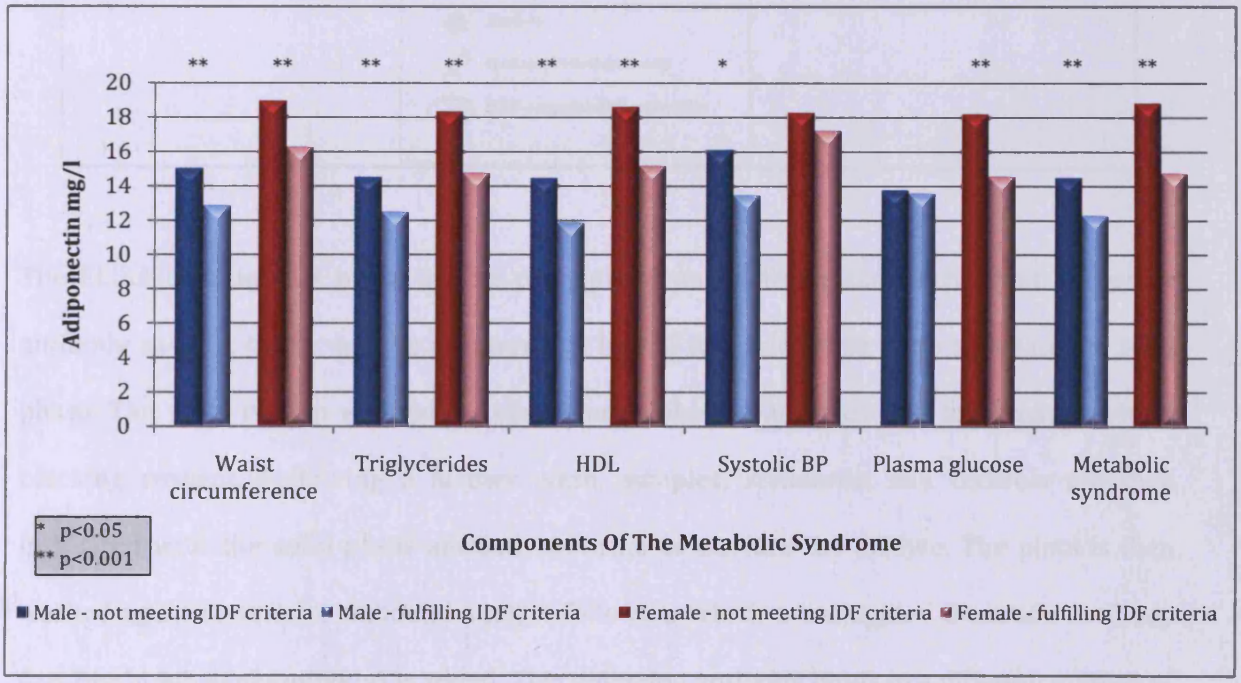
4.1.3 Adiponectin

Another hypothesis to explain the physiological mechanism linking excess body fat to adverse metabolic, and thus health, consequences is thought to be via modification of the

adipocytokine profile, the details of which have been discussed in Chapter 1. A reduction in adiponectin or an elevation in TNFα, IL-6, and leptin is associated with greater insulin resistance. Previous investigators have illustrated the inverse correlation of adiponectin levels with most components of the metabolic syndrome, defined by the IDF criteria (detailed in Chapter 1), as illustrated in Figure 4.1 [218]. Measurement of this key adipocytokine was therefore included in the present studies of CB_1 receptor modulation on adipogenesis.

Figure 4.1: Association Of Reduced Serum Adiponectin With Features Of The Metabolic Syndrome In Males & Females As Defined By The IDF Criteria

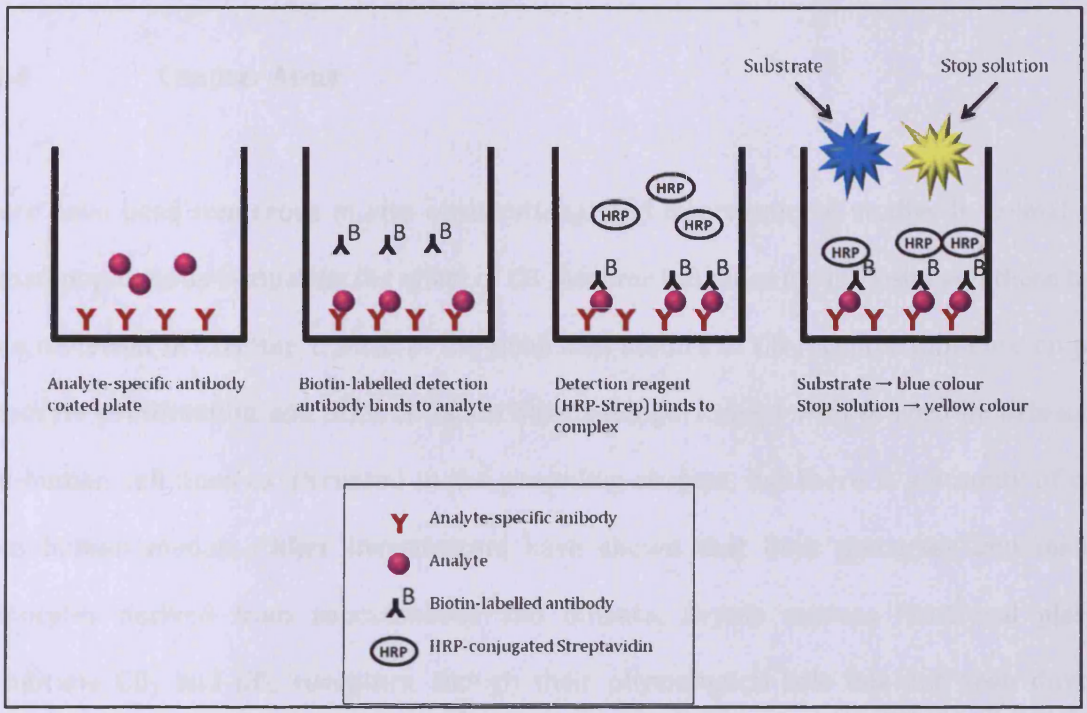
(adapted from Santaniemi et al [218])



4.1.3.1 Enzyme-Linked Immunosorbent Assay (ELSIA)

As previously discussed in Chapter 1, adiponectin is found in low and high molecular weight forms with the latter being metabolically more important. Most of the commercially available assays, including Duoset® ELISA (R&D systems, Oxford, UK) used in this study measure total adiponectin using the following method illustrated in Figure 4.2 [219].

Figure 4.2: Schematic Representation Of The ELISA Technique



The ELISA technique is based on the principle of an 'antibody sandwich'. First, a capture antibody specific to the analyte of interest is bound to a microtitre plate to create the solid phase. The plate is then washed to remove any unbound antibody and then coated with a blocking reagent. Following a further wash, samples, standards, and controls are then incubated with the solid phase antibody in order to capture the analyte. The plate is then washed again to remove unbound analyte following which a conjugated detection antibody (e.g. biotin-labelled antibody) is added. This detection antibody binds to a different epitope of the molecule being measured, completing the sandwich. This process is again followed by a wash to remove unbound detection antibody, after which a detection reagent (e.g. Horseradish-peroxidase (HRP) conjugated streptavidin) is added. The plate is washed and a substrate solution (e.g. tetramethylbenzidine peroxide) is added. The substrate is converted by the HRP to a blue coloured product in proportion to the amount of bound analyte. Colour development is stopped upon the addition of an acidic solution turning the colour yellow, and

the intensity of the yellow colour is measured by reading the optical density at A450 on a microtitre plate reader.

4.1.4 Chapter Aims

There have been numerous *in vivo* observational and interventional studies in animal and human populations evaluating the effect of CB receptor influence on adiposity and these have been reviewed in Chapter 1. Most of the published studies of CB receptor influence on pre-adipocyte proliferation and differentiation have been performed with *in vitro* models using non-human cell lines as discussed in the preceding chapter, but there is a scarcity of data from human models. Other investigators have shown that both precursor and mature adipocytes derived from subcutaneous and omental depots express functional plasma membrane CB_1 and CB_2 receptors, though their physiological role has not been directly examined *in vitro* [220].

In order to address some of the uncertainties regarding the extrapolation of cell line data to humans and the depot specific differences seen in WAT physiology, this chapter aims to assess the biological effect of CB_1 receptor agonism and antagonism on proliferation and adipogenesis using human primary cultures, both to confirm or refute the findings from the previous cell line studies, and to compare these effects in cells derived from subcutaneous and omental adipose tissue.

4.2 MATERIALS AND METHODS

4.2.1 Study Approvals & Patients Studied

Approvals were obtained from the Cardiff and Vale NHS Trust Research and Development Office and the South Wales Research Ethics Committee to approach individuals undergoing elective, open abdominal surgery for non-metabolic causes at the University Hospital of Wales, Cardiff. Following admission to the surgical wards, potential study participants were provided with a patient information sheet (Appendix 1). The exclusion criteria applied were pregnancy or treatment with the oral contraceptive pill, oestrogens, androgens, glucocorticoids, growth hormone or thyroxine therapy due to the well-established effects of these hormones on adipocyte function. Patients with lipodystrophy, and subjects receiving highly active antiretroviral therapy (HAART) for HIV infection or other conditions/treatment with a known effect on adipose tissue were also excluded as were diabetics unless treated with dietary therapy alone. Informed consent was obtained (Appendix 2) and data were collected on the individual's age, past medical and drug history, as well as measurements of height, weight, waist and hip circumference, and body fat percentage (Tanita Body fat monitor/scale TBF-538, Tanita corporation, Tokyo, Japan). The surgeons (Mr. M Puntis, Mr. B Rees and Mr. N Kumar) kindly harvested paired subcutaneous and omental adipose tissue samples during surgery for subsequent extraction of pre-adipocytes.

4.2.2 Reagents And Culture Media

The CB receptor modulators, cell culture materials and methods were identical to those used in the cell line experiments with 3T3-L1 as described in the previous chapter. All culture medium constituents were obtained from BioWhittaker (Belgium) unless otherwise stated.

4.2.3 Explants

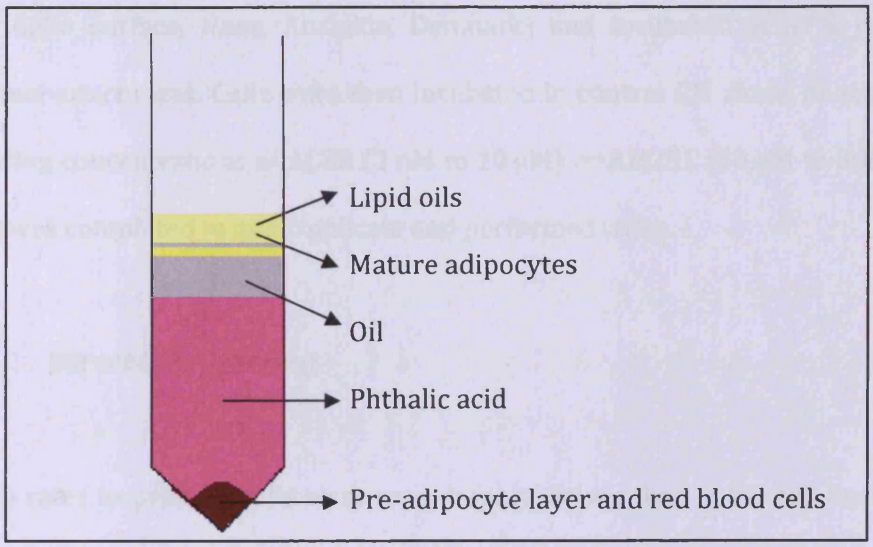
Adipose tissue samples were cut into small fragments (0.5-1 mm diameter), placed into 6 well plates and covered with just enough CM to prevent sample dehydration. A few drops of fresh CM were added every 48 hours. Pre-adipocytes were noted to migrate out of the explants and attach on to the surface of the culture plate, however it would take several weeks to collect enough pre-adipocytes by this process for use in experiments, therefore the collagenase digest method was used instead.

4.2.4 Collagenase Digest

Primary pre-adipocytes were successfully isolated from paired subcutaneous and omental adipose tissue depots (n=8) by collagenase digest followed by centrifugation through phthalic acid dionyl ester. The adipose tissue samples retrieved from theatre were cut into 0.5-1 mm fragments and approximately 3 ml adipose tissue was placed into individual 20 ml universal containers. The tissue was combined with 7 mls Hank's balanced salt solution (HBSS), 2 mls 7.5% w/v bovine serum albumin (BSA, Sigma Aldrich) and 1 ml (30mg/ml) type II collagenase, and incubated for 1 hour in a 37°C water bath while shaking every 5 minutes. The contents were then transferred to a clean 15 ml falcon tube and 2 mls phthalic acid dionyl ester mixed isomer was added to each sample prior to centriguation at 1500 rpm for 5 minutes at 4°C to allow separation into different cell and lipid layers as illustrated in Figure 4.3.

The supernatant was removed and the resultant cell pellet was resuspended in 10 mls CM in a 75 cm^2 Nunclon™ delta surface flask (Nunc, Roskilde, Denmark). Initially red blood cell removal was attempted with ammonia sulphate but this was not very effective therefore after 24 hours the cells were washed twice with 5 mls HBSS to remove any residual red cells, and this process was repeated after a further 24 hours if still required.

Figure 4.3: Isolation Of Primary Pre-Adipocytes Using Collagenase Digest Followed By Centrifugation Through Phthalic Acid Dionyl Ester.



4.2.5 Routine Maintenance Of Human Primary Pre-Adipocytes

All cell culture experiments were performed in sterile conditions at 37°C in an atmosphere containing humidified 5% CO_2 in air. Both subcutaneous and omental pre-adipocytes were grown in 75 cm^2 flasks. Culture medium was refreshed every 48 hours and upon reaching confluence, the cells underwent passage by trypsinisation as described in Chapter 3. It was noted that the primary pre-adipocytes invariably became senescent after the third passage, therefore where possible proliferation experiments were performed after the first, and differentiation experiments after the second passage.

4.2.6 Proliferation Studies

4.2.6.1 Cell Culture Protocol

Subcutaneous and omental pre-adipocytes were grown to confluence and trypsinised as described previously. The approximate number of cells per ml solution was calculated with

the aid of a haemocytometer (Camber counter, Weber Scientific International Ltd, Middlesex, UK). Cells were subsequently diluted and seeded at $1x10^3$ per well in 24 well plates (Nunclon™ delta surface, Nunc, Roskilde, Denmark) and incubated at 37°C overnight to enable optimal attachment. Cells were then incubated in control CM alone, or supplemented with increasing concentrations of ACEA (1 nM to 10 μM) or AM251 (10 nM to 500 nM). Each experiment was completed in quadruplicate and performed twice.

4.2.6.2 Direct Cell Counting

Proliferation rates in primary cells were considerably slower than in the cell lines, therefore the pre-adipocytes were collected for direct cell counting after 72 and 168 hours of treatment. Each well was washed with 0.5 mls of 1% w/v trypsin and then incubated with a further 0.5 mls trypsin until cells were free in suspension. The contents of individual wells were transferred to corresponding cell counting cuvettes containing 10 mls each of Coulter Isoton II Diluent (Beckman Coulter, Gmbh, Germany). Cell densities were measured using a Coulter particle counter (Z2, Beckman Coulter Gmbh, Germany) with particle size set between 3.8 nm and 9.0 nm. Each sample was counted twice and the average value used for statistical analysis.

4.2.7 Adipogenesis Studies

4.2.7.1 Cell Culture Protocol

Subcutaneous and omental pre-adipocytes were plated in 6 well plates and adipogenesis induced in confluent populations of pre-adipocytes by replacing CM with DM as described in Chapter 3. Cells were maintained in DM alone, or supplemented with 100nM ACEA or 100nM AM251, for 21 days with DM changes occurring every 72 hours. Morphological changes of differentiation were monitored by microscopic examination.

4.2.7.2 Isolation Of Total RNA & Reverse Transcription To cDNA

Samples for RNA extraction were collected on days 0, 7, 14 and 21. Total RNA was isolated using Invitrogen's TRIzol reagent as described in the previous chapter. The RNA concentration and quality was assessed by spectrophotometry (GeneQuant, GE Healthcare) and analysis on a 1.2% agarose gel. Samples were stored at -80°C until further use. Also, as per the cell line study methods, the RNA was treated with DNase, reverse transcribed to cDNA, and conventional PCR performed with primers for the human housekeeping gene PGK-1 to confirm the absence of contaminating genomic DNA.

4.2.7.3 QPCR Measurement Of Markers Of Adipogenesis

Real-time QPCR was performed to measure the expression of LPL, a terminal marker of adipogenesis and the housekeeper gene APRT. QPCR was performed using Stratagene's Brilliant® SYBR® Green Master Mix. The reaction components and thermal cycling protocol were identical to those detailed in chapter 3. Standard curves obtained from serial dilutions of PCR amplicons sub-cloned into pGEM-T vectors at 10^6 to 10^2 copies were included for each gene.

4.2.7.4 ELISA Measurement Of Adiponectin

Adiponectin concentrations were measured in conditioned medium collected on days 0, 7, 14 and 21 of the differentiation protocol using the Human Adiponectin Duoset® ELISA development system (R&D Systems, Oxford, UK) according to the manufacturer's instructions. Costar ELI 96 well plates (R&D Systems, Oxford, UK) were coated with 100 µl / well capture antibody (55 µl / ml PBS) and stored overnight. Prior to use the next day, the plates were washed twice with wash buffer (0.05% Tween 20 in PBS, pH 7.4) following which the wells were blocked with diluent (1% w/v BSA in PBS) for 2 hours. After repeating the wash process, 100 µl of serially diluted adiponectin standards (25 to 4000 pg / ml) or 100 µl neat

conditioned medium was added to individual wells and incubated at room temperature for 2 hours. Once again the plates were washed and each well coated with 100 µl horseradish peroxidase conjugated Streptavidin (50 µl / ml), incubated at room temperature, washed again and coated with 100 µl substrate solution (tetramethylbenzidine peroxide). The reaction was terminated by the addition of 50 µl stop solution (10% sulphuric acid) and optical density was measured using an OpsysMR microplate reader (Dynex Technologies, Chantilly, USA) set to 450nm. The intra- and inter- assay coefficient of variation was less than 4%.

4.2.8 Statistical Analysis

Data were analysed using the SPSS (version 14.0) statistical software package.

The data generated by the proliferation and adipogenesis studies were not normally distributed (determined by generating Q-Q and P-P plots of data obtained) and therefore analysed according to median and interquartile ranges (IQR) using non-parametric statistical tests. The Friedman's test was performed on each experiment and the Wilcoxon Signed Ranks test was performed on individual treatments relative to control conditions. The QPCR data were analysed as absolute values per input microgram of mRNA and corrected relative to the appropriate housekeeper gene expression.

4.3 RESULTS

4.3.1 Study Participant Demographics

Table 4.1 lists the anthropometric measurements of the patients who participated in this study. Paired subcutaneous and omental adipose tissue samples were collected from all 8 patients.

Table 4.1: Anthropometric Data Of Study Participants

Patient	Age (years)	Sex	Height (m)	Weight (kg)	BMI	Waist:Hip	Fat %
1	55	M	1.78	69.9	22.1	1.02	10
2	76	M	1.75	62.2	20.3	0.94	8.5
3	75	M	1.78	82	25.9	0.88	26
4	64	F	1.63	74	27.9	0.83	31
5	74	F	1.55	48	20.0	1.0	27
6	74	F	1.63	59.5	22.4	0.85	30
7	30	F	1.55	60	25	-	-
8	66	F	1.52	57	24.7	0.77	35

4.3.2 Primary Pre-Adipocyte Cell Culture

Examples of the microscopic appearance of the primary pre-adipocytes, including the presence of atypical cells in the omental adipose tissue, are given in Figure 4.4. The primary pre-adipocytes were noted to be larger than the cell lines and exhibited much slower proliferation rates in comparison, with a population doubling time of almost 5 days, therefore

the number of cells available for use in experimentation was more limited than with the cell lines.

Figure 4.4A : Subcutaneous Pre-Adipocytes

(magnification 200x)

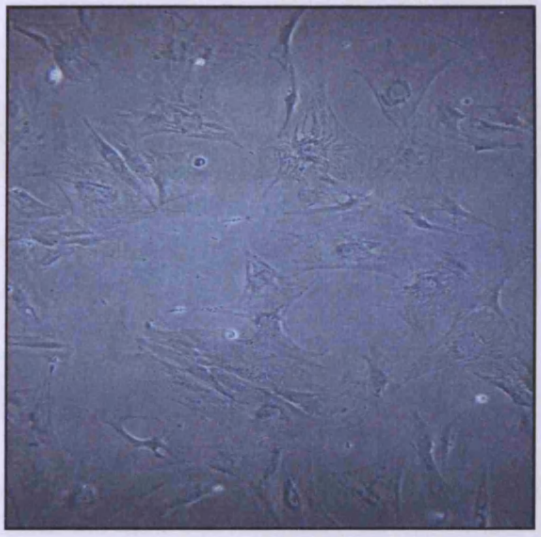
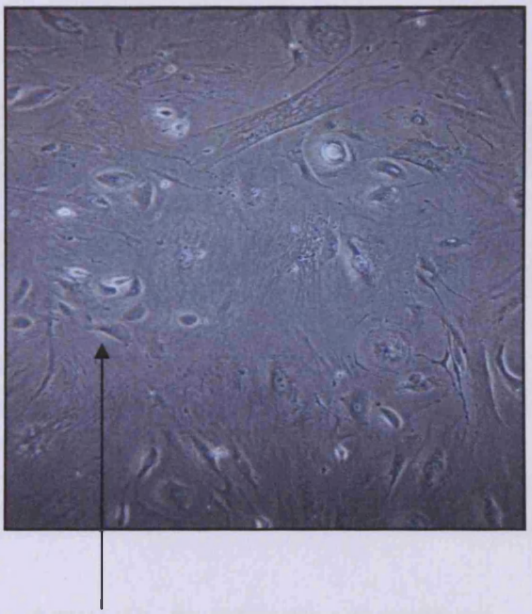


Figure 4.4B: Omental Pre-Adipocytes

(magnification 200x)



Pre-adipocytes derived from all 8 individuals were used in the proliferation experiments but only the first 5 were used in the adipogenesis studies. This was due to difficulties encountered with the initial pre-adipocyte extraction method (explants) where sufficient numbers of cells could not be generated despite prolonged cell culture. Also it was noted in our early experiments that it was not possible to induce differentiation in primary pre-adipocytes beyond the third passage due to cell senescence. Lastly in one individual who underwent surgery for abdominal malignancy, a significant number of atypical cells were noted in the omental samples (Figure 4.4B) and thus could not be used for our studies.

4.3.3 CB_1 Agonist ACEA Stimulates Subcutaneous Pre-Adipocyte Proliferation

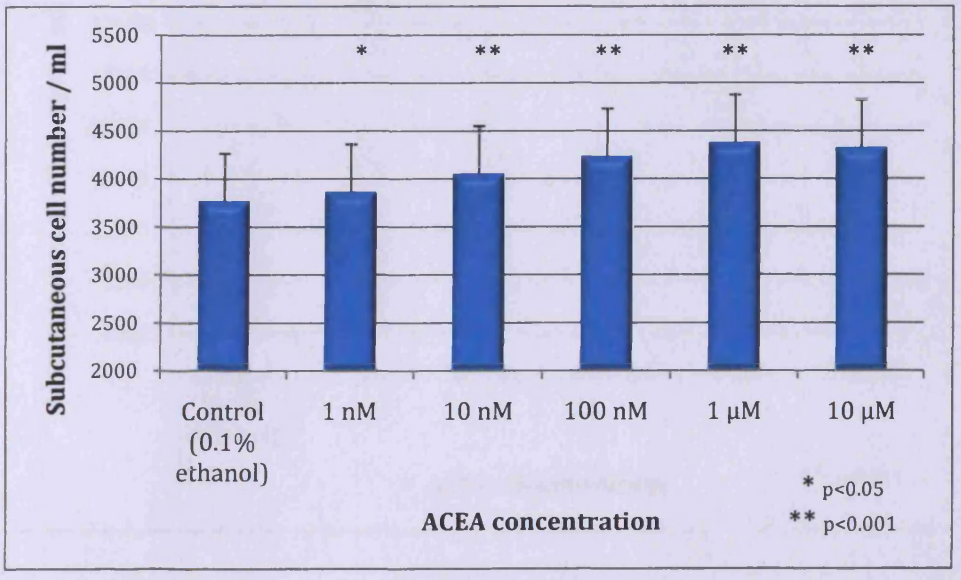
Exposure to the selective CB_1 agonist ACEA for 72 hours was associated with a significant, concentration-dependent increase in proliferation of the subcutaneous pre-adipocytes as illustrated in Table 4.2 and Figure 4.5.

Table 4.2 Mean, Median & IQR For Subcutaneous Cells Treated With ACEA For 72 Hours

ACEA concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	3766	3876	3332-4162	n/a
1 nM	3861	3904	3468-4232	0.029
10 nM	4050	4123	3602-4524	**<0.001**
100 nM	4230	4317	3741-4711	**<0.001**
1 µM	4375	4528	3734-4965	**<0.001**
10 µM	4324	4411	3706-4902	**<0.001**

Figure 4.5: Effect Of ACEA On Subcutaneous Pre-Adipocyte Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



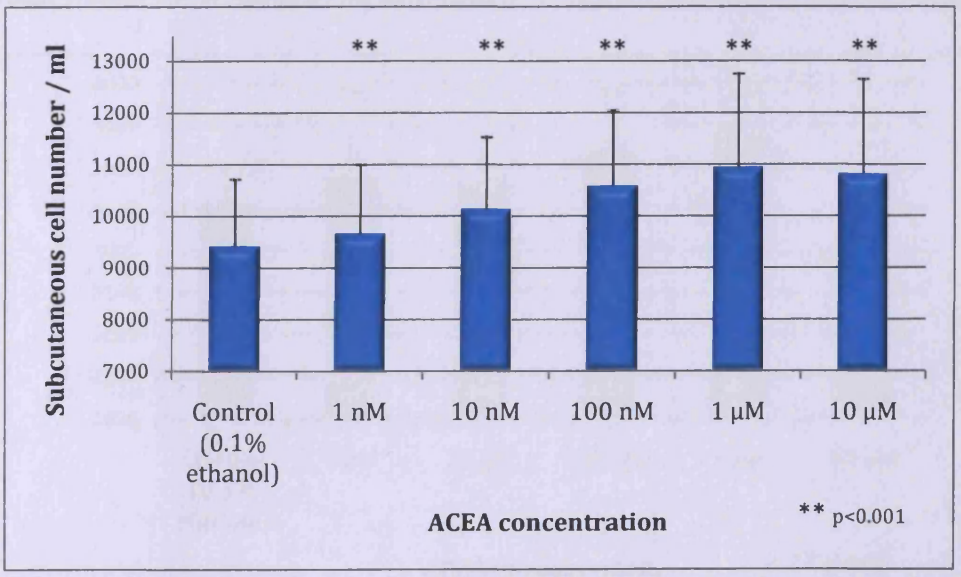
Exposure to the CB_1 agonist ACEA for 168 hours was also associated with a significant, concentration-dependent increase in proliferation of the subcutaneous pre-adipocytes as illustrated in Table 4.3 and Figure 4.6.

Table 4.3 Mean, Median & IQR For Subcutaneous Cells Treated With ACEA For 168 Hours

ACEA concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	9416	9691	8329-10405	n/a
1 nM	9653	9760	8671-10582	<0.001
10 nM	10126	10308	9005-11312	<0.001
100 nM	10576	10793	9353-11779	<0.001
1 µM	10938	11320	9337-12410	<0.001
10 µM	10810	11028	92655-12255	<0.001

Figure 4.6: Effect Of ACEA On Subcutaneous Pre-Adipocyte Proliferation At 168 Hours

Cells were counted 168 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



4.3.4 CB_1 Agonist ACEA Stimulates Omental Pre-Adipocyte Proliferation

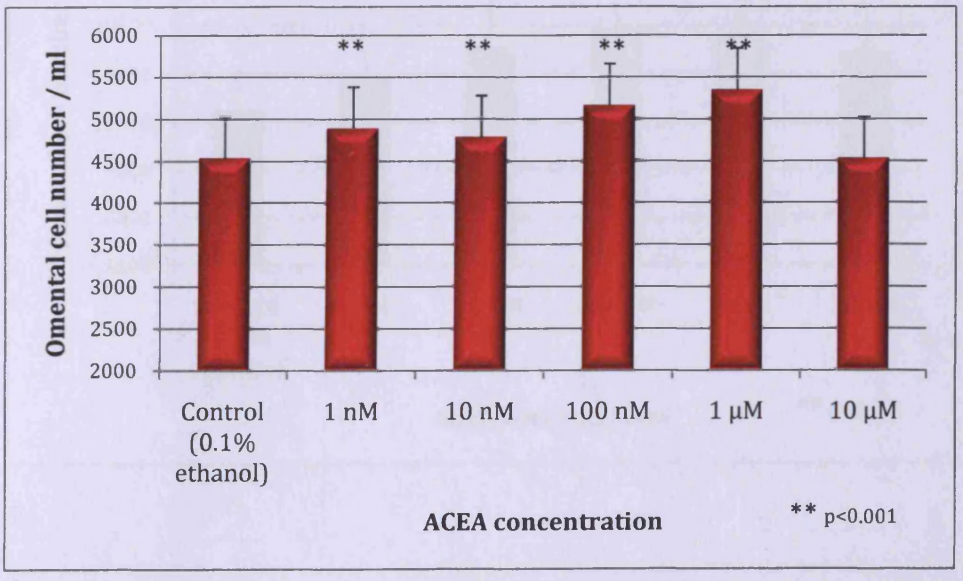
Exposure to the selective CB_1 agonist ACEA for 72 hours was associated with a significant, concentration-dependent increase in proliferation of the omental pre-adipocytes as illustrated in Table 4.4 and Figure 4.7.

Table 4.4: Mean, Median & IQR For Omental Cells Treated With ACEA For 72 Hours

ACEA concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	4531	4564	4193-4937	n/a
1 nM	4882	4830	4500-5294	<0.001
10 nM	4780	4677	4306-5139	<0.001
100 nM	5157	4979	4571-5648	<0.001
1 µM	5344	5312	4913-5780	<0.001
10 µM	4527	4495	4168-4901	0.753

Figure 4.7: The Effect Of ACEA On Omental Pre-Adipocyte Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



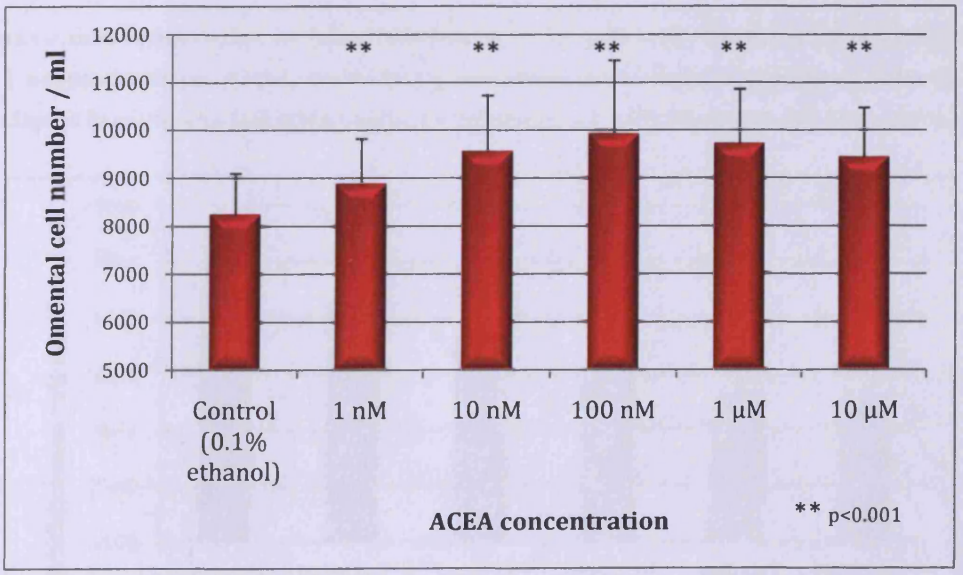
Exposure to the CB_1 agonist ACEA for 168 hours was associated with a significant, concentration-dependent increase in proliferation of the omental pre-adipocytes as illustrated in Table 4.5 and Figure 4.8.

Table 4.5: Mean, Median & IQR For Omental Cells Treated With ACEA For 168 Hours

ACEA concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	8239	8298	7624-8976	n/a
1 nM	8877	8782	8182-9627	<0.001
10 nM	9559	9355	8612-10277	<0.001
100 nM	9919	9575	8792-10862	<0.001
1 µM	9717	9658	8933-10508	<0.001
10 µM	9432	9354	8684-10210	<0.001

Figure 4.8: The Effect Of ACEA On Omental Pre-Adipocyte Proliferation At 168 Hours

Cells were counted 168 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Freedman's test for overall experiment.



4.3.5 CB_1 Antagonist AM 251 Inhibits Subcutaneous Pre-Adipocyte Proliferation

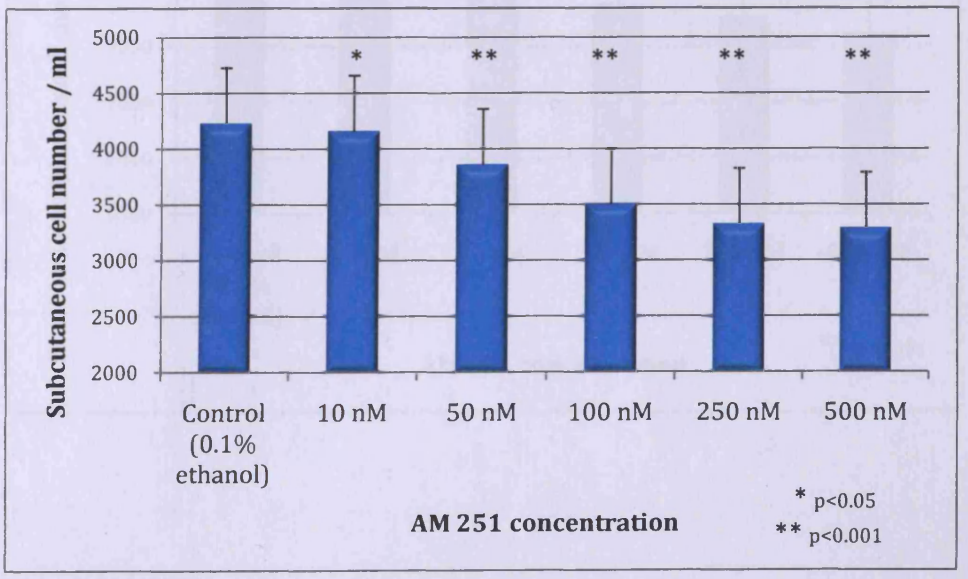
Exposure to the CB_1 antagonist AM 251 for 72 hours was associated with a significant, concentration-dependent reduction in proliferation of the subcutaneous pre-adipocytes as illustrated in Table 4.6 and Figure 4.9.

Table 4.6 Mean, Median & IQR For Subcutaneous Cells Treated With AM 251 For 72 Hours

AM 251 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	4226	4251	3809-4603	n/a
10 nM	4156	4195	3840-4489	0.036
50 nM	3855	3810	3542-4161	<0.001
100 nM	3502	3451	3211-3776	<0.001
250 nM	3322	3216	2954-3608	<0.001
500 nM	3286	3137	2977-3515	<0.001

Figure 4.9: Effect Of AM251 On Subcutaneous Pre-Adipocyte Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



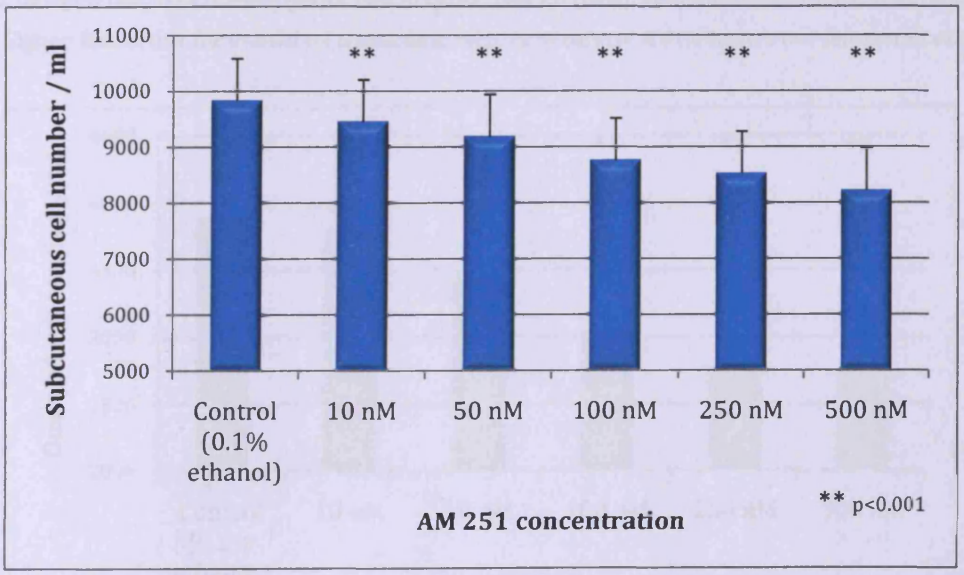
Exposure to the CB_1 antagonist AM 251 for 168 hours was associated with a significant, concentration-dependent reduction in proliferation of the subcutaneous pre-adipocytes as illustrated in Table 4.7 and Figure 4.10.

Table 4.7: Mean, Median & IQR For Subcutaneous Cells After AM 251 For 168 Hours

AM 251 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	9829	9885	8857-10705	n/a
10 nM	9447	9534	8728-10202	<0.001
50 nM	9179	9072	8433-9908	<0.001
100 nM	8755	8629	8027-9440	<0.001
250 nM	8518	8245	7577-9251	<0.001
500 nM	8216	7843	7444-8790	<0.001

Figure4.10 Effect Of AM251 On Subcutaneous Pre-Adipocyte Proliferation At 168 Hours

Cells were counted 168 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



4.3.6 CB_1 Antagonist AM 251 Inhibits Omental Pre-Adipocyte Proliferation

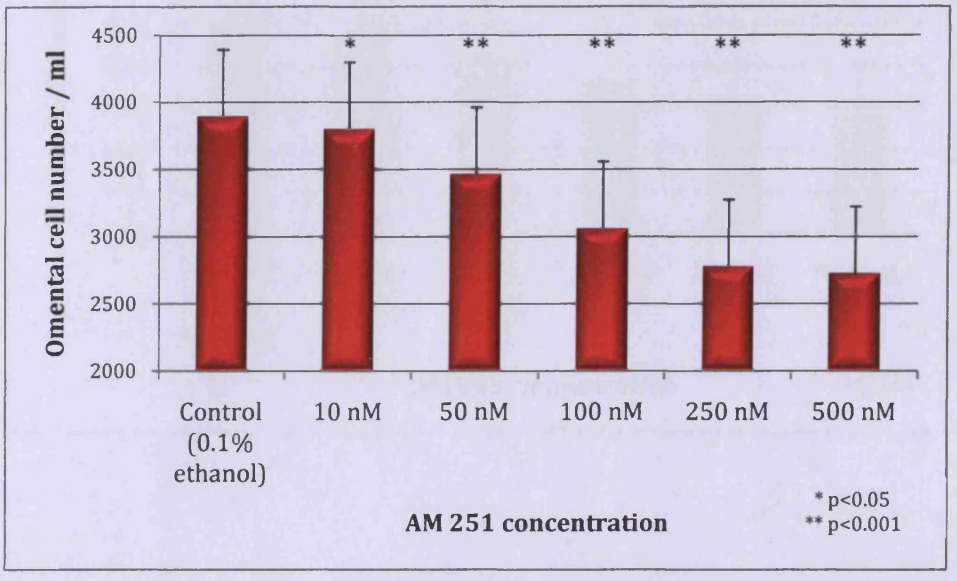
Exposure to the CB_1 antagonist AM 251 for 72 hours was associated with a significant, concentration-dependent reduction in proliferation of the omental pre-adipocytes as illustrated in Table 4.8 and Figure 4.11.

Table 4.8: Mean, Median & IQR For Omental Cells Treated With AM 251 For 72 Hours

AM 251 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	3889	3588	3178-4607	n/a
10 nM	3794	3509	3092-4445	0.001
50 nM	3459	3276	2811-4062	<0.001
100 nM	3055	2926	2601-3450	<0.001
250 nM	2773	2756	2334-3162	<0.001
500 nM	2721	2798	2235-3140	<0.001

Figure 4.11: The Effect Of AM 251 On Omental Pre-adipocyte Proliferation At 72 Hours

Cells were counted 72 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



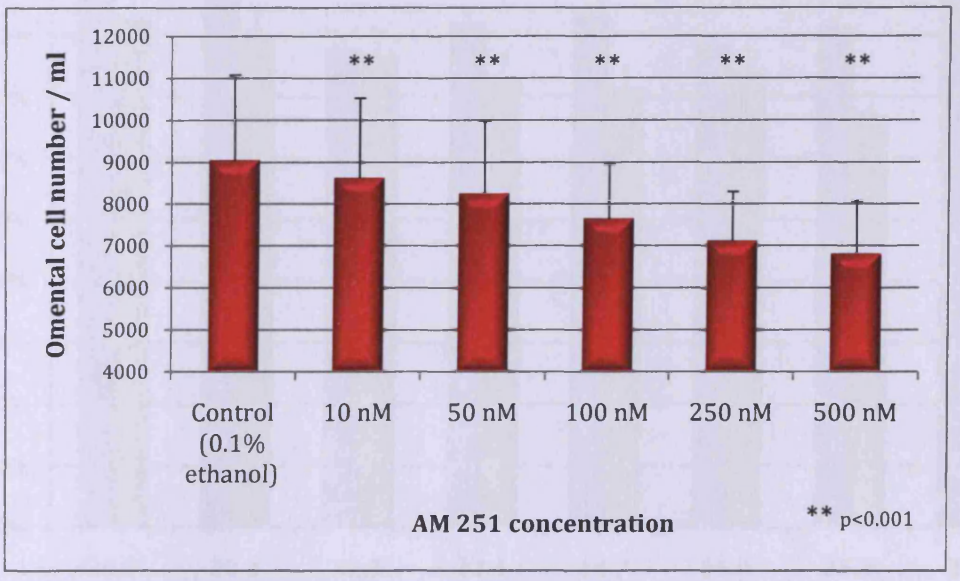
Exposure to the CB_1 antagonist AM 251 for 168 hours was associated with a significant, concentration-dependent reduction in proliferation of the omental pre-adipocytes as illustrated in Table 4.9 and Figure 4.12.

Table 4.9: Mean, Median & IQR For Omental Cells Treated With AM 251 For 168 Hours

AM 251 concentration	Mean Cell number/ml	Median Cell number/ml	IQR Cell number/ml	p-value (vs control)
Control (0.1% ethanol)	9046	8344	7392-10714	n/a
10 nM	8624	7976	7028-10101	**<0.001**
50 nM	8236	7801	6693-9672	**<0.001**
100 nM	7638	7315	6502-8626	**<0.001**
250 nM	7110	7067	5984-8108	**<0.001**
500 nM	6802	6996	5588-7850	**<0.001**

Figure 4.12: Effect Of AM 251 On Omental Pre-Adipocyte Proliferation At 168 Hours

Cells were counted 168 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control & Friedman's test for overall experiment.



4.3.7 CB_1 Antagonist AM 251 May Exert A Preferential Effect On Omental Pre-Adipocyte Proliferation

It was also noted that the inhibitory effect of CB_1 antagonism was greater in omental as compared to the subcutaneous cells obtained for individual patients at any given BMI in most but not all cases (Figure 4.13A). A trend was also observed for a greater reduction in omental preadipocyte number with increasing BMI but not in the subcutaneous populations. CB_1 agonist treatment was associated with an increased proliferation effect in the omental cells in subjects with a higher BMI (Figure 4.13B).

Figure 4.13A: Effect Of 500 nM AM 251 On Primary Pre-adipocyte Proliferation

Cells were counted 168 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for subcutaneous vs. omental for each individual patient.

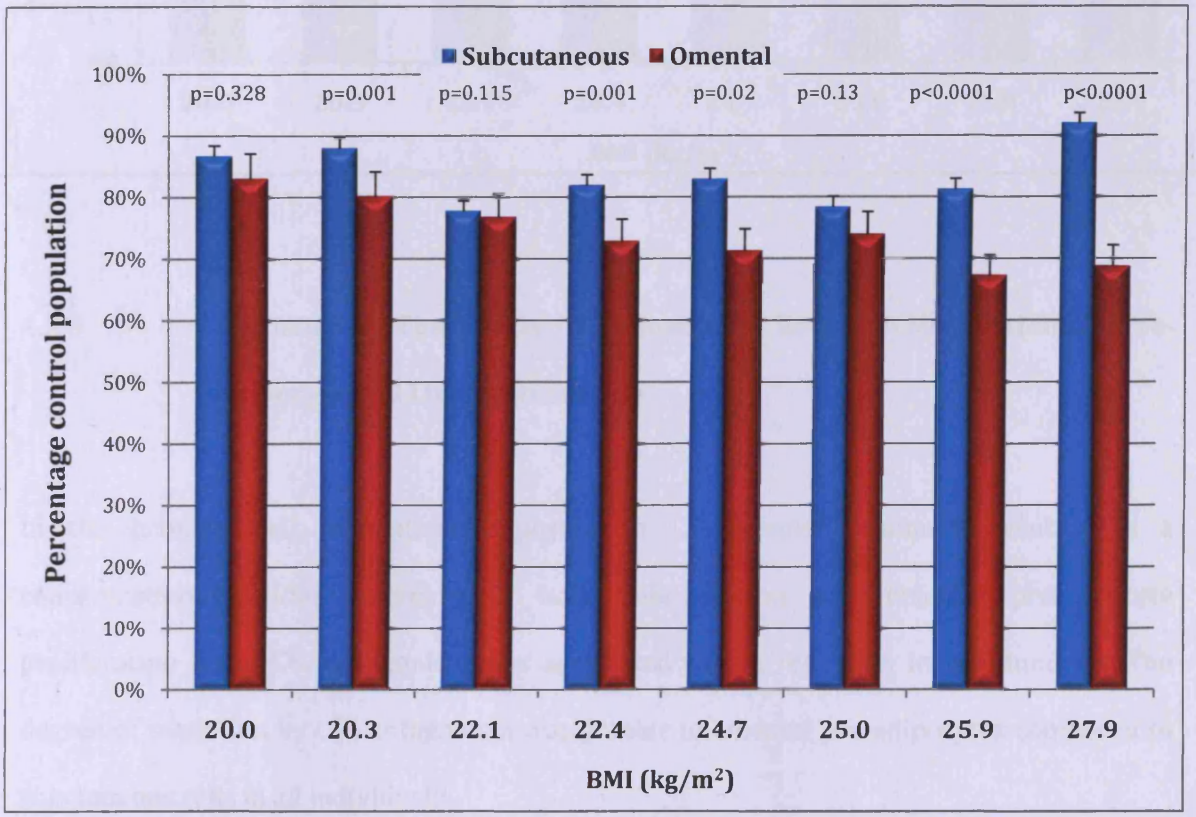
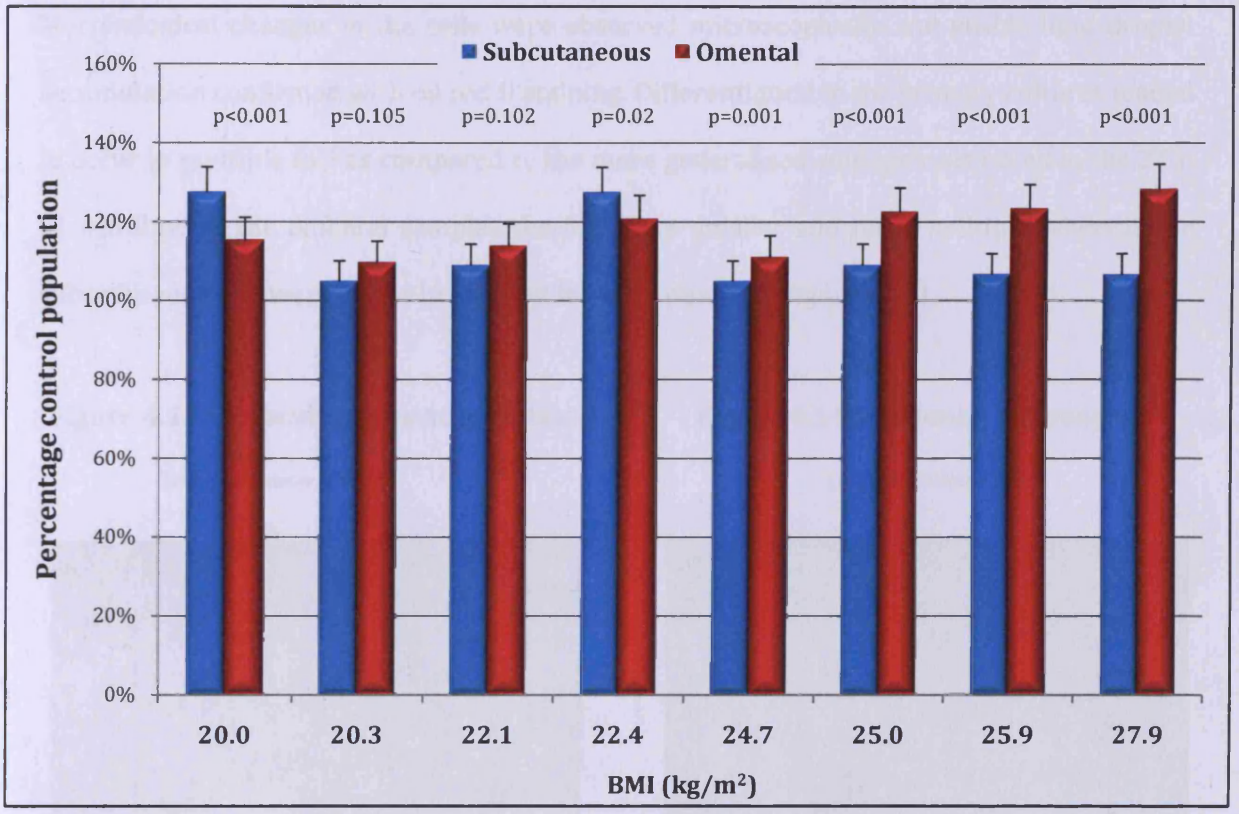


Figure 4.13B: Effect Of 10 μM ACEA On Primary Preadipocyte Proliferation

Cells were counted 168 hours after treatment after plating 1 x 10^3 cells/well. Results represent the mean and SD from 2 independent experiments performed in quadruplicate (n=8). Statistical significance determined by Wilcoxon Signed Ranks test for subcutaneous vs. omental for each individual patient.



4.3.8 Summary Of The Effects Of Cannabinoid Receptor Modulation On Pre-Adipocyte Cell Line Proliferation

In the primary cell populations exposure to CB_1 agonist treatment resulted in a concentration-dependent increase in both subcutaneous and omental preadipocyte proliferation while CB_1 antagonism was associated with a reduction in cell numbers. The degree of inhibition by CB_1 antagonism was greater in omental pre-adipocytes compared to subctaneous cells in all individuals.

4.3.9 Adipogenesis In Primary Subcutaenous & Omental Cell Cultures

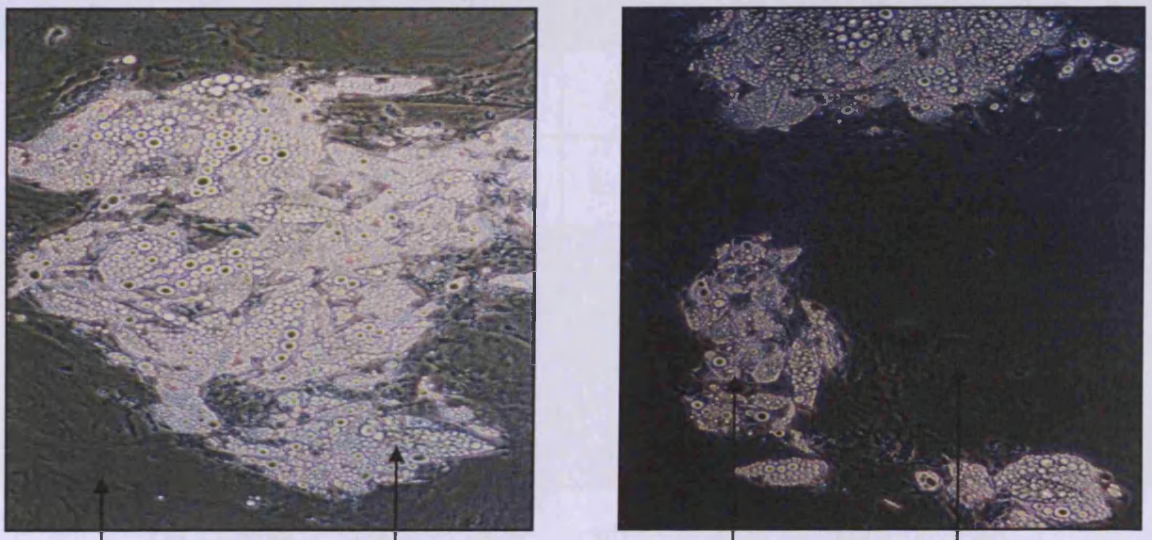
The effect of cannabinoid receptor modulation on adipogenesis was investigated by quantification of LPL expression by QPCR and measurement of adiponectin production. Morphological changes in the cells were observed microscopically and visible lipid droplet accumulation confirmed with oil red O staining. Differentiation in the primary cultures tended to occur in multiple foci as compared to the more generalised adipogenesis noted in the 3T3-L1 cell line. In the omental samples the foci were smaller and more multiple whereas the subcutaneous foci were larger in size and fewer in number (Figure 4.14).

Figure 4.14A Subcutaneous Adipocytes

(magnification 200x)

Figure 4.14B: Omental Adipocytes

(magnification 200x)



4.3.10 The Effect Of CB_1 Agonist ACEA & CB_1 Antagonist AM 251 On LPL Expression In Subcutaneous Adipogenesis

Treatment with the CB1 agonist ACEA had no significant effect on subcutaneous pre-adipocyte differentiation as measured by LPL expression whereas exposure to the CB1 antagonist AM 251 resulted in an increase in LPL expression (Table 4.10 and Figure 4.15).

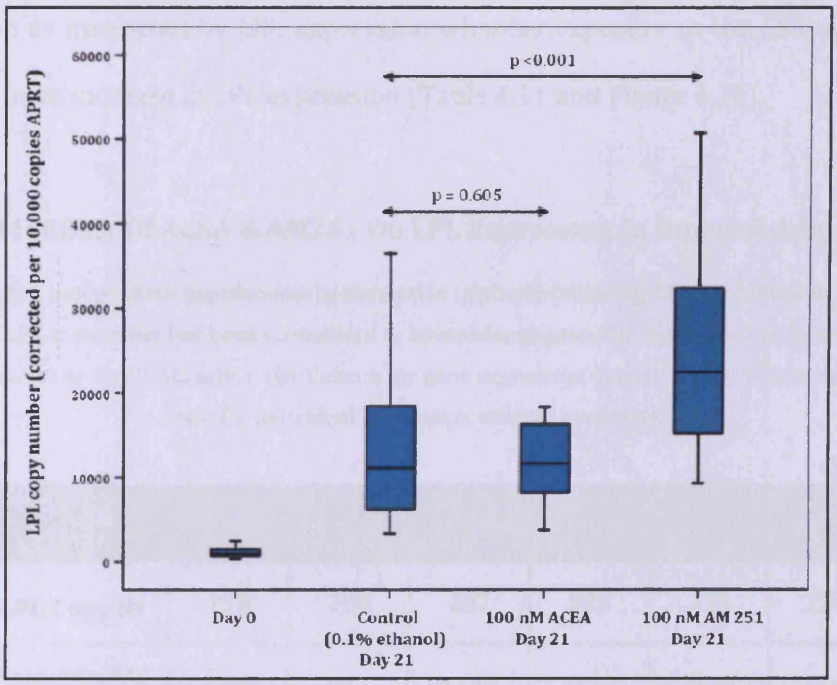
Table 4.10: Effect Of ACEA & AM251 On LPL Expression In Subcutaneous Adipogenesis

Data represent 2 independent experiments performed in triplicate following 21 days culture in DM, ACEA or AM251 (n=5). LPL expression has been normalised to housekeeper gene ARP expression and also expressed as fold-change relative to Day 0. Statistical significance for gene expression determined by Wilcoxon Signed Ranks test for individual treatments relative to control

Patient N		1	2	3	4	5	Mean	p-value
Day 0	**LPL Copy №**	223	255	331	255	270	267	n/a
	Normalised to APRT Copy №	999	1183	1248	1282	1312	1204	
Day 21 Control (0.1% ethanol)	**LPL Copy №**	**2738**	**2913**	**2266**	**3255**	**2201**	**2675**	**n/a**
	Normalised to APRT Copy №	**19257**	**13313**	**10281**	**16997**	**10700**	**14110**	
	FOLD change	**19.3**	**11.3**	**8.2**	**13.3**	**8.15**	**11.7**	
Day 21 100 nM ACEA	**LPL Copy №**	2356	3528	2877	3677	3387	3165	0.605
	Normalised to APRT Copy №	10685	18150	9006	20218	15752	14762	
	FOLD change	10.7	15.3	7.2	15.7	12.0	12.3	
Day 21 100 nM AM 251	**LPL Copy №**	**5741**	**5818**	**5818**	**4947**	**4477**	**5360**	**<0.001**
	Normalised to APRT Copy №	**37791**	**35380**	**30145**	**32070**	**26230**	**32323**	
	FOLD change	**37.8**	**29.9**	**24.2**	**25.0**	**20.0**	**26.8**	

Figure 4.15: Effect Of ACEA & AM251 On Subcutaneous Adipogenesis

Data represent 2 independent experiments performed in triplicate following 21 days culture in DM, ACEA or AM251 (n=5). LPL expression has been normalised to housekeeper gene ARP expression. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control.



4.3.11 The Effect Of CB_1 Agonist ACEA & CB_1 Antagonist AM 251 On UCP-1 Expression In Terminally Differentiated Subcutaenous Adipocytes

QPCR was performed on cDNA derived from RNA collected on day 0 and day 21 of the differentiation protocol and transcript expression for UCP-1 was normalised to housekeeper gene (APRT) expression. Transcript expression was at the limit of QPCR detection (Ct values > 35) and did not alter with adipogenesis in control conditions or when supplemented with ACEA or AM 251.

4.3.12 The Effect Of CB_1 Agonist ACEA & CB_1 Antagonist AM 251 On LPL Expression In Omental Adipogenesis

Treatment with the CB1 agonist ACEA had no significant effect on omental pre-adipocyte differentiation as measured by LPL expression whereas exposure to the CB1 antagonist AM 251 resulted in an increase in LPL expression (Table 4.11 and Figure 4.16).

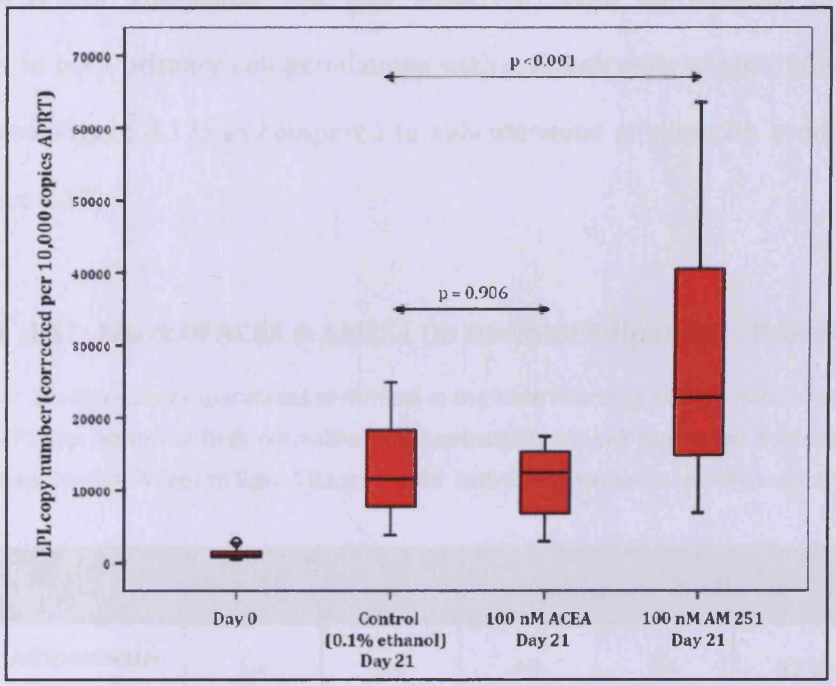
Table 4.11: Effect Of ACEA & AM251 On LPL Expression In Omental Adipogenesis

Data represent 2 independent experiments performed in triplicate following 21 days culture in DM, ACEA or AM251 (n=5). LPL expression has been normalised to housekeeper gene ARP expression and also expressed as fold-change relative to Day 0. Statistical significance for gene expression determined by Wilcoxon Signed Ranks test for individual treatments relative to control.

Patient №		1	2	3	4	5	Mean	p-value
Day 0	LPL Copy №	258	266	282	225	235	253	n/a
	Normalised to APRT Copy №	1368	957	1372	1412	1467	1315	
Day 21 Control (0.1% ethanol)	LPL Copy №	3671	2848	2664	438	237	2024	n/a
	Normalised to APRT Copy №	15209	15012	11943	2008	1099	9297	
	FOLD change	11.1	15.7	8.7	1.5	0.8	7.06	
Day 21 100 nM ACEA	LPL Copy №	2730	2410	3521	361	355	1875	0.906
	Normalised to APRT Copy №	10380	9467	13994	2469	1810	7624	
	FOLD change	7.6	9.9	10.2	1.7	1.2	5.8	
Day 21 100 nM AM 251	LPL Copy №	6149	6062	4678	363	347	3519	<0.001
	Normalised to APRT Copy №	40246	25499	19171	2323	1617	17771	
	FOLD change	29.4	26.6	14.0	1.6	1.1	13.5	

Figure 4.16: Effect Of ACEA & AM251 On Omental Adipogenesis

Data represent 2 independent experiments performed in triplicate following 21 days culture in DM, ACEA or AM251 (n=5). LPL expression has been normalised to housekeeper gene ARP expression. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control



4.3.13 The Effect Of CB_1 Agonist ACEA & CB_1 Antagonist AM 251 On UCP-1 Expression In Terminally Differentiated Omental Adipocytes

QPCR was performed on cDNA derived from RNA collected on day 0 and day 21 of the differentiation protocol and transcript expression for UCP-1 was normalised to housekeeper gene (APRT) expression. Transcript expression was at the limit of QPCR detection (Ct values > 35) and did not alter with adipogenesis in control conditions or when supplemented with ACEA or AM 251.

4.3.14 The Effect Of CB_1 Agonist ACEA & CB_1 Antagonist AM 251 On Adiponectin Production During Subcutaneous & Omental Adipogenesis

Treatment with CB_1 antagonist was also associated with an increase in adiponectin concentration in both primary cell populations with a significantly greater effect in omental (Table 4.12 and Figure 4.17) as compared to subcutaneous adiponectin production (Table 4.13 and Figure 4.18).

Table 4.12: Effect Of ACEA & AM251 On Omental Adiponectin Production

Data represent 2 independent experiments performed in triplicate following 21 days culture in DM, ACEA or AM251 (n=5). LPL expression has been normalised to housekeeper gene ARP expression. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control

Patient №		1	2	3	4	5	p-value
Day 0	**Adiponectin µg/ml**	54	59	49	54	33.0	n/a
Day 21 Control (0.1% ethanol)	**Adiponectin µg/ml**	149.5	960.0	233.5	447.6	187.5	n/a
	FOLD change	2.8	16.2	4.8	8.3	5.7	
Day 21 100 nM ACEA	**Adiponectin µg/ml**	143.0	940.5	211.0	431.5	206.0	0.394
	FOLD change	2.6	15.9	4.3	7.9	6.2	
Day 21 100 nM AM 251	**Adiponectin µg/ml**	1128.5	1417.5	825.5	1024.0	946.5	**0.026**
	FOLD change	20.8	24.0	16.8	18.9	28.6	

Figure 4.17: Effect Of ACEA & AM251 On Omental Adiponectin Production

Data represent 2 independent experiments performed in triplicate following 21 days culture in DM, ACEA or AM251 (n=5). LPL expression has been normalised to housekeeper gene ARP expression. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control

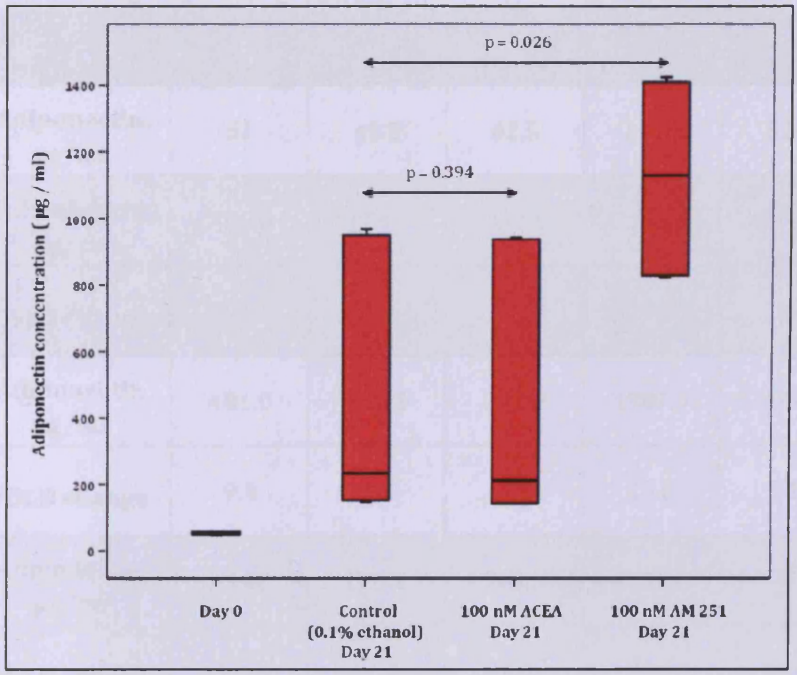


Figure 4.18: Effect Of ACEA & AM251 On Subcutaneous Adiponectin Production

Data represent 2 independent experiments performed in triplicate following 21 days culture in DM, ACEA or AM251 (n=5). LPL expression has been normalised to housekeeper gene ARP expression. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control

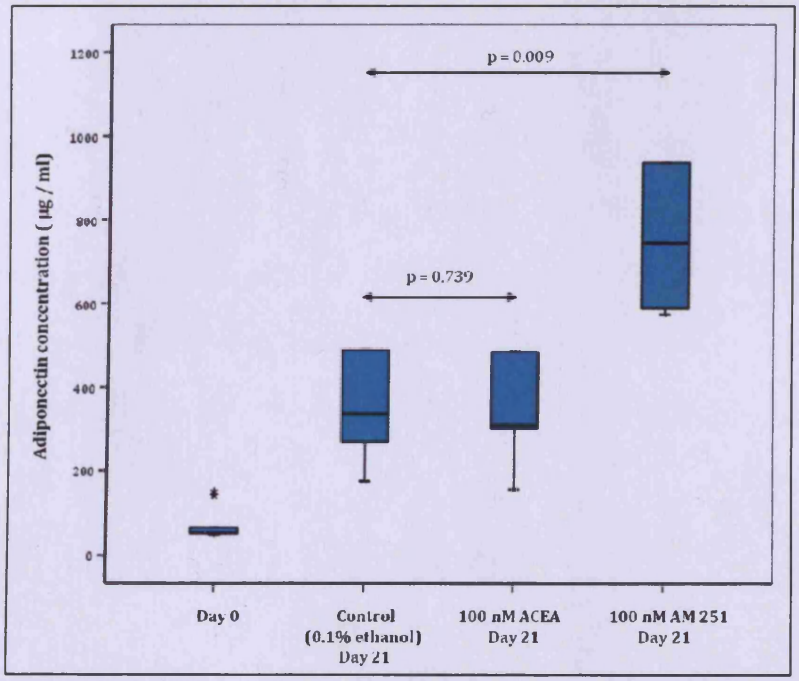


Table 4.13: Effect Of ACEA & AM251 On Subcutaneous Adiponectin Production

Data represent 2 independent experiments performed in triplicate following 21 days culture in DM, ACEA or AM251 (n=5). LPL expression has been normalised to housekeeper gene ARP expression. Statistical significance determined by Wilcoxon Signed Ranks test for individual treatments relative to control

Patient №		1	2	3	4	5	p-value
Day 0	**Adiponectin µg/ml**	51	48.5	62.5	146.5	52.5	n/a
Day 21 Control (0.1% ethanol)	**Adiponectin µg/ml**	486.0	176.5	337.5	2196.5	272.0	n/a
	FOLD change	9.5	3.6	5.4	15.0	5.2	
Day 21 100 nM ACEA	**Adiponectin µg/ml**	481.0	158.5	311.0	1987.0	303.5	0.739
	FOLD change	9.4	3.3	4.9	13.6	5.8	
Day 21 100 nM AM 251	**Adiponectin µg/ml**	935.0	578.0	745.0	4258.0	597.0	**0.009**
	FOLD change	18.3	11.9	11.9	29.0	11.4	

4.4 DISCUSSION

Adipose tissue is now recognised as a dynamic endocrine organ producing biologically active adipocytokines involved in the regulation of energy metabolism and body weight homeostasis. These adipocytokines play a crucial role in development of obesity and the pathophysiology of obesity-related disease processes. Abdominal obesity is associated with an elevated risk of type II DM and CVD. The ECS has been implicated in obesity, promoting adverse metabolic processes resulting in weight gain, lipogenesis, insulin resistance and dyslipidaemia. Clinical studies have confirmed that intermediate-term CB_1 receptor blockade is associated with loss of body weight and waist circumference and an improvement in the metabolic profile including reductions in triglycerides, fasting insulin, HbA1c, blood pressure and an increase in HDL and adiponectin [77-80, 221].

The previous chapter demonstrated an increase in pre-adipocyte proliferation with CB_1 agonism in studies using the murine white pre-adipocyte cell line, 3T3-L1, whereas CB_1 antagonism had the opposite consequence. The anti-proliferative effect was associated with a block at the G1/S phase of the cell cycle. These findings are in keeping with those published elsewhere in the literature [207]. In this chapter these experiments have been repeated using human primary pre-adipocytes derived from both subcutaneous and omental adipose tissue depots. The same effects on proliferation were noted in both the subcutaneous and omental cell populations with a significantly greater effect of CB_1 antagonist-induced inhibition in the omental pre-adipocytes in all paired samples. As such this represents a novel observation since no other *in vitro* studies directly examining the effect of CB modulation in human primary subcutaneous and visceral pre-adipocytes are evident. These results may in part explain some of the anti-obesity effects observed with CB_1 antagonist treatment, including a reduction in waist circumference.

Sarzani et al have described an altered pattern of CB_1 receptor expression in adipose tissue in overweight individuals [222]. The investigators have demonstrated the highest mRNA expression of CB_1 to be in subcutaneous adipose tissue in normal weight subjects (BMI < 25 kg/m^2) whereas CB_1 expression was increased in omental adipose tissue in individuals with a BMI ≥ 25 kg/m^2 suggesting CB_1-mediated overactivity of the ECS in visceral adipose tissue in obesity [222]. This role is further supported by evidence from Matias et al that patients with obesity or hyperglycaemia caused by type 2 DM exhibit higher concentrations of endogenous endocannabinoids in visceral fat or serum, respectively, than their corresponding controls [76]. The differences in receptor expression may explain the possible differential effect of CB1 receptor antagonism observed in the current experiments in cells derived from the subcutaneous and omental adipose tissue depots.

The anti-proliferative and cell cycle blocking effects of CB_1 antagonism are not limited to adipose tissue. Recent *in vitro* studies using SR 141716 have illustrated inhibition of human breast cancer cell proliferation in highly invasive metastatic MDA-MB-231 cells [223]. This effect was not accompanied by cell apoptosis or necrosis, and was characterised by arrest in the G1/S phase of the cell cycle [224]. Similarly CB_1 antagonism has been shown to inhibit proliferation of peripheral blood mononuclear cells, also associated with G1/S transition blockade [224]. Cell line studies of murine white pre-adipocytes indicate inhibition of MAP kinase activity by CB_1 blockade which may be one of the mechanisms involved in inhibition of cell proliferation [207, 208].

In addition to the anti-proliferative effects, in the preceding chapter we have also described an increase in mRNA expression of terminal markers of mature adipocytes following CB_1 antagonist treatment. These findings are also seen in the human primary cultures, where an increase in LPL expression was observed in both the subcutaneous and omental adipocytes. CB_1 agonist treatment did not appear to have an effect on primary adipogenesis. We also observed significantly greater levels of adiponectin produced by the cultured subcutaneous

and omental adipocytes after exposure to CB_1 antagonist treatment. These results are also in keeping with the cell line data in Chapter 3 as well as those published from other *in vitro* cell line studies in the literature [207, 225]. Alterations in the adipocytokine profile, as well as the anti-proliferative effects described previously, provide evidence for direct modulation of adipocyte function as part of the mechanism of action of the ECS on energy metabolism.

The data presented in this thesis do not demonstrate any increase in UCP-1 expression in the WAT cell line 3T3-L1 or in human primary cultures and thus do not support CB_1 blockade-mediated transdifferentiation towards a brown fat phenotype as part of the mechanism to explain the weight loss and improvement in metabolic parameters observed with CB_1 antagonism. This is in contrast to a recent study by Perwitz et al who demonstrated that CB_1 blockade with the antagonist SR 141716, or receptor knock down with small interfering RNA (siRNA), induces UCP-1 expression in murine white adipocytes in a time- and concentration-dependent manner at both the RNA and the protein level [209]. Whether these discrepancies are due to the differing cell types and CB_1 antagonist used for study, or secondary to the limitation of our QPCR technique for low copy number detection, still requires further clarification.

The first pharmacological CB_1 antagonist to be developed, SR 141716 or rimonabant, was associated with a significant adverse event profile and its clinical use has therefore been discontinued. Nevertheless, CB_1 receptor blockade still represents a new alternative for managing obesity and its associated risks. Further work is still required to fully elucidate the mechanism behind its observed clinical effects and also to explore the possibilities for body fat redistribution and white to brown fat transdifferentiation for greater metabolic benefit. Development of future agents will however require further clarification of both long-term effectiveness and an acceptable safety profile. Furthermore, any pharmacological intervention for the treatment of obesity can only be justified when accompanied by individuals' dietary and lifestyle modification to ensure benefits are sustained long-term.

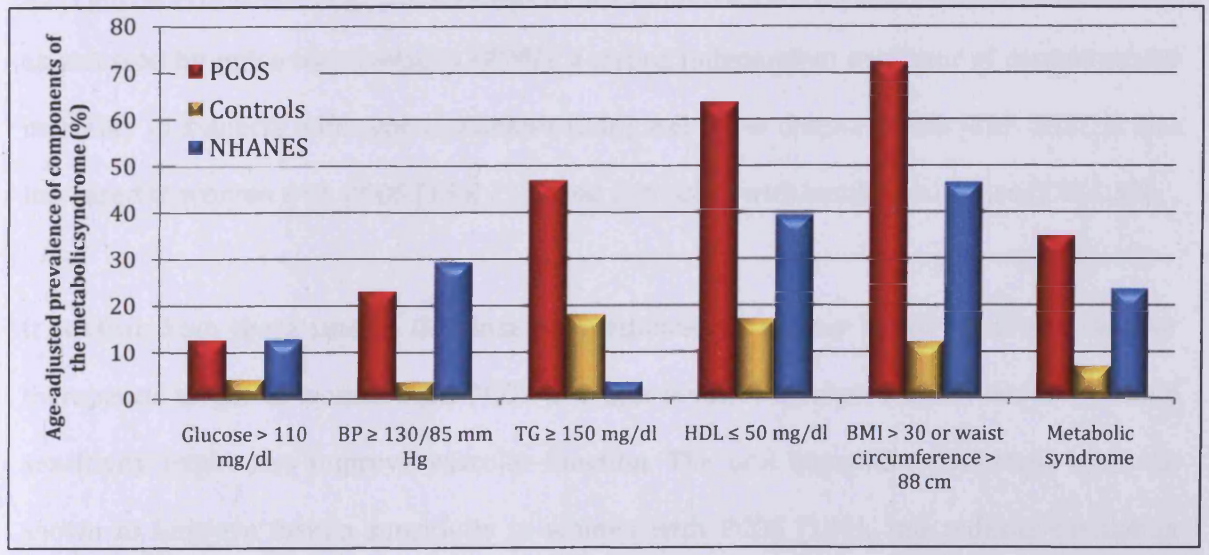
CHAPTER 5: EFFECT OF METFORMIN ON VASCULAR FUNCTION, BODY COMPOSITION & METABOLIC PROFILE IN PCOS

5.1 INTRODUCTION

PCOS is the commonest endocrinopathy in women of reproductive age, affecting approximately 7% of the pre-menopausal population [226]. In addition to its effects on reproductive health it is now well recognized that PCOS is a metabolic disorder characterized by increased insulin resistance [88] which leads to an excess lifetime risk of type II diabetes [84, 85]. Patients with this condition display a cluster of metabolic disturbances, including obesity [227], dyslipidaemia [89], impaired fibrinolysis [228] and hypertension [87], which are often apparent at a young age, and it is therefore unsurprising that a number of studies have highlighted an increased prevalence of the metabolic syndrome amongst women with PCOS ranging from 15 to 50%.

Figure 5.1: Age-Adjusted Prevalence Of Features Of The Metabolic Syndrome In Women With PCOS, Age-Matched Controls And Women From the NHANES Study.

(Adapted from Dokras et al, 2005 [127])



For example, as illustrated in Figure 5.1, Dokras et al reported a 47.3% prevalence rate of the metabolic syndrome, according to the WHO criteria, in PCOS in contrast to 4.3% in an age-adjusted control population and 23.4% in comparison to the National Health and Nutrition Examination Survey (NHANES) study cohort [127].

Although epidemiological studies have thus far failed to demonstrate an increase in cardiovascular mortality in women with PCOS, these abnormalities are likely to contribute to an increased risk of cardiovascular disease, based on data from studies using surrogate markers such as carotid intima media thickness [134, 229]. Developing therapeutic strategies to minimise this vascular burden is therefore important.

Insulin resistance is intricately linked to large blood vessel compliance [230, 231] and endothelial function [232], and could contribute to the vascular dysfunction observed in patients with PCOS. For example, the inability of insulin to reduce aortic stiffness (as measured by augmentation index) in obese insulin-resistant subjects correlates closely with whole-body insulin resistance [233, 234], implying that defects in the metabolic action of insulin are accompanied by abnormal vascular function. Endothelial dysfunction, an early marker of vascular damage, has been demonstrated in women with PCOS [90, 91, 131, 135, 235] and is accompanied by reduced insulin-mediated vasodilatation [135]. Arterial stiffness, as assessed by pulse wave velocity (PWV), a strong independent predictor of cardiovascular mortality in subjects with type II diabetes [236] and other disease states [237, 238], is also increased in women with PCOS [135, 235], and correlates with insulin resistance [235, 239].

It is clear from these studies that insulin resistance *per se* may be an important vascular therapeutic target in women with PCOS and that medical therapies which improve insulin sensitivity might also improve vascular function. The oral biguanide metformin has been shown to improve insulin sensitivity in women with PCOS [139], and reduces circulating concentrations of endothelin-1 [140], a marker of endothelial dysfunction. More recently,

some studies have also shown that metformin can improve endothelial function in women with PCOS [240-243] while others reported either a neutral effect on vascular function [244, 245] or variable improvement or worsening dependent on smoking status [246]. However, interpretation of these studies is difficult due to their observational [240, 242-244] or open label [241, 245, 246] study designs. In light of this uncertainty and to minimise the potential for bias, the following studies sought to determine whether short-term metformin therapy could improve arterial stiffness and endothelial function in patients with PCOS using a randomized, double-blind, placebo-controlled crossover design.

5.1.2 Arterial Stiffness

5.1.2.1 Mechanisms Of Arterial Stiffness

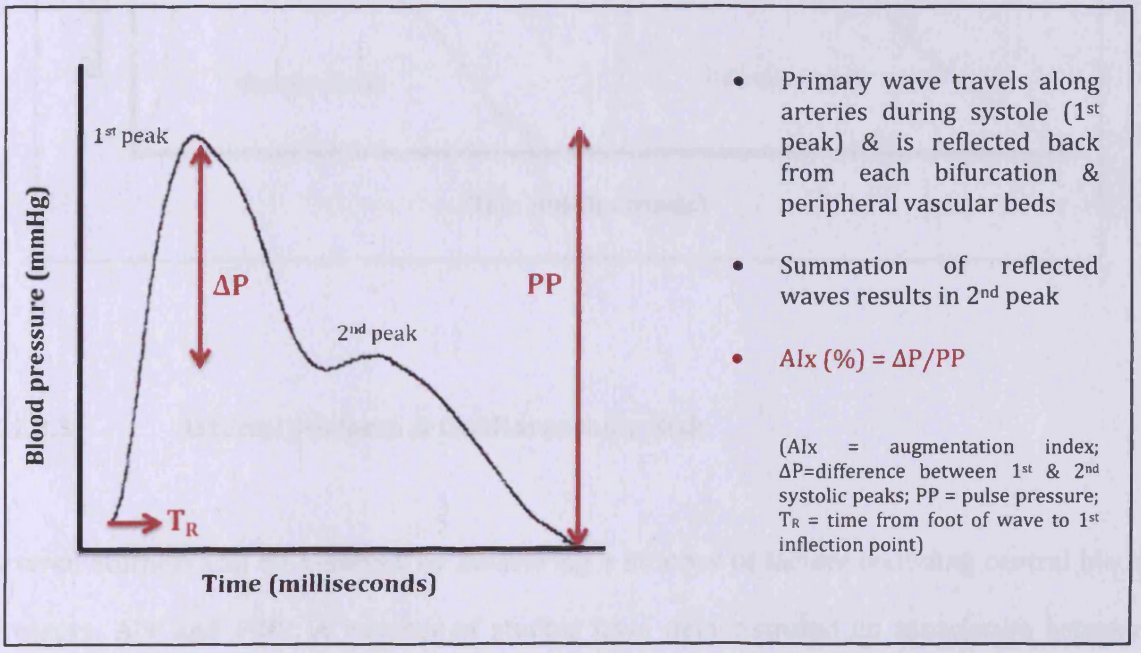
The arterial wall is comprised of three layers – the outer adventitia consisting of connective tissue and the vessel's vascular supply, the smooth muscle and elastin-containing media which is the major determinant of elasticity, and the inner endothelium which regulates vascular tone, haemostasis and inflammatory processes. The Windkessel theory describes the circulation as a central elastic reservoir, comprised of compliant and expansile large arteries, into which the heart pumps, from which blood is transported to the tissues via relatively non-elastic peripheral vessels [247]. The elasticity of the proximal large arteries is largely the result of the high elastin to collagen ratio within the media layer of the vessel wall. Arterial stiffness can therefore arise as a consequence of structural changes within the vessel, for example secondary to age-related progressive elastin fibre degeneration, as well as disease states affecting vascular endothelial function such as hypertension and diabetes. A number of genetic factors have also been implicated including polymorphic variations in the fibrillin-1 [248] and endothelin receptor genes [249].

5.1.2.2 Arterial Pressure Waveform

The arterial pressure waveform is a composite representation of the pressure waves generated during ventricular systole (1st waveform peak) which then travels through the circulation until resistance is encountered at the peripheries when it is reflected back upon itself during diastole. The 2nd waveform peak represents a summation of the reflected waves as illustrated in Figure 5.2. Augmentation index (AIx), a composite measure of wave reflection and systemic arterial stiffness, is defined as the difference between the first and second systolic peaks of the central pressure waveform, expressed as a percentage of the central pulse pressure (Figure 5.2).

Figure 5.2: Schematic Representation Of The Arterial Pressure Waveform

(adapted from Oliver et al , 2003 [247])

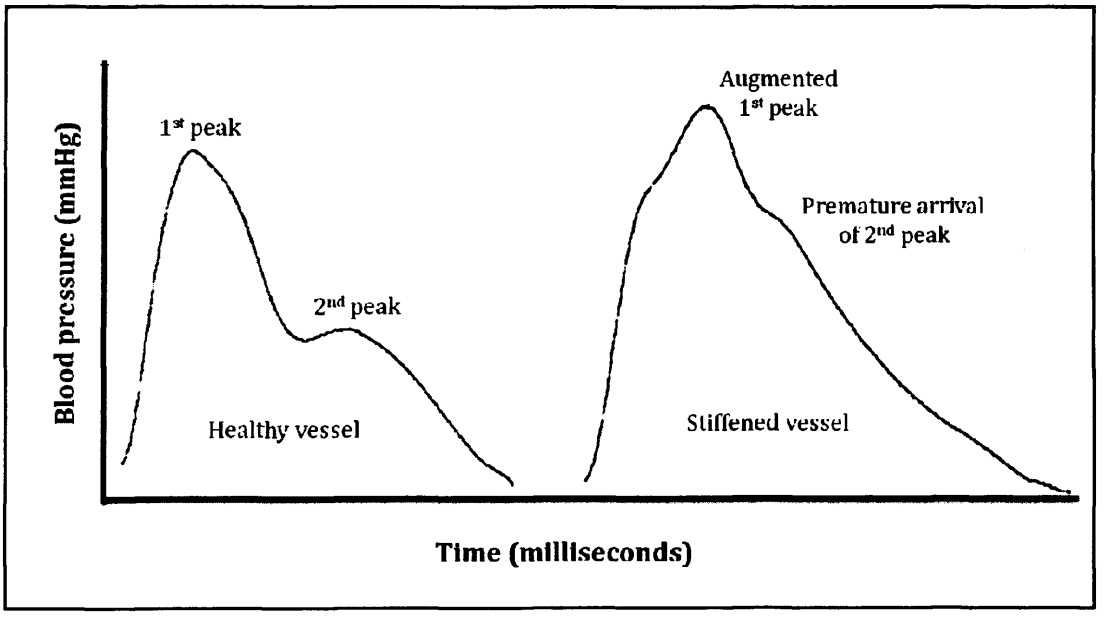


With increasing arterial stiffness the speed with which the left ventricular ejection pressure wave travels through the arterial circulation increases, thus resulting in an earlier return of the reflected wave from the peripheries (Figure 5.3). If the reflected wave continues to return

less in diastole and more in systole itself, this can augment the late systolic pressure on the left ventricle (afterload) and reduce the coronoary arterial perfusion pressure during diastole. This process leads to a greater risk of ischaemic heart disease (due to decreased coronary perfusion in diastole), cerebrovascular disease (due to increased central systolic pressure) and cardiac failure (due to increased left ventricular load).

Figure 5.3: Schematic Representation Of Healthy & Stiffened Arterial Waveforms

(adapted from Oliver et al, 2003 [247])



5.1.2.3 Arterial Stiffness & Cardiovascular Risk

Arterial stiffness can be assessed by measuring a number of factors including central blood pressure, AIx, and PWV. A number of studies have demonstrated an association between these indices of arterial stiffness and other cardiovascular risk factors. For example, Wilkinson and colleagues showed increased AIx and aortic PWV (aPWV) in subjects with hypercholesterolaemia [250] and type I diabetes [251]. Chronic smoking is associated with higher AIx and central systolic blood pressure (cSBP) when compared to matched non-smoking controls [252], while increased aPWV is also seen in individuals with essential

hypertension meeting the criteria for the metabolic syndrome [253]. A positive correlation has also been demonstrated between the inflammatory marker high-sensitivity C-reactive protein (hsCRP), and both aortic and brachial PWV [254], while declining endothelial function is also associated with elevated large artery stiffness and central systolic blood pressure (cSBP) [255]. There is also evidence for an independent association between manifestations of increased arterial stiffness derived from non-invasive pulse wave analysis (PWA), and the presence of CAD in individuals undergoing coronary angiography [256]. Arterial stiffness is independently predictive of cardiovascular mortality in a number of disease states, including renal failure, hypertension, and glucose intolerance/type II diabetes [236-238], and interventions to reduce stiffness indices are associated with improvements in survival. For example, survival of patients with end-stage renal disease is significantly better for patients whose aortic PWV declines in response to BP lowering intervention [257].

These studies illustrate the importance of arterial stiffness in predicting cardiovascular risk, thus making this a significant parameter with potential clinical application when evaluating patient risk, disease progression and response to intervention. The development of quick, reproducible, and non-invasive tools to measure arterial stiffness may also facilitate incorporation into diagnostic and therapeutic clinical trials.

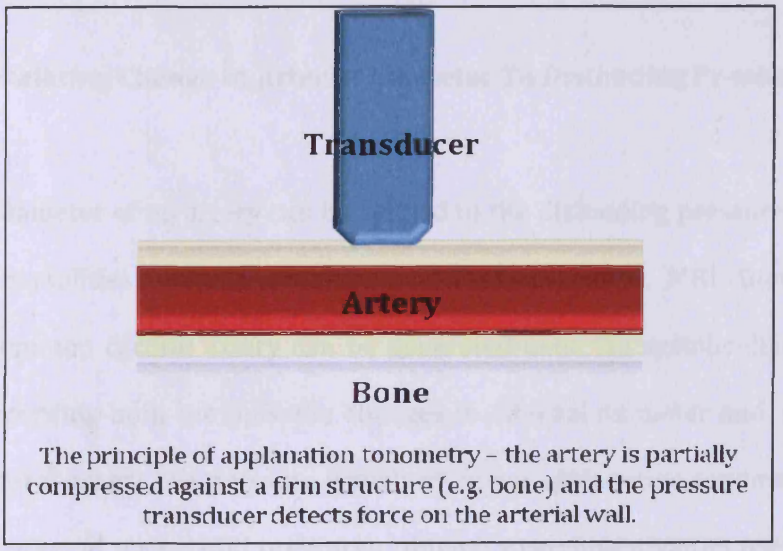
5.1.3 Non-Invasive Assessment Of Arterial Stiffness

There are numerous invasive and non-invasive methodologies that have been employed to measure arterial elasticity *in vivo*. While invasive techniques are more accurate, their widespread use is limited in research and clinical practice. The non-invasive measures can broadly be divided into three categories - analysis of arterial waveforms, measurement of pulse wave velocity, and relating change in arterial diameter to distending pressure.

5.1.3.1 Analysis Of Arterial Waveforms

A number of technologies, including the SphygmoCor™ apparatus (AtCor Medical Ltd, Sydney, Australia) used in this study, utilise analyses of specific components of the arterial pressure waveform (Figure 5.2) to measure arterial stiffness. Peripheral arterial pressure waveforms from, for example, the radial, femoral, or carotid artery, can be acquired using applanation tonometry [258]. This involves placing a highly sensitive pressure transducer on the skin over the site of the peripheral pulse whilst compressing the artery against a firm structure such as bone. The sensor is then able to detect dynamic pressure and volume changes in the underlying vessel to generate the peripheral arterial waveform (Figure 5.4). This waveform is calibrated to conventionally measured peripheral blood pressure and the system software applies a validated mathematical transfer function to generate the central arterial waveform and measure the central blood pressure [259]. Pulse wave analysis (PWA) can then be performed to calculate AIx and T_R as described in the previous section.

Figure 5.4: Schematic Representation Of Applanation Tonometry



The principle of applanation tonometry – the artery is partially compressed against a firm structure (e.g. bone) and the pressure transducer detects force on the arterial wall.

5.1.3.2 Measurement Of Pulse Wave Velocity

PWV refers to the speed with which the arterial wave travels across a specified distance, and can be measured by a number of methods. Doppler ultrasound [260] or applanation tonometry [258], can be used to record the arterial waveform from a proximal site, e.g. the carotid, and a more distal site, e.g. the femoral artery. In order to travel between these two locations the arterial wave needs to traverse most of the abdominal aorta, an artery particularly prone to atherosclerotic change. The time-delay for the arrival of the foot of the arterial wave at these two sites can be calculated by either simultaneous measurements at both sites, or by gating to the peak of the R-wave of the ECG [247]. The distance between the two sites is measured externally and PWV calculated as distance/time in metres per second.

The manual measurement of distance between these two sites is an approximation of the true distance and depends upon the subject's body habitus and vessel tortuosity. A more accurate measurement can be obtained using magnetic resonance imaging (MRI) though this is significantly more expensive and not always readily available.

5.1.3.3 Relating Change In Arterial Diameter To Distending Pressure

The change in diameter of an artery can be related to the distending pressure and measured using imaging modalities such as ultrasound or, less commonly, MRI. Diameter-pressure curves of the common carotid artery can be generated over the systolic-diastolic range by continuously recording both the pulsatile changes in internal diameter and, simultaneously on the contralateral artery, the pressure waveform using applanation tonometry [261]. This allows determination of the carotid pressures, though some investigators use brachial blood pressures instead. Distensibility can then be compared at the mean arterial pressure and at a fixed distending pressure e.g. 100 mm Hg, with decreasing distensibility indicating a higher degree of arterial stiffness.

5.1.3.4 Comparison Of Non-Invasive Measures Of Arterial Stiffness

Table 5.1 summarises the relative strength and weaknesses of the major methods used to measure arterial stiffness.

Table 5.1: Advantages & Disadvantages Of Non-Invasive Measures Of Arterial Stiffness

(adapted from Oliver et al, 2003 [247])

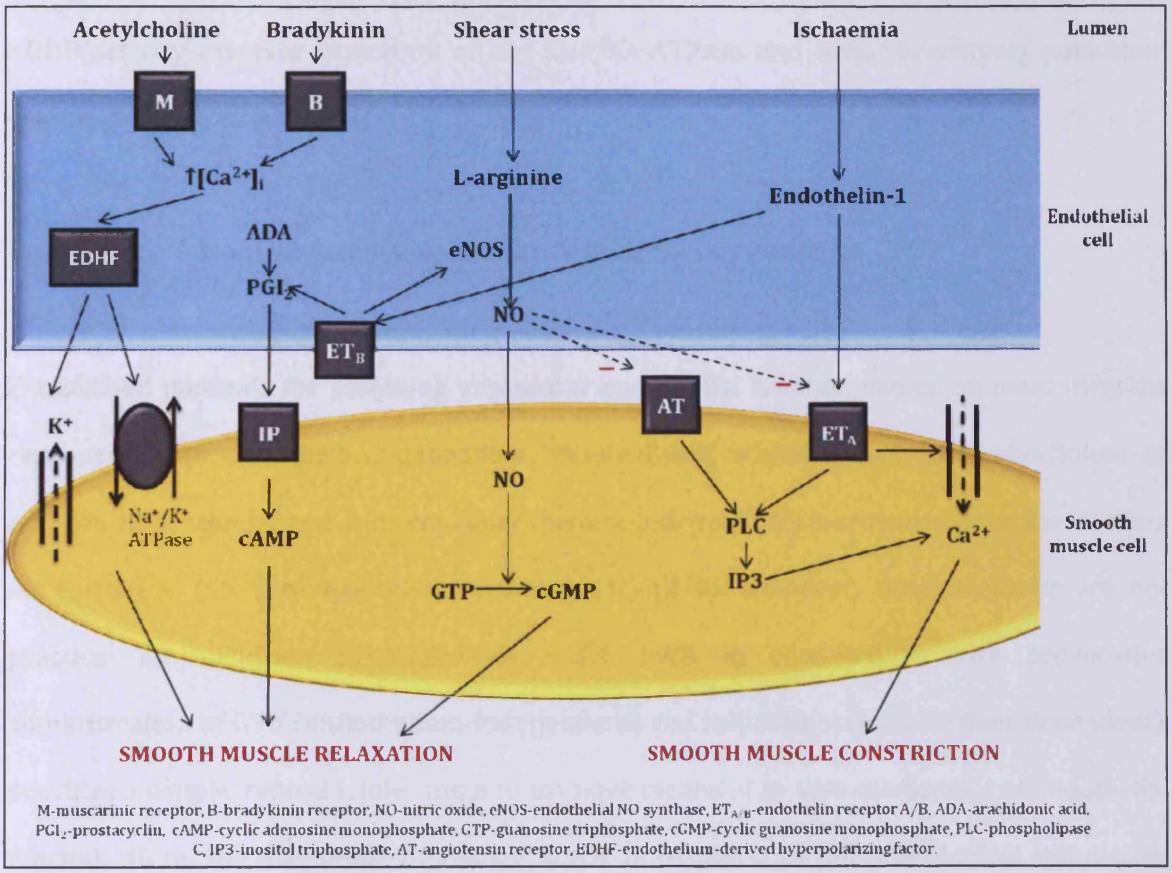
	AIx (using PWA)	Arterial Ultrasound	PWV (PWA or ultrasound)	PWV (using MRI)
Ease of use	+++	+	++	+
Affordability	+++	++	+++	+
Quality of validation	++	++	++	+++
Freedom from operator bias	++	+	++	+++
Evidence of prognostic value	++	++	+++	+
Endothelial function testing	+++	+++	+	+

Of these techniques, measurement of AIx by PWA using applanation tonometry requires the least training, can be rapidly measured and thus easily incorporated into clinical trials. PWV and ultrasonography are more time-consuming and the latter in particular requires additional training and dedicated staff. MRI measures are also time-consuming and are further limited by affordability and availability. Both PWV and AIx can be measured with a high degree of reproducibility [258] and have been shown to carry significant prognostic value. For these reasons, as well as local availability, it was decided to measure PWX and AIx using PWA to assess arterial stiffness in the present clinical trial. Furthermore, this technique was also used for endothelial function testing and will be discussed in greater detail below.

5.1.4 Vascular Endothelium & Endothelial Dysfunction

The vascular endothelium lining the luminal surface of all blood vessel walls plays an integral role in the regulation of vascular tone, haemostasis and permeability, mediated by a number of endothelium-derived factors (Figure 5.5). Of the vasoactive factors that the endothelium can release, prostacyclin (PGI_2), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF) are the most significant [262]. NO is synthesized from L-arginine by NO synthases (NOS) present in the endothelium (eNOS), or inflammatory cells (iNOS). NO is a lipophilic agent and is thus able to traverse cell membranes causing vasodilatation via activation of soluble guanylyl cyclase generating cyclic guanosine monophosphate within vascular smooth muscle cells [263]. NO production by eNOS can be stimulated directly by shear stress, as well as by muscarinic or bradykinin receptor-mediated calcium channel activation [264]. NO also inhibits the vascoconstrictive actions of angiotensin-II (AT II) and endothelin-1 (ET-1). AT-II binds to the G-protein-coupled angiotensin receptor on vascular smooth muscle cells which stimulates phopholipase C (PLC) mediated inositol triphosphate (IP3) production leading to an increase in intra-cellular calcium and consequent vasoconstriction [265]. ET-1, generated within the endothelial cells, acts upon the endothelin A receptor on smooth muscle cells, which in turn is also linked to PLC as well as voltage operated calcium channels leading to vasoconstriction [266]. When testing endothelial function, nitrate-containing pharmacological agents such as glyceryl trinitrate (GTN) can be used as direct, or endothelium-independent vasodilators due to direct delivery of NO to the vascular smooth muscle cells. Endothelium-dependent function can be examined using shear stress or drugs that depend upon NO generation via eNOS, for example, salbutamol [267].

Figure 5.5: Modulators Of Vascular Tone [262-266]



PGI_2 is synthesized from arachidonic acid by cyclooxygenase (COX) isozymes expressed in the vascular endothelium and is thought to contribute to the maintenance of vascular homeostasis. PGI_2 elicits smooth muscle relaxation by activating specific cell-surface receptors that are G-protein-coupled to adenylyl cyclase thereby elevating cyclic adenosine monphosphate (cAMP) levels and inducing smooth muscle relaxation [268]. PGI_2-mediated vasodilation can also be stimulated by ET-1 via stimulation of the endothelin B receptor [266].

The identity of EDHF remains unclear, although several candidates have been proposed including K+ ions, cAMP, cytochrome p450 2C products, H_2O_2, spread of electrotonic current and, most recently, C-type natriuretic peptide (CNP) [262, 269]. It is, however, accepted that

vasorelaxation resulting from EDHF follows opening of endothelial calcium-activated potassium channels, and that hyperpolarization of vascular smooth muscle associated with EDHF activity involves activation of the Na+/K+-ATPase and inward-rectifying potassium channels (K_{IR}) [262].

5.1.5 Non-Invasive Assessment Of Endothelial Function

Established methods for assessing vasomotor endothelial function centre on measuring the response to an endothelium-dependent, NO-mediated stimulus such as acetylcholine or reactive hyperaemia, and a direct (endothelium-independent) nitrovasodilator like sodium nitroprusside (SNP) or glyceryl trinitrite (GTN) [270]. However, these methods are not practical for inclusion in large-scale trials. PWA in combination with provocative administration of GTN (endothelium-independent) and salbutamol (endothelium-dependent), provides a simple, reproducible, and non-invasive means of *in vivo* assessment of endothelial function. Here, the magnitude of change in AIx in response to pharmacological stimulation represents the functional state of the endothelium.

Endothelial function can also be assessed by radiological measurements, usually by ultrasound, of vessel dilatation in response to pressure-controlled forearm blood flow (reactive hyperaemia test, or flow-mediated dilatation) [91]. This technique requires considerably more training than PWA, and comparative studies have demonstrated a significant correlation between both these methods [270].

5.1.6 Clinical Trial Design

The clinical trial described in this chapter compares the effect of our investigational medicinal product (IMP), metformin, to that of placebo using a randomised, double-blind, crossover study design. The main advantage of the crossover study design is that each individual acts as

his or her own 'control,' thus reducing the influence of confounding variables. Removing patient variation in this way makes crossover trials potentially more efficient than similar sized, parallel group trials in which each subject is exposed to only one treatment – IMP or control [271]. In theory treatment effects can be estimated with greater precision given the same number of subjects. Another significant advantage is that a substantial within-subject correlation is expected, i.e. an individual's tendency to vasodilate in response to GTN should be consistent. The main drawback is the potential to carry-over a treatment effect from the 1st treatment phase to the 2nd. This can be overcome to a large extent by ensuring an adequate wash-out period and performing baseline measures prior to each treatment phase.

5.1.7 Chapter Aims

This chapter aims to describe the results obtained from a clinical trial analysing the effects of metformin on vascular function, body composition and metabolic profile in PCOS. The primary outcome measures for this study were defined as changes in markers of arterial stiffness (AIx, T_R, bPWV, aPWV), endothelial function and blood pressure (peripheral and central). The secondary outcome measures were to evaluate the effects of metformin therapy on anthropometric measurements of body composition (weight, BMI, fat percentage, waist and hip circumference), changes in serum androgens (testosterone, sex hormone binding globulin (SHBG), free androgen index (FAI)), insulin resistance (fasting insulin, glucose, HOMA-IR), lipid profile (TC, HDL, LDL, TG) biochemical markers of vascular risk (hsCRP, PAI-1), and adiponectin.

5.2 MATERIALS AND METHODS

5.2.1 Trial Approval & Funding

Prior to commencement of the clinical trial, the project was approved by the Research and Development Department at the University Hospital of Wales (Ref 05/CMC/3456E), the South Wales Reserch Ethics Committee (Ref 06/WSE04/33), and the United Kingdom Medicines and Healthcare Products Regulatory Authority (MHRA) (Ref 2005-005471-69). The study was sponsored by Cardiff University (Ref SPON CU 153) and placed on the International Standard Randomised Controlled Trial Number register and ascribed the registration ISRCTN 61785174 (http://www.controlled-trials.com/isrctn/).

The study was supported by the Lewis Thomas Gibbon Jenkins of Briton Ferry Fellowship Award from the Royal College of Physicians. The funding source had no input in the design, conduct, management, analysis or interpretation of the study.

5.2.2 Trial Participants - Inclusion & Exclusion Criteria

Patients were recruited from the endocrine and dermatology clinics at the University Hospital of Wales (UHW). A diagnosis of PCOS was made according to the Rotterdam criteria [93]. Specifically, patients with androgen excess (clinical symptoms of hyperandrogenism and/or elevated testosterone) with ovulatory dysfunction (fewer than 6 menstrual cycles per year) were recruited, supported by ovarian ultrasound where available (polycystic ovaries present in 24/30 subjects on imaging). Congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumours, hyperprolactinemia and thyroid disease were excluded by biochemical testing.

Patients were aged between 18 and 35 years and were excluded from participation if they were pregnant or planning to become pregnant, breastfeeding, had a history of current or recent (within 6 months) use of oral contraceptives, antidiabetics or antiandrogens, or had any contraindications to metformin therapy including renal or hepatic impairment. Patients with a history of known hypertension, hyperlipidemia or diabetes were also excluded. As metformin is known to improve ovulation rates in women with PCOS, study participants were advised to use barrier methods of contraception if they were sexually active; pregnancy tests were performed at each visit with a plan for study withdrawal if pregnancy was confirmed.

5.2.3 Consent

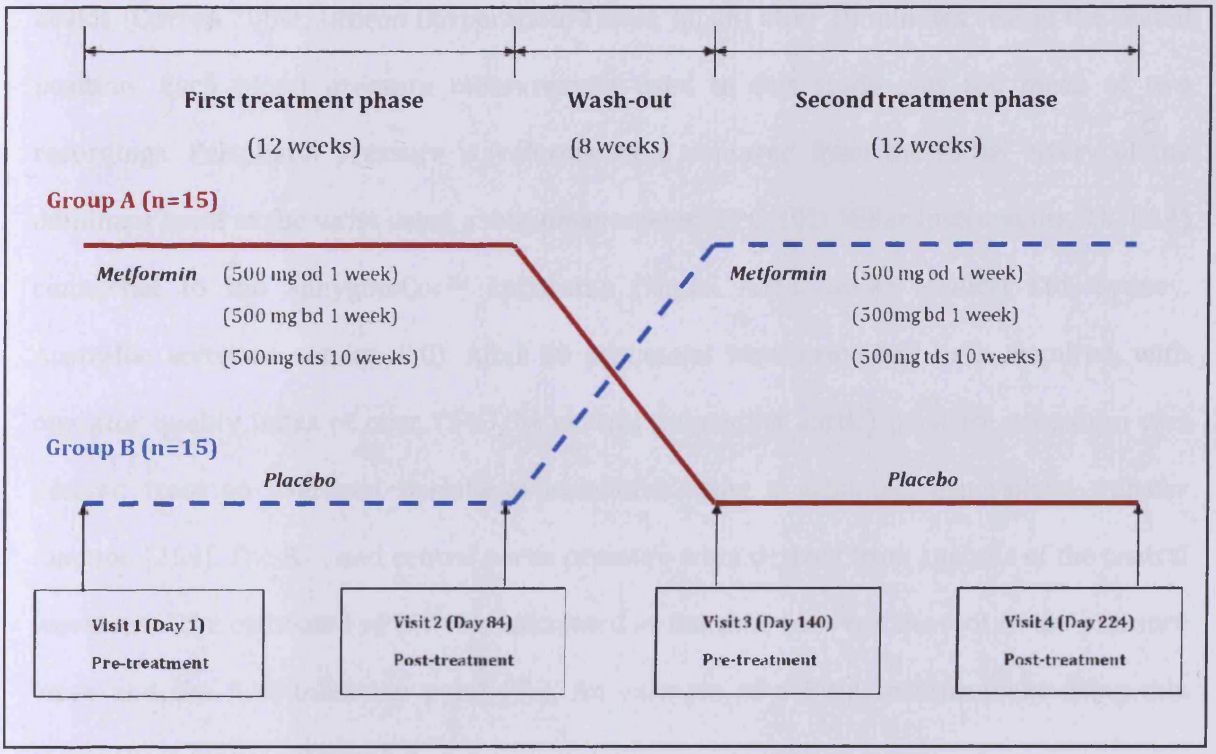
Initial contact was made by providing each subject with a patient invitation letter and information sheet explaining the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it may entail (Appendix 3). This was followed by a telephone call a week later to determine interest in study participation and confirm eligibility. A personal visit at the Clincal Research Facility in UHW was then arranged where written informed consent was obtained (Appendix 4) prior to study commencement. Each subject was informed that participation in the study is voluntary and she may withdraw from the study at any time and that withdrawal of consent will not affect her subsequent medical treatment or relationship with the treating physician.

5.2.4 Treatments & Interventions

Thirty participants were randomized to receive consecutive 12 week treatment periods of either metformin followed by placebo tablets of identical appearance or placebo followed by metformin administration. To minimise gastrointestinal side-effects metformin was given in a dose of 500mg once daily for 1 week, 500mg twice daily for 1 week then 500mg three times daily for the remaining 10 weeks (matched with equal tablet numbers in the placebo phase).

A washout interval of 8 weeks separated the two treatment phases. Metformin and placebo capsules were both supplied by Merck-Santé (Lyon, France) and packaged in a licensed pharmaceutical laboratory (St Mary's Pharmaceutical Unit, Cardiff, UK). The clinical trial study design is illustrated in Figure 5.6.

Figure 5.6: Summary Of Clinical Trial Design



5.2.5 Measurements

Participants were assessed in the Clinical Research Facility at the UHW at four points: baseline tests at visit 1 and at the end of the wash-out phase (visit 3) and after each treatment (metformin or placebo) phase (visits 2 and 4). On each occasion blood samples were collected after an overnight fast, followed by assessment of body composition, arterial stiffness and endothelial function. A full clinical history was obtained at the first visit and any changes recorded upon each subsequent visit. An example of the study visit case report form can be found in Appendix 5.

5.2.5.1 Measurement Of Arterial Stiffness & Endothelial Function

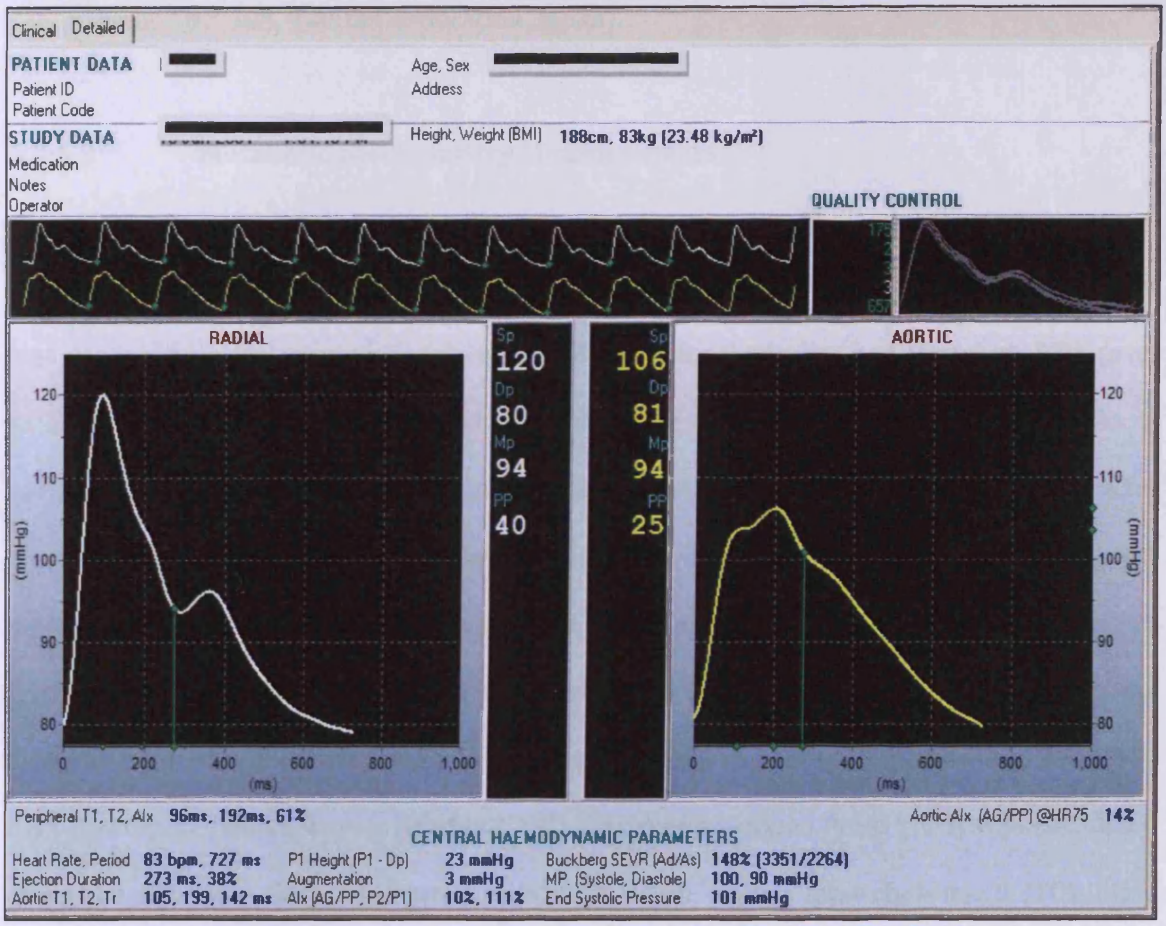
All measurements, were performed in a quiet, temperature-controlled room, and taken exclusively by the author of this thesis to avoid any inter-observer variability. Smokers were asked to refrain from smoking on the morning of their visit. Blood pressure was measured at the brachial artery of the dominant arm using a validated semi-automated oscillometric device (Omron 705IT, Omron Corporation, Tokyo, Japan) after 10 minutes rest in the seated position. Each blood pressure measurement used in this study was the mean of two recordings. Peripheral pressure waveforms were captured from the radial artery of the dominant hand at the wrist using a micromanometer (SPC-301; Millar Instruments, TX, USA) connected to the SphygmoCor™ apparatus (Model MM3, AtCor Medical Ltd, Sydney, Australia; software version 8.0). After 20 sequential waveforms had been acquired, with operator quality index of over 75%, the central (ascending aortic) pressure waveform was derived from an averaged peripheral waveform using a validated, generalized transfer function [259]. The AIx, and central aortic pressure were derived from analysis of the central waveform. The estimated aPWV was calculated as the time between the foot of the pressure wave and the first inflection point (T_R). An example of a PWA measurement using this technique is given in Figure 5.7.

Pulse wave velocity (PWV) was measured by the Sphygmocor system by sequentially recording ECG-gated carotid and femoral waveforms (for aortic PWV; aPWV) and carotid and radial waveforms (for brachial PWV; bPWV) as described previously.

Endothelial function was assessed using a validated, non-invasive method whereby the change in AIx in response to the administration of nitroglycerin, (GTN, an endothelium-independent nitrovasodilator), and salbutamol (an endothelium-dependent β_2-adrenoceptor agonist) is determined by pulse wave analysis. Briefly, a 500 μg tablet of GTN (Alpharma, Barnstable, UK) was placed under the tongue for 3 minutes and then removed;

haemodynamic recordings (pulse, blood pressure and AIx) were then made at 3, 5, 10, 15, 20 and 30 minutes after administration. At 30 minutes after GTN administration, 400 μg salbutamol (IVAX pharmaceuticals, London, UK) was given by inhalation through a spacer device and haemodynamic recordings were taken at 5, 10, 15 and 20 minutes after salbutamol administration. The response to GTN or salbutamol was defined as the maximum change in AIx after drug administration. Each of the above measurements was performed twice and averaged prior to data entry.

Figure 5.7: Example Of SphygmoCor™ Generated PWA



5.2.5.2 Anthropometric Measurements

Body height was measured to the nearest 0.5 cm using a stadiometer and body weight (in light clothing without shoes) to the nearest 0.5 kg on a balance beam scale. Waist circumference was measured at minimal respiration and reported to the nearest 0.5 cm by positioning a flexible anthropometric tape parallel to the floor and immediately above the iliac crest. Hip circumference was measured at the maximum circumference over the buttocks and recorded to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Percentage fat was measured by bioimpedance (Tanita Body Fat analyser TBF-305, Tanita Corporation, Japan).

5.2.5.3 Metabolic Biochemistry Measurements

Blood samples were collected on the morning of each visit following an overnight fast. Serum was prepared by centrifugation of blood at 4000 rpm for 8 minutes and stored at -20°C prior to analysis in a single laboratory at the UHW. The intra- and interassay coefficients of variation (all less than 9%), lower limits of assay detection and reference ranges used for biochemical testing, are listed in Table 5.2. Serum sex hormone binding globulin (SHBG) was measured using an Elecsys E170 automated immunoassay analyser (Roche SHBG assays, Roche Diagnostics, Lewes, UK), and testosterone, oestradiol, and progesterone using the Siemens Centaur automated immunoassay analyser (ADVIA Centaur Testosterone/Estradiol-6/Progesterone assays, Bayer, Newbury, UK). The free androgen index (FAI) was calculated using the equation: FAI = (testosterone/SHBG) × 100. Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL), and triglycerides were assayed using an aeroset automated analyser (Abbott Diagnsotics, Berkshire, UK); LDL cholesterol (LDL) was calculated using Friedewald's formula [272]. Insulin was measured using an immunometric assay specific for human insulin (Invitron, Monmouth, UK). Glucose was measured using the Aeroset chemistry system (Abbott Diagnsotics, Berkshire, UK). High sensitivity C-reactive

protein (hsCRP) was assayed via nephelometry (BN™ II system, Dade Behring, Milton Keynes, UK) and PAI-1 was measured using a tissue plasminogen activator coated ELISA plate that assays only active PAI-1 (Zymutest, Hyphen Biomed, Nodia, Amsterdam). Insulin resistance was estimated using the homeostasis model assessment method (HOMA-IR) calculated by the formula: fasting serum insulin (μU/ml) × fasting serum glucose (mmol/l)/22.5 [273]. Serum adiponectin was measured in duplicate by ELISA (R&D Systems, Oxford, UK) as described in Chapter 4.

Table 5.2: Biochemical Assay Information

Test	Coefficient of variation	Lower limit of detection	Reference range
Insulin	4.1-4.9%	1 mU/l	N/A
Glucose	1.5-2.2%	0.1 mmol/l	N/A
Triglycerides	0.8-1.1%	0.07 mmol/l	0.6-2.0 mmol/l
HDL	5.5-1.4%	0.13 mmol/l	0.7-1.7 mmol/l
Total cholesterol	0.8-1.6%	0.16 mmol/l	N/A
SHBG	1.1-4.0%	0.35 nmol/l	19.8-122 nmol/Ll (pre-menopausal)
Testosterone	2.7-7.6%	0.35 nmol/l	<2.5 nmol/l (females)
Oestradiol	7.9-8.3%	0.37 nmol/l	69.4-905.5 (follicular) 130.3-2094.8 (mid-cycle) 82.9-939.5 (luteal)
Progesterone	4.1-6.7%	0.67 nmol/l	0.48-4.45nmol/l (follicular) 10.62-81.28 nmol/l (luteal) 14.12-89.14 nmol/l (mid-luteal)
hsCRP	2.5-4%	0.1 mg/l	0-3.0 mg/l

5.2.6 Randomisation & Blinding

The treatment assigned to each participant was determined according to a computer generated randomization list produced by an independent external pharmacist (St Mary's Pharmaceutical Unit, Cardiff, UK). The participant study drug boxes were labelled with unique patient identification numbers. When a participant was found eligible for the study, she was allocated a unique patient identification number in sequential, chronological order. The participant was then treated with the medication labelled with the same number. If a participant was withdrawn from the study, her medication was not reallocated. All participants and investigators remained blind to treatment allocation for the duration of the study.

5.2.7 Outcome Measures

The primary outcome measures were defined as changes in arterial stiffness (AIx, T_R, bPWV and aPWV), endothelial function, and blood pressure (peripheral and central). Secondary outcome measures were defined as changes in serum androgens (testosterone, SHBG and FAI), body composition (weight, BMI, percentage fat, waist and hip circumference) and metabolic biochemistry (TC, HDL, LDL, Triglycerides, PAI-1, adiponectin, hsCRP, HOMA-IR).

5.2.8 Sample Size & Power Calculation

Sample size advice was obtained from Professor R. Newcombe, Department of Primary Care and Public Health, School of Medicine, Cardiff University. Subsequently the aim was to recruit 32 subjects into the study in order to provide over 80% power to detect a shift of 0.5 times the standard deviation of within-group changes at the 5% alpha level using a paired t-test. Because the main rationale for using a crossover study was that a substantial within-subject

correlation was anticipated, it was therefore expected that the power should be well in excess of this figure.

5.2.9 Statistical Methods

All statistical analyses were performed using SPSS version 14 for Windows (SPSS, Chicago, IL). All analyses were performed as per pre-established protocol and in line with previous guidance on analysis of crossover trials [274]. This assessment of treatment efficacy compares the within-subjects period I minus period II differences between the treatment sequence groups AP (active-placebo) and PA (placebo-active) by unpaired t-test. In the event of serious departure from Gaussian distributional form, scale transformation was used. Point and interval estimates of the treatment difference (estimated as above) are given as well as p-values. 5% and 1% levels of significance were set for the primary and secondary outcome measures, respectively.

5.2.10 Procedures & Instructions

5.2.10.1 Adverse Event Reporting

An adverse event was any undesirable sign, symptom or medical condition occurring after the patient has given informed consent. A serious adverse event (SAE) was defined as an undesirable sign, symptom or medical condition which:

1. was fatal or life-threatening
2. required or prolongs hospitalisation
3. was significantly or permanently disabling or incapacitating
4. constituted a congenital anomaly or a birth defect

5. was medically significant, jeopardised the subject or required medical or surgical intervention to prevent one of the outcomes listed above.

Information about all non-serious and SAEs, irrespective of causality, whether volunteered by the subject, discovered by investigator questioning, or detected through physical examination, laboratory test or other means, was collected and recorded. Adverse events were followed as appropriate. Where possible, each adverse event was described by:

1. Its duration
2. The severity grade (mild, moderate, severe)
3. Its relationship to study drug (suspected, not suspected)
4. The action(s) taken

Pregnancy was managed as a SAE and followed up to determine details of birth, outcome, including spontaneous or voluntary abortion, the presence or absence of any birth defects or congenital abnormalities, or any maternal/newborn hazards. Periodic safety reports were provided to all regulatory authorities. All SAEs were reported immediately to the trial sponsor (Cardiff University), regardless of the causal relationship and expectedness, as well as the MHRA as per European Union requirements and in accordance with Good Clinical Practice.

5.2.10.2 Emergency Procedure For Unblinding

The randomisation codes for each patient were held at the Pharmacy Department at UHW in individual sealed envelopes for emergency use only. Emergency unblinding was considered in the event of a SAE provided that knowledge of the subject's treatment allocation would be of benefit in her management.

5.2.10.3 End Of Study Unblinding

After completion of the final subject's final visit, a complete electronic database was created which contained all the data collected on vascular, anthropometric and metabolic parameters. A copy of this database was supplied on a CD-ROM to a research governance officer from Cardiff University who undertook a final study closure inspection and confirmed source verification of the data. The Pharmacy department was then given permission to release the randomisation codes so data analysis could be performed.

5.2.11 Project Management & Administrative Procedures

The author of this thesis was responsible for the day-to-day conduct of the study, and therefore co-ordinated recruitment, obtained consent, performed the blood sampling and measured PWV and AIx in all study participants. In the event of an emergency, the author was contactable 24 hours a day. Contact information was provided to all study participants and was printed on all study medication containers.

Formal meetings were undertaken on a weekly basis with Dr Aled Rees, the Principal Investigator, to discuss all aspects of the study and to plan the work for the forthcoming week. Informal discussions regarding any difficulties that arose with the project took place as required.

All data was entered into a secure electronic database kept locked within the department. Study data will be stored for 15 years after completion of the clinical trial. Dr Rees will act as custodian of this data and only the study investigators will have access to the data. In addition the trial master files and case report forms have been stored in hard copy form at the Cardiff University off-site archiving facility.

5.2.11.1 Auditing Procedure

As part of Good Clinical Practice the study protocol and all documentation were closely monitored. The study was conducted as outlined in the study protocol and in accordance with all applicable government regulation. All study documentation was made available for inspection at any time by appropriate regulatory authorities including internal audits by the Cardiff and Vale NHS Trust and Cardiff University Research and Development audit officers.

5.2.11.2 Handling Of Study Medication

All study medication was dispensed by the Pharmacy Directorate at UHW. Drug supplies were stored in an appropriate, secure area and all drug supplies were used only for this protocol and not for any other purpose.

5.2.12 Intra-Observer Variability

Formal training was undertaken for all aspects of pulse wave analysis at an 'Arterial Stiffness Theory & Practice' course at the Vascular Research Unit in Addenbrooke's Hospital, Cambridge, UK. Prior to commencement of the clinical trial, intra-observer variability was established by performing PWA on 20 healthy volunteers at two time points 30 minutes apart, to measure AIx. The mean overall intra-operator variability was less than 2% (Table 5.3).

Table 5.3: Intra-Operator Variability

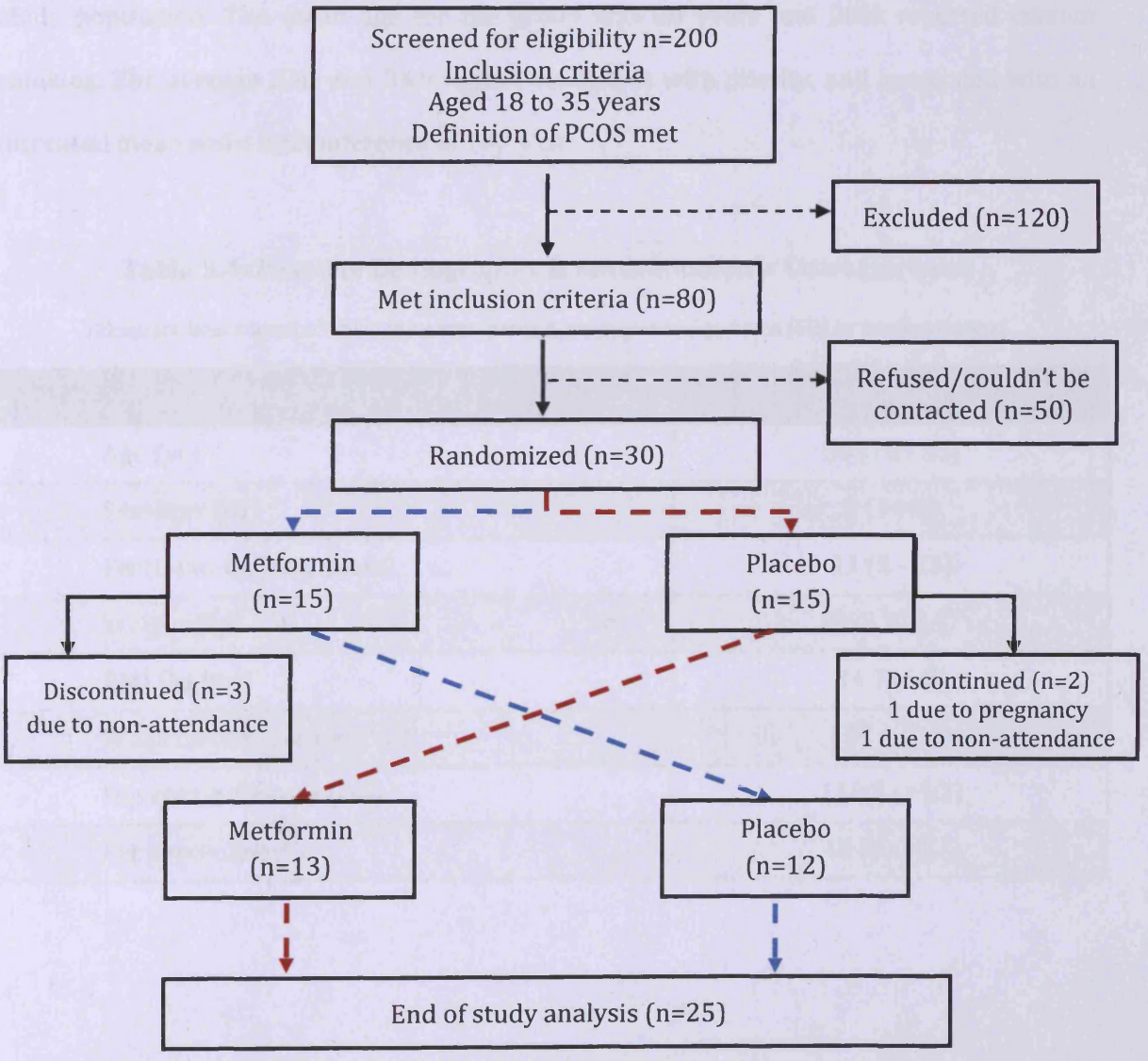
Subject	Gender	Age (yrs)	Height (cm)	Weight (kg)	BP (mmHg)	AIx 1 (%)	AIx 2 (%)	Difference AIx 1 & 2
1	M	42	180	102	132/74	23	22.5	0.50
2	M	38	175	71	128/64	15	13.5	1.50
3	M	37	166	70	127/84	24	24	0.00
4	M	35	175	71	120/81	14.5	14	0.50
5	M	35	173	78	134/83	14.5	13.5	1.00
6	M	33	173	80	120/80	7	5	2.00
7	F	56	163	57	138/88	29	28	1.00
8	F	40	161	58	98/70	17	16	1.00
9	F	40	156	59	127/81	29	29	0.00
10	F	38	158	80	144/104	41	38	3.00
11	F	37	164	123	131/68	17.5	15	2.50
12	F	37	160	53	121/94	31	27	4.00
13	F	36	158	75	118/75	30	27	3.00
14	F	34	146	74	116/75	24	22	2.00
15	F	31	160	78	132/78	31	27.5	3.50
16	F	29	160	69	119/88	18	16	2.00
17	F	28	165	83	141/93	-4.5	-5	0.50
18	F	26	167	72	110/71	11	11	0.00
19	F	22	180	69	122/67	-6	-8	2.00
20	F	20	167	78	132/73	19	16	3.00
Mean intra-operator variability (SD)								**1.65(1.2)**

5.5 RESULTS

5.3.1 Subject Recruitment And Trial Completion

Figure 5.8 summarises the flow of participants through each phase of the study. 200 patients from the Endocrine and Dermatology clinics at the University Hospital of Wales, Cardiff, were screened for trial eligibility of which 80 fulfilled all the inclusion criteria. Of these a final 30 women consented to trial participation and were randomised accordingly.

Figure 5.8: Flow Of Participants Through Each Phase Of The Clinical Trial



25 subjects completed the full protocol. This gave a slightly reduced power of 70% to detect a shift of 0.5 times the SD of within-group changes. Five subjects were withdrawn during the course of the study: 1 participant because of pregnancy and 4 individuals because of protocol violation (non-attendance for study visits). The data analysed are therefore for the 25 subjects who completed the study. No significant carryover effect was noted in any of the measurements.

5.3.2 Baseline Demographic & Anthropometric Data

Table 5.4 summarises the baseline demographic and anthropometric characteristics of the study population. The mean age for the group was 30 years and 20% reported current smoking. The average BMI was 34.9 kg/m^2, consistent with obesity, and associated with an increased mean waist circumference of 107.6 cm.

Table 5.4: Baseline Demographic & Anthropometric Characteristics

Values are based upon all 30 study participants & are expressed as mean (SD) or median (range).

Characteristic	Mean SD
Age (yr)	30 (18 - 35)
Smokers (n)	6 (20%)
Ferriman-Gallwey score	11 (2 - 23)
Weight (kg)	96.5 (23.4)
BMI (kg/m^2)	34.9 (6.8)
Waist circumference (cm)	107.6 (21)
Hip circumference (cm)	119.5 (19.2)
Fat percentage	48 (21 - 57)

5.3.3 Baseline Vascular Parameters

Table 5.5 summarises the baseline vascular markers for the study population. Reference values for AIx using the SphygmoCor apparatus have been provided by the manufacturer (Atcor medical) derived from unpublished data by Cockroft et al (Cardiff, UK) available at http://www.atcormedical.com. Based upon their data the mean AIx for a similar mean aged population is quoted to be 3.03% (95% CI -15.57 to 24.5) compared with a value of 18.5% in our PCOS study population, which may suggest increased baseline arterial stiffness. However, the demographic and anthropometric characteristics of this reference population are not stated in the manufacturer's literature. The baseline vascular parameters for PWV and blood pressure are comparable to those reported in similar studies in PCOS cohorts [245].

Table 5.5: Baseline Vascular Parameters Of Study Participants

Values are based upon all 30 study participants & are expressed as mean (SD).

Characteristic	Mean SD
T_R (msec)	145.7 (11.4)
AIx (%)	18.5 (10.5)
bPWV (m/sec)	7.7 (1.1)
aPWV (m/sec)	7.0 (1.2)
Peripheral SBP (mm Hg)	127 (11)
Peripheral DBP (mm Hg)	85 (9)
Central SBP (mm Hg)	117 (11)
Central DBP (mm Hg)	85 (9)
Fall in AIx after GTN (%)	-16.8 (5)
Fall in AIx after salbutamol (%)	-14.6 (6.4)

5.3.4 Baseline Metabolic Biochemical Parameters

Table 5.6 summarises the baseline metabolic parameters for the study population.

Table 5.6: Baseline Metabolic Biochemical Parameters Of Study Participants

Values are based upon all 30 study participants & are expressed as mean (SD) or median (range).

Characteristic	Mean (SD) or Median (range)
Adiponectin (pg/ml)	474.3 (95.3 - 1915.3)
hsCRP (mg/liter)	3.4 (0.16 - 14.4)
Insulin (pmol/liter)	20.7 (5.1 - 42.2)
Glucose (mmol/liter)	5.1 (0.5)
HOMA-IR	2.5 (0.7 - 4.8)
TC (mmol/l)	4.8 (0.7)
HDL (mmol/l)	1.2 (0.3)
LDL (mmol/l)	3.0 (0.5)
Triglycerides (mmol/liter)	0.9 (0.4 - 5.2)
PAI-1 (ng/ml)	1.75 (0.5 - 23.9)
Testosterone (nmol/liter)	2.6 (0.8)
SHBG (nmol/liter)	33.2 (18.5 - 174.3)
FAI	7.6 (0.8 - 17.4)
Oestradiol (pmol/l)	156 (70-707)
Progesterone (nmol/l)	3.5 (2-31.5)

5.3.5 Prevalence Of Features Of The Metabolic Syndrome In Study Population

There was an increased prevalence of features of the metabolic syndrome based on the 2006 IDF definition amongst the study population (Table 5.7). The commonest abnormality was an increased waist circumference, a marker of visceral adiposity, in 93% of the subjects. 60% had evidence of hypertension, 53% low HDL, 20% hypertriglyceridaemia, and 13% impaired

fasting glucose. 47% fulfilled the criteria for the metabolic syndrome with ≥ 3 features present. These findings are in keeping with reports in the literature by Dokras et al [127] who reported a prevalence of 34% of the metabolic syndrome in PCOS, and 43% by Apridonidze and colleagues [129] as discussed previously.

Table 5.7: Prevalence Of Features Of The Metabolic Syndrome In PCOS Based On the 2006 IDF Criteria

Metabolic Syndrome Criteria	Prevalence № (%)
Waist circumference (≥ 80 cm)	28 (93 %)
Triglycerides (≥ 1.7 mmol/l)	6 (20 %)
HDL cholesterol (< 1.29 mmol/l)	16 (53 %)
BP (Systolic ≥ 135 or diastolic ≥ 85 mmHg)	18 (60 %)
Dysglycaemia (Fasting glucose ≥ 5.6 mmol/l)	4 (13 %)
Central obesity + 2 other criteria	14 (47 %)

5.3.6 Treatment Compliance & Adverse Events

All adverse events were recorded in the trial case report forms and reported to the Cardiff and Vale NHS Trust Research and Development Department as well as Cardiff University (study sponsor). Annual safety reports were submitted to the South Wales Resarch and Ethics Committee as well as the Medicines and Healthcare Regulatory Authority.

Overall the study medication (active and placebo) was well tolerated. Five adverse events were reported during the course of the study, three of which were expected (gastrointestinal discomfort during the active treatment phase) but mild and self-limiting. One subject described a headache (while on metformin) and this was also mild in intensity and self-limiting. Another participant became pregnant (while on placebo) and was immediately

withdrawn from the study. The pregnancy was followed to term with no maternal or foetal complications.

5.3.7 Effect Of Metformin On Vascular Function

Metformin therapy significantly improved all markers of arterial stiffness in our study population (bPWV, aPWV, AIx; all $p<0.001$) with the exception of T_R which did not change (Figures 5.9 and 5.10).

Figure 5.9: Effect Of Metformin Therapy On Augmentation Index In PCOS

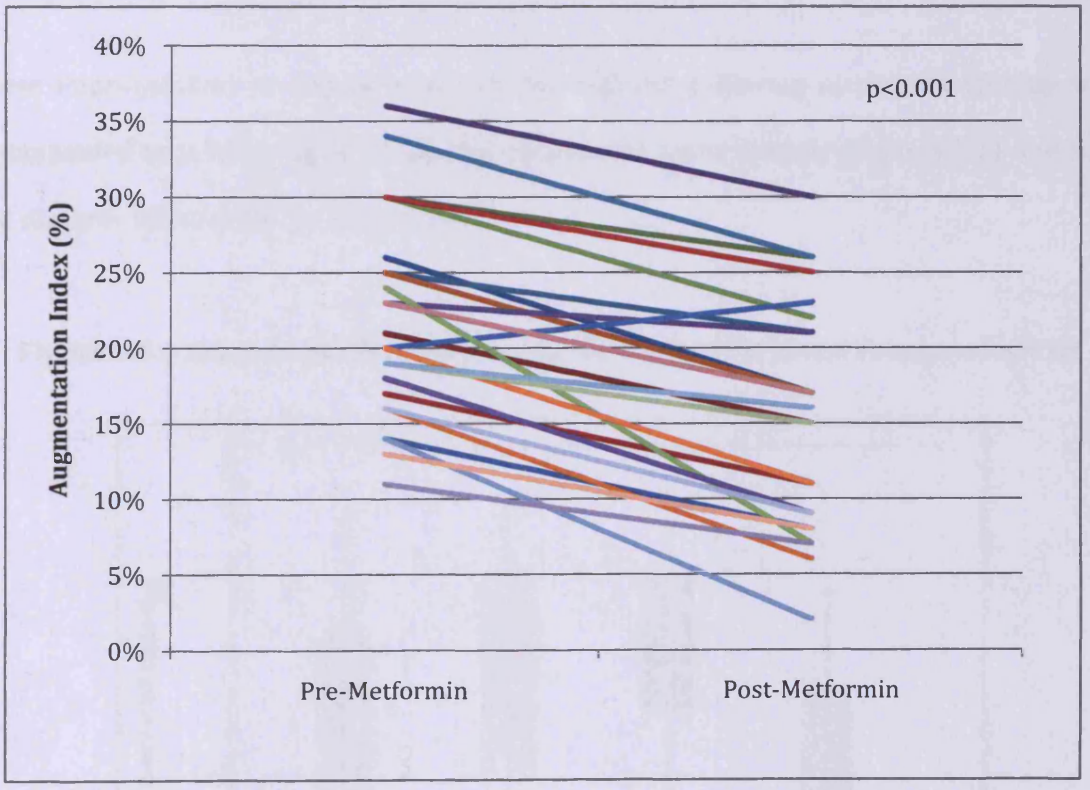
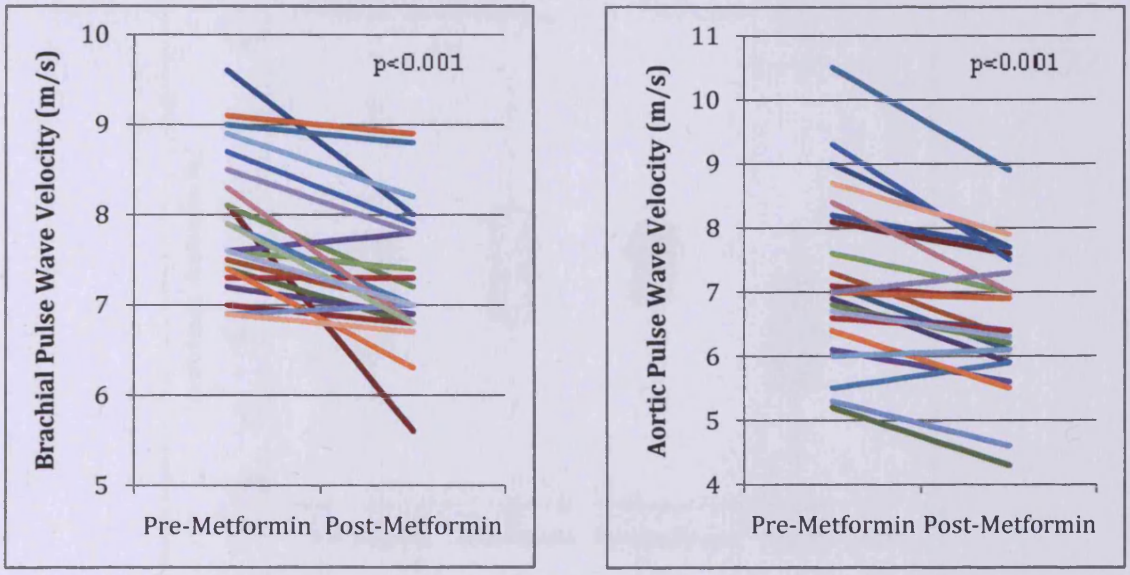


Figure 5.10: Effect Of Metformin Therapy On Brachial & Aortic PWV In PCOS



These improvements in measures of arterial stiffness following metformin therapy were accompanied by a lowering of peripheral and central aortic systolic (Figures 5.11 and 5.12) and diastolic blood pressure (all $p<0.001$).

Figure 5.11: Effect Of Metformin Therapy On Peripheral Blood Pressure In PCOS

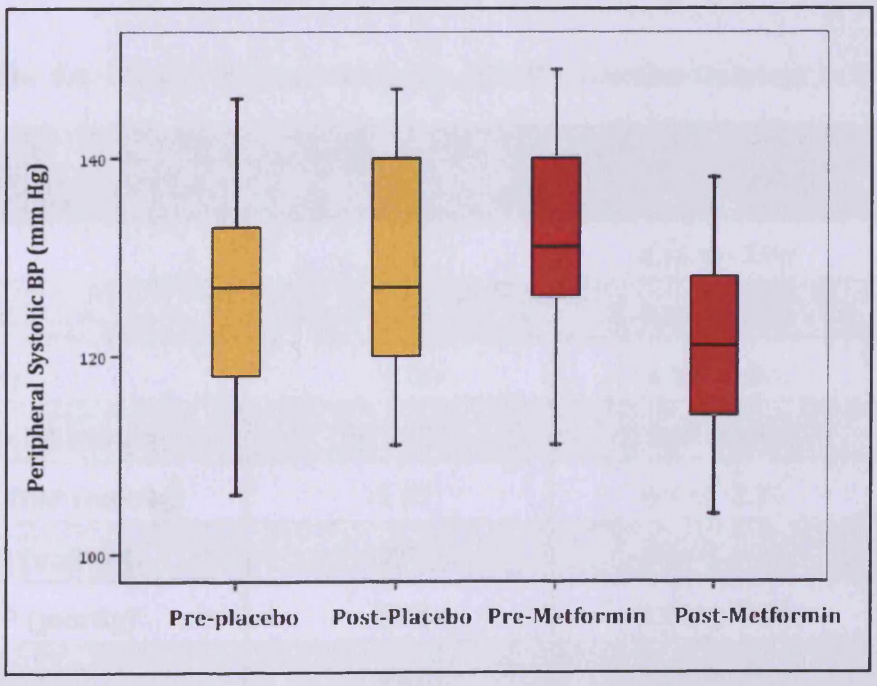
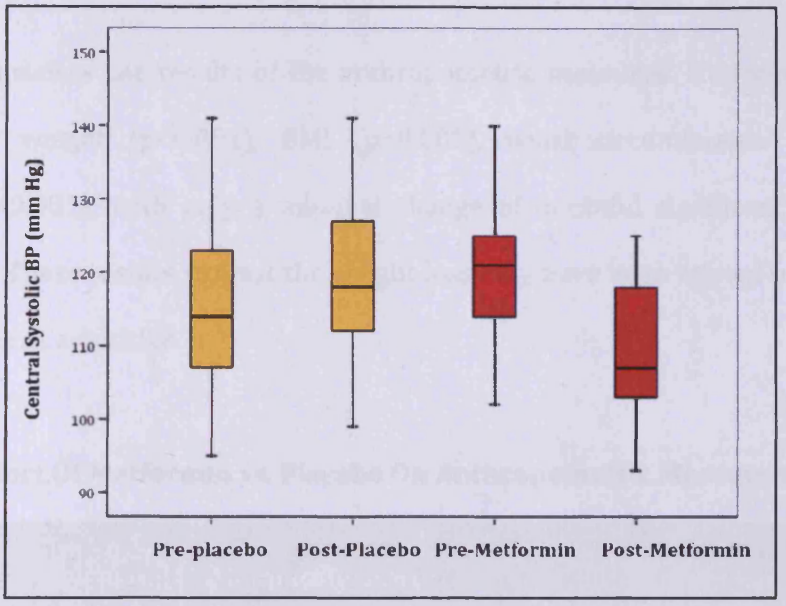


Figure 5.12: Effect Of Metformin Therapy On Central Systolic Blood Pressure In PCOS



Metformin also improved endothelium-dependent and endothelium-independent vascular responses as evidenced by an increased reduction in AIx post-salbutamol (p=0.003) and post-GTN (p<0.001) respectively. A summary of the primary vascular outcome measures for the study is given in Table 5.8.

Table 5.8: Effect Of Metformin *vs.* Placebo On Vascular Outcome In PCOS

	Mean difference	95% CI for the difference	p-value
AIx (%)	-6.1	-8.46 to -3.74	**<0.001**
bPWV(m/s)	-0.73	-1.09 to -0.38	**<0.001**
aPWV (m/s)	-0.76	-1.12 to -0.4	**<0.001**
Peripheral SBP (mmHg)	-7.65	-10.47 to -4.84	**<0.001**
Peripheral DBP (mmHg)	-5.83	-8.4 to -3.22	**<0.001**
Central SBP (mmHg)	-9.02	-12.05 to -6	**<0.001**
Central DBP (mmHg)	-5.96	-8.74 to -3.17	**<0.001**
AIx post-GTN (%)	-4.68	-6.93 to -2.43	**<0.001**
AIx post-salbutamol (%)	-5.38	-8.68 to -2.07	**0.003**

5.3.8 Effect Of Metformin On Anthropometric Measurements

Table 5.9 summarises the results of the anthropometric measures. Metformin significantly reduced body weight ($p<0.001$), BMI ($p<0.001$), waist circumference ($p<0.001$), fat percentage ($p<0.001$), with only a minimal change of doubtful significance noted in hip circumference. These results suggest the weight loss may have been related to a reduction in central, or visceral, adiposity.

Table 5.9 : Effect Of Metformin *vs.* Placebo On Anthropometric Measurements In PCOS

	Mean difference	95% CI for the difference	p-value
Weight (kg)	-2.05	-2.9 to -1.2	**<0.001**
BMI (kg/m²)	-0.75	-1.06 to -0.43	**<0.001**
Fat percentage (%)	-2.46	-3.26 to -1.65	**<0.001**
Waist circumference (cm)	-4.88	-6.96 to -2.81	**<0.001**
Hip circumference (cm)	-0.82	-1.62 to -0.02	**0.045**

5.3.9 Effect Of Metformin On Metabolic Biochemical Parameters

Metformin significantly increased adiponectin concentrations ($p=0.001$) as illustrated in Figure 5.13 and was also associated with a fall in plasma triglycerides. There were no changes observed with metformin therapy in hsCRP, insulin, glucose, HOMA-IR, total cholesterol, HDL, LDL, PAI-1, testosterone, SHBG, FAI, oestradiol or progesterone. Table 5.10 summarizes the results of the metabolic biochemistry.

Figure 5.13: Effect Of Metformin Therapy On Adiponectin In PCOS

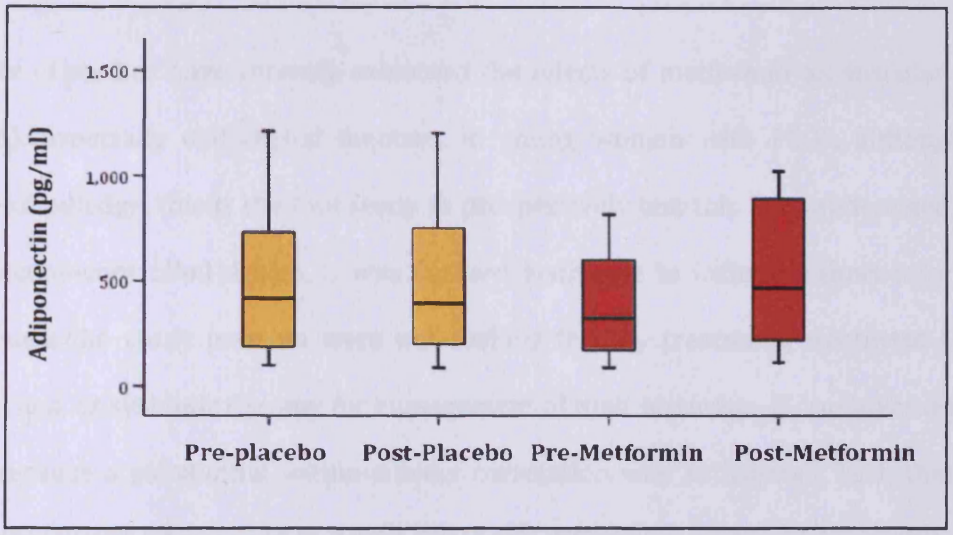


Table 5.10: Effect Of Metformin *vs.* Placebo On Metabolic Markers In PCOS

	Mean difference	95% CI for the difference	p-value
Adiponectin (pg/ml)	+81.5	+18.7 to +144.3	**0.001**
hsCRP (mg/l)	-0.13	-0.34 to +0.86	0.216
Insulin (pmol/l)	-0.08	-4.75 to +4.58	0.971
Glucose (mmol/l)	-0.13	-0.46 to +0.21	0.43
HOMA-IR	-0.46	-2.21 to +1.3	0.583
TC (mmol/l)	-0.75	-2.42 to +0.92	0.35
HDL (mmol/l)	-0.69	-2.36 to +0.99	0.39
LDL (mmol/l)	-0.67	-2.35 to +1.02	0.41
Triglycerides (mmol/l)	-0.16	-0.26 to -0.56	**0.004**
PAI-1 (ng/ml)	+0.08	-1.69 to +1.84	0.3
Testosterone (nmol/l)	-0.81	-2.48 to +0.86	0.31
SHBG nmol/l	-3.31	-17.25 to +10.63	0.62
FAI	-1.26	-3.34 to +0.82	0.22
Oestradiol (pmol/l)	+15.34	-25.24 to +55.93	0.44
Progesterone (nmol/l)	+2.66	-2.85 to +8.18	0.33

5.4 DISCUSSION

A number of studies have recently examined the effects of metformin on vascular function [240-246], especially endothelial function, in young women with PCOS, although to the author's knowledge, this is the first study to prospectively test this in a randomized, double-blind, placebo-controlled design. It was deemed justifiable to include a short-term placebo arm because the study patients were not seeking fertility treatment, treatment for oligo-/amenorrhea, or systemic therapy for management of their hirsutism. A crossover design was chosen because a substantial within-subject correlation was anticipated, such that smaller sample sizes would be required to detect significant differences, and used a washout period of 8 wk to minimize the potential for a carryover effect. Although endothelial function has been found to vary according to phase of the menstrual cycle [275], we were unable to apply these temporal limitations to the vascular measurements because of the need for equal treatment duration in each phase of the crossover design. Nevertheless, it is not considered likely that any potential confounding influences of metformin on improved menstrual cyclicity [245] might account for the changes observed in vascular function because estradiol and progesterone levels did not differ after the metformin and placebo phases.

The data demonstrated that metformin significantly improved endothelial function in the PCOS cohort, an observation consistent with some [240-243], but not all [244-246], previous studies in young women with PCOS. The latter studies employed flow-mediated dilatation of the brachial artery as their method to assess endothelial function. An alternative technique based on a similar principle was used in the present study, whereby changes in AIx in response to endothelium-dependent (salbutamol) and endothelium-independent (GTN) pharmacological challenges are measured by pulse wave analysis [270]. Although this technique may offer reduced precision compared with flow-mediated dilatation in measuring endothelial function [276], it provides a more global assessment of endothelial function, including conduit and resistance vessel endothelial function, and correlates strongly with gold

standard measures [270]; hence, it was anticipated that the current findings would be comparable to other methodologies.

Endothelial dysfunction is an important process in the progression of atherosclerosis and independently predicts cardiovascular morbidity [277, 278]. An improvement in large artery stiffness was observed with metformin therapy as evidenced by a reduction in AIx and slowed brachial and aortic PWV. A slowing of aortic PWV in response to metformin is especially noteworthy given that this measure of arterial stiffness is independently predictive of cardiovascular mortality in a number of disease states, including renal failure, hypertension, and glucose intolerance/type II diabetes [236-238]. A 1 m/sec increase in aortic PWV was associated with a 39 and 8% increase in mortality in patients with renal failure [237] and type II diabetes [236] respectively; hence, the observed reduction of almost 0.8 m/sec is likely to be clinically important. In contrast, Meyer and colleagues [245] failed to detect a change in PWV with 6 months of metformin treatment in their population of women with PCOS but did note an increase in PWV with a high-dose oral contraceptive (35g ethinyl estradiol/2 mg cyproterone acetate), related principally to increased insulin resistance. These divergent effects on arterial stiffness of the two most commonly prescribed treatments for young women with PCOS thus has important practical implications when considering choice of therapy in the clinic. The study population in Meyer et al. had a similar mean age (31 years), BMI (36.3 kg/m^2), systolic blood pressure (121mmHg), and aortic PWV (7.47 m/sec) to that described here; hence these discrepancies cannot be accounted for on the basis of differences in patient characteristics at baseline. Furthermore, although their sample size calculations were performed primarily for changes in insulin sensitivity, their study was also adequately powered to detect a 3–4% difference in PWV [245]. However, in contrast to the present findings, the participants in Meyer and colleagues' study did not lose weight, nor did their blood pressure change, which suggests that the vascular improvements observed in this thesis with metformin might be dependent at least in part on changes in these variables. In support of this, weight loss alone (8% over 1 yr) has been shown to reduce aPWV by 0.5

m/sec in subjects with type II diabetes [279], and a blood pressure reduction of 24/9 mm Hg with valsartan and hydrochlorothiazide is associated with a reduction in aortic PWV by 1.8 m/sec [280].

A fall in peripheral blood pressure with metformin therapy has been reported previously in women with PCOS [281, 282], although the reduction observed here in central aortic blood pressure is likely to have even greater clinical relevance because this has been shown to be a more powerful predictor of mortality than brachial blood pressure in patients with CVD [283]. The 2-kg reduction in weight and 0.75 kg/m^2 fall in BMI is consistent with the findings of a recent systematic review of insulin-sensitizing drugs on weight loss in women with PCOS in which a weighted mean difference of 0.68 in BMI was found compared with placebo [284]. The reduction in waist circumference suggests a predominant action on the visceral fat compartment, which others have confirmed using computerized tomography [285]. In keeping with this, in the present trial an increase in adiponectin was noted, which has previously been reported with metformin in overweight women with PCOS [286]. This rise may in itself have contributed to the improvements in vascular function because adiponectin enhances nitric oxide production by endothelial cells with attendant benefits on endothelial function [287].

It was a surprise that insulin resistance as measured by HOMA-IR did not improve with metformin, in contrast to a number of other studies that demonstrate improved insulin action with metformin in women with PCOS [139, 245]. These studies typically employed more sensitive measures of insulin resistance than HOMA-IR, and it is possible that an improvement in insulin resistance with metformin treatment might have been observed had a more robust measure been employed. Nevertheless, it is worth noting that the baseline values of HOMA-IR in this study were relatively low, suggesting only a modest degree of insulin resistance in the trial subjects despite their elevated BMI. Hence, the possibility that

metformin might be exerting its beneficial actions on the vasculature by a mechanism independent of improved insulin sensitivity cannot be entirely discounted.

Metformin was also noted to lower triglyceride levels but did not alter HDL, LDL, or TC. A proatherogenic dyslipidemia, characterized by raised triglyceride levels and reduced HDL concentrations accompanied by increased small, dense LDL particles, is common in women with PCOS [115]. This trial did not undertake any qualitative lipoprotein analysis; hence, an effect of metformin on LDL subclass cannot be excluded, but these findings of reduced triglycerides, although modest, are in agreement with some reports that have shown a reduction with metformin treatment, albeit with large variation among individuals [236, 288]. No changes in testosterone, FAI, or SHBG were observed with metformin therapy, suggesting that the beneficial effects of metformin on vascular function are independent of changes in androgen status. The impact of androgens on vascular function in women with PCOS is controversial, with some studies demonstrating an association of endothelial dysfunction with androgen level [90, 140] and others suggesting a favourable effect of androgens on vascular function [239, 289].

The improvements noted in vascular function raise the question as to how metformin might be exerting its benefits. The crossover design of the present study precluded the use of regression analysis to explore the major contributors to the improved vascular action. However, the magnitude of change (mean difference/SD of the active-placebo differences) for each variable significantly affected by metformin therapy was very similar, suggesting that these were all being influenced by the intervention to a similar degree and hence possibly by the same pathway. It may be speculated that this pathway might involve the key energy sensor AMP kinase (AMPK) [290]. In addition to metformin's ability to activate AMPK in the liver and in muscle, with consequent benefits on enhanced glucose uptake and reduced hepatic glucose output, metformin has also been shown to activate AMPK in the endothelium and in vascular smooth muscle, where it mediates vascular relaxation through the α1 subunit

in an endothelium-independent manner [291, 292]. These findings may thus explain our observations whereby metformin enhanced endothelium-independent (NTG) as well as endothelium-dependent (salbutamol) vascular responses.

In conclusion, the data presented in this chapter support an important action of metformin in improving vascular function in young overweight women with PCOS, but further studies are needed to confirm these findings and to delineate the potential mechanisms in operation. In particular, this study was limited by the lack of a weight-loss control group; hence, further trials that include this as a control arm are required to fully assess whether metformin therapy has additional benefits on vascular function beyond weight loss alone. Furthermore, overweight, anovulatory women with PCOS may be particularly at risk of insulin resistance and vascular dysfunction. Therefore, trials in normal weight and eumenorrheic subpopulations are also required before these findings can be generalized to all women with PCOS. Additional studies should also explore the potential for additive or synergistic interactions with other agents known to improve vascular function, such as statins, or exercise regimens, which also have the potential to activate AMPK.

CHAPTER 6: GENERAL DISCUSSION

6.1 Background

Global cardiometabolic risk represents the overall likelihood of developing type II diabetes, cardiovascular disease, or both, due to a cluster of modifiable adverse factors [21]. These include classic risk factors such as smoking, high LDL cholesterol levels, hypertension and elevated blood glucose, in addition to others closely associated with abdominal obesity, such as insulin resistance, low HDL cholesterol, high triglycerides and elevated inflammatory markers [21]. The rationale for the concept of the metabolic syndrome arises from the observation that a clustering of these adverse metabolic and vascular risk factors represents a high-risk group requiring careful attention and treatment following an integrated approach.

Treatment approaches to date have largely been directed at individual traits such as hypertension and dyslipidaemia. However, the pathogenesis of the components of the metabolic syndrome is largely linked to underlying obesity, especially an accumulation of central or visceral adiposity, and insulin resistance. Therefore therapies targeting these factors should help minimise the increasing health burden arising from CVD and type II diabetes attributable to obesity.

6.2 Thesis Aims

The aims of this thesis were to examine the effects of existing (metformin) and novel (CB_1 antagonist) pharmacological agents that target components of the metabolic syndrome using appropriate models of the syndrome *in vivo* (PCOS) and *in vitro* (adipogenesis) by:

1. Evaluating the actions of cannabinoid receptors on proliferation, differentiation and adipokine production in models of *in vitro* adipogenesis.

2. Determining the *in vivo* effects of metformin therapy on arterial stiffness, endothelial function, adipokine profile and insulin sensitivity in young women with PCOS.

It was hypothesised that these agents may exert their benefits not only by improving insulin sensitivity and obesity, but also through indirect mechanisms involving a switch in the adipokine profile.

6.3 Current Pharmacological Therapies For The Management Of Obesity

Obesity trends amongst adults in the UK continue to increase. The National Heart Forum report published in February 2010 predicts that by 2020 the proportion of adults who will be overweight aged 20-65 is 40% for men and 32% for women [293]. The team also predict a 23% rise in the prevalence of obesity-related stroke, a 34% rise in obesity-related hypertension, a 44% rise in obesity-related coronary heart disease and a 98% rise in obesity-related diabetes [293].

Effective weight management requires lifestyle modification to encourage healthy eating habits and increased levels of physical activity to ensure that energy expenditure is at least equal to, if not greater than energy intake. A number of studies including the Diabetes Prevention Programme (DPP) and the Finnish Diabetes Prevention Trial (FDPT) have provided convincing evidence for the benefits of lifestyle interventions facilitating weight loss in the prevention of diabetes [294, 295]. However, such approaches are not always sufficient to achieve and maintain weight loss outside of clinical trial settings. Given the health risks associated with increased adiposity, there is a demand for safe and effective anti-obesity agents to act as an adjunct to lifestyle modification in both promoting and maintaining weight loss, to help improve co-morbidity.

The early drugs used for weight loss were centrally-acting appetite suppressants including amphetamines but these are no longer an option due to their high addiction potential. 5-hydroxytryptamine (5-HT) uptake inhibitors such as fenfluramine have been used in the past as well but have been removed from the market due to an association with pulmonary hypertension and valvular heart disease [296].

Until recently there have only been two drugs available as pharmacological adjuncts for weight loss with license for use in the developed world. Orlistat can cause modest weight loss and acts by inhibiting the absorption of ingested fat within the gastrointestinal tract, though its use can be limited by the side effects of faecal incontinence and interference with the absorption of fat soluble vitamins [297]. Sibutramine, a centrally acting drug that stimulates the sensation of satiety and increases energy expenditure, was the alternative agent. However, in January 2010, the MHRA announced the suspension of the marketing authorisation for sibutramine following a review by the European Medicines Agency (EMA). It was determined that sibutramine's cardiovascular risks outweigh its benefits, with emerging evidence to suggest an increased risk of non-fatal heart attacks and strokes with this medicine [298].

A third agent, the first-in-class CB_1 receptor blocker drug, rimonabant was available from 2006 to 2008, and represented a novel approach targeting both central and peripheral sites in the regulation of weight and energy homeostasis. However, due to safety concerns (detailed below) the drug is no longer available but the endocannabinoid system still remains a promising therapeutic target for further research and development, and will be discussed in greater detail in the following section.

6.4 The ECS As A Therpeutic Target In The Management Of Obesity & The Metabolic Syndrome

6.4.1 The Role Of CB_1 Receptors In The Regulation Of Metabolism

In one of the earliest animal studies examining the effect of CB_1 receptor blockade on appetite suppression by Colombo and colleagues, chronic administration of rimonabant resulted in a transient reduction in food intake as well as a longer lasting reduction in body weight, suggesting a beneficial metabolic effect of this treatment independent of energy intake [299]. This has been supported by further observations in CB_1 receptor-deficient mice who exhibit hypophagia and leanness compared to their wild-type littermates, and also maintained a lean phenotype in comparison to the wild-type mice despite identical paired feeding, indicating possible altered energy expenditure and metabolism [59]. Diet induced obesity in mice is accompanied by dyslipidaemia, insulin resistance and fatty liver, and can thus be considered a murine model of the metabolic syndrome [21]. Once daily treatment with rimonabant at 10 mg/kg for forty days, has been shown to result in a 50% reduction in adipose tissue mass in mice with diet-induced obesity [300]. Furthermore, CB1 receptor knockout mice are resistant to diet induced obesity and maintain a more favourable metabolic profile when compared to the wild-type mice who do become obese on an identical high calorie diet [58].

The human clinical trials of rimonabant in obesity (RIO) have been divided into four main groups: RIO-North America [79], RIO-Europe [221], RIO-Lipids [78] and RIO-Diabetes [80]. In these studies up to two years treatment with rimonabant resulted in significant weight loss that was also associated with improvements in the metabolic profile including lower plasma TG, increase in HDL, reductions in the elevations of plasma insulin and leptin, and an increase in adiponectin concentration [78-80, 221]. An interesting observation is that the majority of the weight loss occurred during the first year of treatment whereas improvements in lipid and glycaemic profiles continued even in the second year, and thus may not be attributable to

weight loss alone [221, 301]. These findings support an additional role of the ECS in peripheral sites, including adipose tissue, to produce beneficial metabolic effects beyond weight loss in obese patients.

6.4.2 *In Vitro* Studies Supporting A Role Of Peripheral CB_1 Receptors

The relevance of the ECS to adipocyte physiology is supported by studies, including the experiments described in this thesis, demonstrating CB_1 receptor expression in pre-adipocytes which changes dynamically through adipogenesis, becoming more prominent in mature adipocytes and is closely associated with PPARγ receptor profiles during adipocyte differentiation [76, 207]. In keeping with these findings, Bouboula et al demonstrated PPARγ transcriptional activation in the presence of endogenous endocannabinoids in murine 3T3-L1 though this remains to be confirmed in human *in vitro* models [302]. Perhaps more interesting is the observation that CB_1 receptors are differentially expressed in WAT derived from different depots [222]. The current studies confirmed a more significant effect of CB_1 receptor antagonism in cells originating in omental adipose tissue, thus modifying an important adverse cardiovascular risk marker. Similarly, higher levels of the endocannabinoid 2-AG have been detected in the intra-abdominal visceral, but not subcutaneous fat of obese subjects [303], and an increase in circulating 2-AG has been shown to positively correlate with important cardiometabolic risk factors, such as BMI, waist circumference, fasting plasma triglyceride and insulin levels, low HDL cholesterol, and adiponectin levels [304].

CB_1 activation in adipose tissue is not limited to promoting cell proliferation but also acts to increase energy storage. Endocannabinoids stimulate lipogenesis by inducing triglyceride accumulation through the inhibition of adenylate cyclase and the consequent reduction of lipolysis, and via activation of lipoprotein lipase to provide exogenous fatty acids for the adipocytes [59, 300]. In addition, endocannabinoids increase the expression and activity of

enzymes involved in fatty acid and triglyceride biosynthesis thus promoting *de novo* lipogenesis [59]. CB_1 activation in adipocytes also modulates insulin signalling and glucose uptake in order to increase energy storage and modify insulin sensitivity [303]. Therefore it would be interesting to expand the *in vitro* studies detailed in the previous chapters to examine the effects of CB_1 receptor blockade upon energy storage within adipose tissue and to determine if a differential effect exists between WAT derived from different fat depots.

Adipose tissue is now recognised as an endocrine organ capable of secreting a number of adipokines which influence adipocyte physiology. The present studies have illustrated an increase in adiponectin production arising from CB_1 antagonism treatment in *in vitro* models of adipogenesis using human primary cultures, which may in part explain the changes in insulin sensitivity observed with rimonabant therapy. Further studies looking at the effects upon other adipokines including visfatin, leptin and IL-6, for example, may improve our understanding of the mechanisms by which CB_1 antagonists exert their metabolic effects.

Recent studies have found that the administration of CB_1 antagonists in diet-induced obese Wistar rats induces an increase in energy expenditure, thus identifying another possible peripheral mechanism contributing to a reduction in adipose mass [305]. This is thought to be mediated by CB_1 antgonist-mediated generation of futile, or substrate, cycles, where two metabolic pathways (in this instance glycogen synthesis and glycolysis) run simultaneously in opposite directions resulting in the dissipation of energy in the form of heat [300]. Although in the studies presented in this thesis CB_1 modulation did not alter UCP-1 expression, a marker of BAT, Perwitz et al have highlighted a rimonabant-induced transdifferentiation in white adipocytes towards a mitochondria-rich brown fat phenotype [209]. CB_1 receptor antagonist treatment may therefore also lead to an eventual increase in energy expenditure via mitochondrial thermogenesis by increasing brown-type adipocytes. Though the presence of BAT has recently been described in adult humans [181, 182], the functional significance is not yet well established and still demands further study.

CB_1 receptor blockade has also been shown to increase mitochondrial biogenesis in cultured white adipocytes by inducing the expression of eNOS [306] and is accompanied by the prevention of high-fat diet-induced fat accumulation, without concomitant changes in food intake [306]. A possible mediator of these functions may be the AMPK system. Endocannabinoids decrease AMPK activity in adipose tissue, contributing to a rise in adiposity and lipogenesis and resulting in decrease in energy expenditure, and induction of AMPK activity has been found to increase mitochondrial content in adipocytes. [306]. Thus, the anti-obesity effects associated with CB_1 blockade may also result from enhanced β-oxidation of free fatty acids elicited by AMPK activation and by eNOS-dependent mitochondrial biogenesis.

The aforementioned studies and the experiments described in this thesis support a significant role for the ECS in the periphery in targeting obesity and thus the metabolic syndrome. This is of importance when considering the future of CB_1 antagonist therapy given the adverse events associated with the first generation drug in this class, rimonabant which have largely been attributable to central CB_1 blockade.

6.4.3 Adverse Events Associated With Rimonabant

The reduction in weight and changes in metabolic parameters observed with rimonabant therapy have unfortunately been accompanied by a significant side effect profile. Pooled data obtained from analyses of the RIO studies following 1 year of treatment with rimonabant highlights adverse effects which can be broadly categorised into three main groups: psychiatric symptoms (1.9% vs. 0.8% in treatment group vs. placebo), gastrointestinal disturbances predominantly in the form of nausea (1.4% vs. 0.1%), and neurological alterations including headaches and vertigo [301]. Altogether, 13.8% of patients receiving 20 mg rimonabant daily discontinued treatment in comparison to 7% of those receiving placebo [301].

In October 2008, the EMA recommended suspending its marketing authorisation for Sanofi-Aventis's first-in-class CB_1 receptor blocker rimonabant, due to an increased risk of psychiatric side-effects including severe depression, anxiety and stress disorders which outweighed the potential benefits [307]. This led to the decision by Sanofi-Aventis in November 2008 to withdraw rimonabant from the market world-wide and discontinue clinical trials of rimonabant for all clinical indications [308]. This move was followed closely by termination of development programmes and clinical trials of other CB_1 receptor blocking compounds by competing pharmaceutical companies including Pfizer (otenabant) and Merk (taranabant) [309].

Previous studies have shown that up to 48% of obese individuals exhibit features of depression and that obese women in particular are 20% more likely to have experienced suicidal ideation and 23% more likely to have actually attempted suicide in comparison to their non-obese counterparts [310]. Whilst in clinical trial settings subjects are rigorously screened for such conditions, it is not clear how robustly this is undertaken in general clinical practice, and may have also contributed to the increase in mood-related adverse effects. Since overweight individuals represent one of the target populations for this class of drugs, any future drug development will need to address the potential for psychiatric disturbance.

However, abandoning the ECS as a potential therapeutic target in weight management may be a little premature, and recently some efforts have been made to circumvent the psychiatric side-effects currently limiting the use of this class of drugs. This has largely focused on targeting the ECS in peripheral tissues which may still preserve some of the metabolic benefits described in the previous section.

6.4.4 Future Drug Development Targeting The ECS

The initial approach towards modulating the ECS to affect weight has predominantly focused on targeting central CB_1 receptors with the aim of appetite suppression [299, 311], though later studies, including the experiments described in this thesis, have supported the additional role of peripheral mechanisms contributing to the reduction in adiposity [207, 225]. Initially drug development included optimising the compound for high brain penetration and antagonism of central CB_1 receptors, thus also contributing to the CNS-mediated psychiatric side-effects. New strategies now include developing agents that preferentially target peripheral CB_1 receptors located within, for example, adipose tissue, liver, muscle and the gastrointestinal tract, with the aim of maintaining the peripherally-mediated beneficial effects whilst minimising the central adverse consequences.

Pavon et al have developed a novel CB_1 receptor antagonist, LH-21, a triazol derivative with poor penetration into the central nervous system [312]. When given acutely it decreases food intake and enhances the anorectic actions of oleoylethanolamide, a feeding suppressant lipid that acts on peripheral sensory terminals, in a similar way to rimonabant [312]. However, unlike rimonabant, chronic administration of LH-21 (3 mg/kg) reduces feeding but does not improve hypertriglyceridaemia or hypercholesterolaemia; nor does it reduce liver fat deposits in Zucker rats suggesting that antagonism of central CB_1 receptors may be necessary for the metabolic benefits of CB_1 blockade, but not for the appetite reduction [312].

Another scientific group working at Jenrin Discovery have developed two CB_1 receptor antagonists, JD 2114 and JD 5006 whose structures are yet to be disclosed, that have been chemically modified to possess little potential to cross the blood-brain barrier [313]. Both these substances have been shown to have high affinity for CB_1 receptors (< 30 nM) with greater selectivity versus CB_2 receptors [313]. Animal studies have been conducted with these compounds using once daily oral administration at doses of 10 mg/kg in diabetic and

dyslipidaemic obese mice, and compared to control populations treated with rimonabant. Significant weight loss was observed with all these substances as well as a reduction in fasting blood glucose, insulin and triglycerides within seven days of treatment thus demonstrating beneficial effects of CB_1 receptor antagonism by targeting peripheral receptors, though further work is still required to establish efficacy and safety in humans [313].

An alternative avenue that is being pursued by some research groups focuses on targeting a binding site on the CB_1 receptor by developing ligands that act as allosteric inhibitors of agonist signalling efficacy. One of the first *in vivo* reports using this class of agents describes the use of PSNCBAM-1, a novel allosteric antagonist at the CB_1 receptor, which in an acute rat feeding model decreased food intake and body weight [314]. Another technique in development is to bypass the CB receptors and modulate the endocannabinoid system by different means. One such approach involves targeting the enzyme fatty acid amide hydrolase, thus affecting the breakdown of endogenous endocannabinoids [315]. Whether these approaches will result in weight reduction and improvements in metabolic profiles in humans, whilst maintaining a satisfactory risk-benefit ratio, remains to be determined.

6.5 Metformin In PCOS – An *In Vivo* Model Of The Metabolic Syndrome

PCOS is the commonest endocrinopathy affecting women of reproductive age and a significant proportion of the affected women fulfil the criteria for the metabolic syndrome, a finding confirmed in the clinical trial reported in this thesis [316]. PCOS therefore represents a useful model to evaluate treatment effects upon cardiometabolic risk. In the presented clinical trial, metformin reduced arterial stiffness and improved endothelial function in young women with PCOS [317]. The improvements noted in vascular function do however raise the question as to how metformin might be exerting its beneficial effects.

Metformin is an oral biguanide and the most commonly prescribed insulin sensitiser used for the treatment of type II diabetes. Its principal metabolic action is thought to be mediated via suppression of hepatic gluconeogenesis and lipogenesis while improving peripheral glucose uptake and glycolysis [138]. Increasing recognition of its pleiotropic actions on a variety of tissues including the liver, adipose tissue, skeletal muscle and endothelium, have led to recent interest in expanding the therapeutic potential of this old drug originally used only for the management of established diabetes [138].

6.5.1 Action Of Metformin In Adipose Tissue

Although adipose tissue is not thought to be the principal site of action of metformin, there is evidence for modest effects on this tissue. There is conflicting evidence regarding the effect of metformin therapy on insulin-mediated glucose transport in adipose tissue [318, 319], but there are data supporting its effects on adipose tissue lipolysis. In primary rat adipocytes stimulated with TNFα and/or high glucose concentrations, metformin treatment inhibits agonist-induced lipolysis [320]. The resulting reduction in available systemic FFAs could also contribute to the insulin sensitising effects of metformin.

In experiments using murine 3T3-L1 cells, metformin has been associated with counteracting adipose tissue expansion via a direct inhibition of adipogenesis through AMPK-dependent stimulation of FA oxidation and inhibition of lipogenesis [321]. This observation may in part explain the weight loss noted in the clinical trial presented in this thesis via a mechanism independent of metformin's actions upon insulin sensitivity.

Metformin may also affect adipose tissue endocrine function by modulating adipokine synthesis and secretion [138]. Leptin deficiency is associated with hyperphagia, a reduction in energy deficiency and morbid obesity [322], and a positive correlation between insulin resistance and hyperleptinaemia has been noted in women with PCOS [323]. In a brown

adipocyte model, metformin treatment resulted in a dose-dependent inhibition of leptin secretion through the stimulation of MAPK [324]. Adiponectin is an insulin-sensitising adipokine secreted exclusively from adipocytes, whilst resistin has been implicated in the pathogenesis of obesity and type II diabetes [325]. Lewandowski and colleagues have demonstrated a reciprocal correlation between adiponectin and resistin independent of insulin resistance in PCOS, though the role of the resistin in PCOS and cardiometabolic risk in this group is not yet fully understood [326, 327].

6.5.2 Action Of Metformin In The Vascular Endothelium

The endothelium is a major target of insulin and endothelial function is considered a marker of insulin sensitivity in the vasculature, thus insulin plays an important role in linking metabolic and cardiovascular pathophysiology [138]. Treatment with insulin sensitising agents such as metformin have been associated with improvements in endothelial function [240]. However, metformin may also exert direct and beneficial effects on the endothelium independent of glucose lowering and insulin sensitisation as suggested by the improvements in vascular function noted in this thesis without a significant change in insulin sensitivity. In addition to metformin's ability to activate AMPK in the liver and in muscle, with consequent benefits on enhanced glucose uptake and reduced hepatic glucose output, metformin has also been shown to activate AMPK in the endothelium and in vascular smooth muscle, where it mediates vascular relaxation through the α1 subunit in an endothelium-independent manner [291, 292]. Additionally, AMPK may stimulate FA oxidation thereby reducing FFAs, and inhibit glyceride synthesis and thus alleviate endothelial lipotoxicity, a known factor contributing to increased superoxide production and impaired NO activity [328]. Further work is however still required to ascertain these underlying mechanisms.

6.5.3 Direct Effect Of Adiponectin On Endothelial Function

Levels of adiponectin inversely correlates with weight, and a reduction in serum adiponectin is closely related to peripheral arterial endothelial dysfunction [329], and is considered to be an independent predictor of cardiovascular disease [330]. Hypoadiponectinaemia observed with weight gain is thus hypothesised to be a potential underlying regulator for the impaired vascular function associated with obesity. A recent study by Deng et al provided evidence that the diminished ability of endothelium-dependent vasodilation in high-fat diet induced obese rats-derived aortic segments can be partially restored by incubation with adiponectin [331]. This process not only improved endothelium-dependent relaxation but also increased total NO production as well as enhanced eNOS activity thus supporting a role for a direct endothelial effect of adiponectin [331].

A significant rise in serum adiponectin concentration associated with metformin therapy was observed in the trial presented in the previous chapter, and appeared independent of the insulin sensitising effects of metformin. This change in the adipokine profile in the PCOS subjects could therefore underlie some of the effects noted with metformin therapy. Similarly, adiponectin has also been shown to activate AMPK and thereby stimulate FA oxidation and glucose uptake thus improving endothelial function as decribed above [332].

6.5.4 Future Studies

This discussion has concentrated on the potential effects of pharmacotherapy with metformin to improve cardiometabolic risk. What is not clear however is whether metformin produces these additional effects beyond weight loss alone. Future studies are required to compare the effects of metformin therapy with and without lifestyle modification with a control arm of subjects managed with lifestyle change alone using a randomised, double-blind, placebo-

controlled parallel group design trial. This would help clarify the effects of weight loss alone and direct effects of metformin in improving cardiometabolic risk.

6.6 Conclusion

Owing to the long-term health risks associated with increased adiposity and the limited efficacy of existing anti-obesity agents, there is an urgent need for further development of alternative therapeutic options to serve as an adjunct to diet and exercise to promote weight loss, and thus reduce the risk of developing obesity-related illnesses. This thesis has described benefits with both novel (CB_1 antagonism) and existing (metformin) treatment approaches targeting models of the metabolic syndrome, though further work is still required to elucidate the mechanism underlying the observed effects. Although the first CB_1 receptor antagonist rimonabant carried an unacceptable psychiatric risk profile, this should not deter future research in this field since the benefits observed with this drug and preliminary data emerging from the newer generation of agents in this class indicate that there is still potential to more selectively target the ECS in the management of obesity, and thus the metabolic syndrome. Both the *in vitro* and *in vivo* studies have demonstrated an increase in adiponectin, either by direct effect on adipose tissue (CB_1 antagonist studies) or associated with accompanying weight loss (metformin effect in PCOS). Treatments targeting a switch in the adipokine profile as the therapeutic goal may therefore represent a new approach to addressing global cardiometabolic risk associated with the metabolic syndrome. Furthermore, the AMPK pathway is also emerging as a key player in regulating energy balance, and both CB_1 antagonists and metformin, as well as adipokines such as adiponectin exert metabolic effects at least in part through this system, providing yet another potential future target for treatment of the metabolic syndrome.

Appendix 1

PATIENT INFORMATION SHEET
Version 2 May 2006

1. Title of study
Physiological and pharmacological modulation of adipose/connective tissue remodelling.

2. Introduction
You are being invited to take part in a research study. Before you decide whether you wish to become involved it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.
Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

3. What is the purpose of this study?
A number of hormones can change the amount of fat that is stored in the body. Our research group is interested in studying the effects of some of these hormones, including thyroid hormones, growth hormone and a hormone called DHEA which is made in the adrenal gland, on the function of fat cells in the laboratory. Not all fat reacts in the same way. In some parts of the body hormones may cause fat to build up while in others it may break down.
In order for us to fully understand how these hormones affect fat cells, we are asking people to donate a small sample of their fat to us for our research studies. This sample will be obtained during your planned operation. We will measure how the cells grow and work in response to treatment with hormones.
We are also interested in studying whether changes in genes controlling growth hormone levels and function can have an effect on fat cells. In order for us to test this we will also ask you to donate a blood sample for us to analyse your growth hormone genes.

4. Why have I been chosen?
You have been chosen for this study as your surgeon has identified you as someone who will be undergoing an operation soon. A total of 100 patients will be studied.

5. Do I have to take part?
No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. If you withdraw your permission after we have obtained your samples, any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

6. What will happen to me if I take part?
A sample, about the size of one sugar cube, will be taken from the fat under your skin during your planned surgery. If you are having abdominal surgery we would also plan to remove a further sample of fat of the same size from the fat tissue that sits around your bowel (called

omental or visceral fat). This procedure will not affect your operation scar. The fat samples would be used in laboratory experiments for further study. During these we would break up the fat and separate it into different cell types. We will measure how the cells grow and work in response to treatment with hormones.
The tests to examine your growth hormone genes (called GH1 and GHR) would involve us taking an extra sample of blood (equivalent to roughly two tablespoons). We would plan to take this sample while you are under anaesthetic during your operation so that you do not feel any discomfort.

7. What do I have to do?
There are no lifestyle or dietary restrictions and you can continue your daily activities normally. There will be no changes to the way in which you need to prepare for your operation and your surgical team will fully instruct you on this as normal. With your permission we will check with you and in your records on your previous medical health in case you have a medical condition or are taking any medication which may affect our ability to analyse your fat cells.

8. What are the possible disdvantages of taking part?
Occasionally patients may experience discomfort (temporary pain, swelling, bruising and rarely infection) caused by the collection of blood. Removing a small sample of fat from under the skin or from around the bowel is unlikely to cause any additional complications to those which could be expected from your type of operation.

The genetic blood sampling will test for variation in your growth hormone genes. These changes occur extremely commonly in the general population and can be seen as a variation of normal. The samples will be fully anonymised and the results of these genetic tests will have no implications for you in terms of inherited risk, reproductive decisions or insurance status.

9. What are the possible benefits of taking part?
The study may provide us with important information in identifying how hormones affect fat cell function. While there are unlikely to be any direct benefits to you, we hope that the results of these studies may help in the future treatment of patients with weight-related conditions such as obesity and diabetes.

10. What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Dr Aled Rees 02920 745002). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.
You are unlikely to be harmed as a result of taking part in this research project, but if you are you should be aware that there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay your legal costs. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

11. Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.
With your permission your GP will be informed of your participation in this study.

If you join the study, some parts of your medical records and the data collected for the study may be looked at by the research team, by representatives of regulatory authorities and by authorised people from the Cardiff and Vale NHS Trust to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site. Our procedures for handling, processing, storage and destruction of data are compliant with the Data Protection Act 1998.

12. What will happen to any samples I give?
After the sample of fat has been obtained at surgery the research doctors will immediately freeze this sample and store it for later analysis, or break it up and grow the fat cells in the laboratory. The blood samples will be used to test your growth hormone genes. Both the fat and blood samples will be stored securely in the Section of Endocrinology at the University Hospital of Wales (Room 168, 2nd Floor). The research investigators, Dr Aled Rees and Dr Marian Ludgate, will act as the day-to-day custodians for these samples. Only Dr Rees and Dr Ludgate, and immediate members of their research team will have access to the use of these samples. In addition, all the samples will be coded and anonymised such that your personal details will not be identifiable. We propose to store the fat and genetic samples for up to 15 years. After this time the samples will be destroyed by incineration.

It is possible that we may wish to use your fat sample for future studies looking at how other hormone systems could affect fat cell function. If this is the case then a further application will be made to the Research Ethics Committee for consideration. Similarly, we may wish to conduct genetic studies on genes other than the growth hormone system in the future. If this is the case then this will also be submitted to an Ethics Committee for consideration. The results of these tests done for research purposes are unlikely to have any direct clinical implications for you. It is possible that these studies may be carried out by researchers other than Dr Rees or Dr Ludgate, including researchers working for commercial companies.

There is little prospect that the current studies will have any commercial significance but if future information from our research using your tissue samples shows any likelihood of commercial benefit, such as the development of a new treatment or medical test, then you would not benefit financially.

13. What will happen to the results of the research study?
The results of the research study will be prepared for publication in appropriate medical journals together with presentation at medical conferences. Patients participating in the study will be able to obtain a copy of the results after they have been published in the relevant journal(s). Patients will not be identified in any report/publication.

14. Who is organising and funding the research?
The study is being organised by Dr Aled Rees and Dr Marian Ludgate in the Section of Endocrinology. Funding for the study is provided from the Section of Endocrinology and the doctors conducting the research are not being paid for including and looking after patients in the study, nor will they be able to sell for profit any samples that are collected.

15. Who has reviewed the study?
The study has been reviewed by the Cardiff and Vale NHS Trust Research and Development Office and by the South East Wales Local Research Ethics Committee.

16. Contact for further information
Should you have any further questions or queries regarding this research study, then please do not hesitate to contact me on 029 20745002. I can also be contacted via email (reesda@cf.ac.uk).

Thank you for considering taking part and taking time to read this sheet.

Aled Rees
Principal Investigator/Senior Lecturer in Endocrinology

You will be given a copy of this Patient Information Sheet and a signed consent form to keep

Appendix 2

CONSENT FORM

Version 2, May 2006.

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Patient Identification Number for this study:

Title of project: Physiological and pharmacological modulation of adipose/connective tissue remodelling

Name of researchers: Dr Aled Rees and Dr Marian Ludgate

Contact details for research team: Centre for Endocrine and Diabetes Sciences, University Hospital of Wales, Heath Park, Cardiff CF14 4XN. Telephone: 02920 742182.

Please initial boxes

1. I have read the attached information sheet on this project, dated May 2006 (version 2), and have been given a copy to keep. I have been able to ask questions about the project and I understand why the research is being done and any risks involved. ☐

2. I agree to give a sample of fat for research in this project. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected. ☐

3. I give permission for someone from the research team to look at my medical records to get information on my previous medical health. I understand that the information will be kept confidential. ☐

4. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

5. I know how to contact the research team if I need to, and how to get information about the results of the research. ☐

6. I consent to my GP being informed of my participation in the study. ☐

7. Consent for storage and use in possible future research projects

I agree that the sample I have given and the information gathered about me can be stored by Dr Aled Rees/Dr Marian Ludgate at the Centre for Endocrine and Diabetes Sciences, School of Medicine, Cardiff University for possible use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than Dr Aled Rees/Dr Marian Ludgate who ran the first project, including researchers working for commercial companies. ☐

8. Consent for genetic research

A. I give permission for growth hormone 1 (GH1) and growth hormone receptor (GHR) genotyping to be carried out on the samples I give, as part of this project ☐

B. I understand that the project/future research using the samples I give may include genetic research aimed at understanding the genetic influences on fat cell regulation but that the results of these investigations are unlikely to have any implications for me personally. ☐

Name of patient (BLOCK CAPITALS)	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Name of Researcher	Date	Signature

Would you like to be sent information about the progress of the project? ☐ Yes ☐ No

Thank you for agreeing to participate in this research

1 copy for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 3

EFFECTS OF METFORMIN ON VASCULAR FUNCTION IN POLYCYSTIC OVARY SYNDROME

PATIENT INFORMATION SHEET
(Version 3 May 2006)

1. Title of study

A randomised, double-blind, placebo-controlled crossover trial of the effects of metformin therapy on vascular and metabolic risk in young women with polycystic ovary syndrome.

2. Introduction

You are being invited to take part in a clinical research study. Before you decide whether you wish to become involved it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please do not hesitate to ask us if there is anything that is not clear or if you would like more information. Take time to consider whether or not you would wish to take part.

Thank you for reading this.

3. What is the purpose of this study?

Polycystic ovary syndrome (PCOS) is the commonest hormone condition in women of reproductive age, affecting up to 10% of the premenopausal population. In addition to its well-recognised effects on weight gain, excessive hair growth and infertility, it is becoming increasingly apparent that PCOS is associated with long-term health risks including diabetes and arterial (blood vessel) disease, both of which are related to impaired action of insulin throughout the body, also known as insulin resistance. Metformin, a drug which acts by improving the action of insulin in the body, is helpful in regularising the menstrual cycle in such patients and restoring fertility.

There have not been many studies on the effects of metformin on the function of blood vessels in PCOS, but this may be an important area to study as research in the middle-aged and elderly without PCOS, has shown that insulin resistance is associated with an increased risk of blood vessel dysfunction, and thus heart disease. Other studies have shown that treating insulin resistance with metformin improves the function of their blood vessels, and therefore reduces this risk.

It is unclear at present whether metformin is able to reduce the risk of diabetes and blood vessel disease in PCOS. We aim to address this question by studying the effects of short-term metformin therapy on blood vessel function and insulin sensitivity in a group of women with PCOS.

This study will involve four visits to the Endocrinology department at the Heath hospital and will last for a total of 32 weeks.

4. Why have I been chosen?

You have been chosen for this study as your endocrinologist has identified you as having PCOS, and you have not been on any treatment for this in the recent past. A total of 36 patients will be studied.

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

6. What will happen to me if I take part?

The study will last for a total of 32 weeks and you will be asked to attend the Clinical Investigation Unit at the Henry Wellcome Biomedical Sciences building on 4 separate occasions. Each of these visits will last approximately 3 hours.

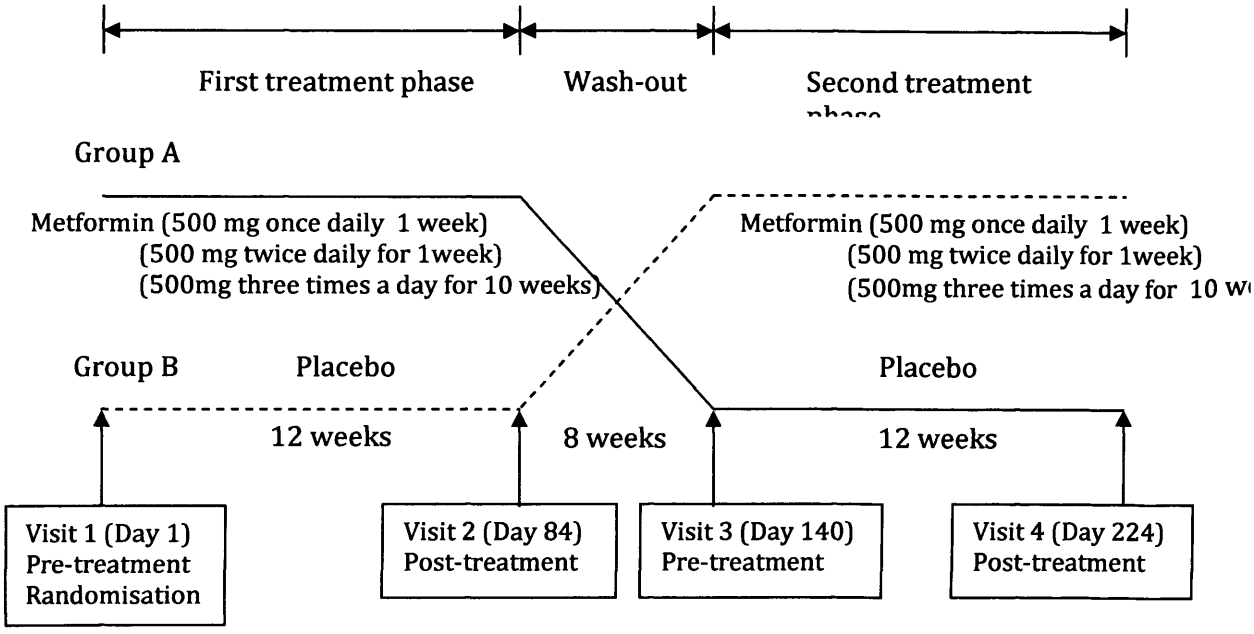
In order to know whether metformin changes blood vessel function we need to make comparisons. Metformin will be compared in this study with a placebo tablet. A placebo is a dummy treatment such as a pill which looks like the real thing but is not. It contains no active ingredient.

People will be put into two groups and then compared. The groups are selected by a computer which has no information about the individual i.e. by chance (or randomised). Half the patients will be put into group A and will receive metformin treatment for 12 weeks. The other half will be put into group B and will receive placebo treatment. These groups will be compared.

Neither you nor your doctor will know in which treatment group you are (although your doctor is able to find out if he/she needs to do so). This is to make sure that the results cannot be influenced in any way.

At the end of 12 weeks of treatment you will have a break of 8 weeks before taking the other treatment for a further 12 weeks (placebo treatment if you received metformin for the first part of the study and metformin if you received placebo initially). The break of 8 weeks between treatments will ensure that the first drug is cleared from your body before you start the new treatment.

A summary of what will happen and when the study visits will take place is shown in the following flow chart.



On each visit the measurements performed are designed to measure the stiffness of your blood vessels. The techniques that we use are completely painless and 'non-invasive'. The main equipment we use is called a 'Sphygmocor' machine. This consists of a small pencil-like probe placed over the wrist, which measures the waveform of the pulse. During the measurements you will also be asked to take a puff of a salbutamol inhaler (like that used in asthma) and a GTN tablet under your tongue (like that used in angina). Both these drugs act to change the shape of the pulse waveform that we measure. Both drugs are safe and are unlikely to produce any side effects although you may experience a headache and flushing after the GTN.

During each visit we will also take blood samples (approximately three tablespoons) to check your hormones, cholesterol, insulin, clotting factors and CRP (a non-specific marker of inflammation in the body). As well as this we take measurements such as your height, weight, and blood pressure.

We will also require you to have a pregnancy test at each study visit before carrying out the above measurements.

If you wish then we will be able to reimburse any travelling expenses / car parking fees incurred while attending for a study visit.

7. What do I have to do?

It is important that you take your regular medication in the normal way without altering the dose or timing of these during the course of the study. You should inform us of any dose adjustments. You will be asked to take the study medication regularly for the duration of the

study. The tablets will be taken once a day for the first week, twice a day for the second week and then three times a day for the next ten weeks during each cycle of treatment.

There are no lifestyle or dietary restrictions and you can continue your daily activities normally. We request that you report any illnesses to us as they may influence the timing of the test visits.

You should inform us immediately if you become pregnant.

For each of the study visits, we ask that you attend the Clinical Investigation Unit at 8 o'clock in the morning having fasted from midnight the previous night. You can drink water freely.

8. What is the drug that is being tested?

Metformin is a drug that is usually used to treat diabetes, but many patients with PCOS are now taking this treatment on a regular basis. It acts by improving the action of insulin in the body and in PCOS, is helpful in restoring menstruation and fertility. The drug comes in tablet form and we will provide you with a card to carry explaining the details of this trial. You should carry this with you at all times.

9. What are the side effects of Metformin?

Metformin is a widely used drug and most patients tolerate it well, though some will experience gastrointestinal side effects such as nausea, vomiting, diarrhoea and abdominal pain. This is usually transient and we aim to minimise this by building up the dose gradually over the first three weeks of treatment. The side effects can also be reduced by taking the medication with, or immediately after food. You will be told if any new side effect is found as a result of this study or any other studies using metformin.

If you become in any way concerned you should contact Dr Neera Agarwal on telephone number 02920 748251.

10. What are the possible disadvantages and risks of taking part?

Metformin can rarely cause a low level of acidity in the blood, but this tends only to be in people whose liver, kidneys, or heart do not work properly. Other than possible discomfort (temporary pain, swelling, bruising and rarely infection) caused by the collection of blood, no other side effects are anticipated from the study procedures.

The risks to an unborn human foetus or a breastfed child from metformin are not fully known. Women who are pregnant or breastfeeding a child may therefore not participate in this trial, neither should women who plan to become pregnant during the study. All women will be asked to have a pregnancy test at each study visit. Women who could become pregnant must use an effective contraceptive during the course of this study. We would request that barrier methods are used, as the oral contraceptive pill and implantable contraceptives may influence some of the measurements we are taking. Any woman who finds that she has become pregnant while taking part in the study should immediately tell her research doctor.

11. What are the potential benefits of taking part?

Some patients experience restored menstruation and fertility, and we hope to demonstrate improved blood vessel function. However, this cannot be guaranteed. The information we get from this study may help us to treat future patients with PCOS better.

12. What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the drug that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

13. What happens when the research study stops?

If you feel you would wish to continue metformin therapy at the end of the study you will be able to discuss this with your Endocrinologist.

14. What if something goes wrong?

This study is being indemnified by Cardiff University. If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

15. Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. With your permission your GP will be informed of your participation in this study. With your permission we may also look at sections of your medical notes which are relevant to the research study.

16. What will happen to the results of the research study?

The results of the research study will be prepared for publication in appropriate medical journals together with presentation at medical conferences. Patients participating in the study will be able to obtain a copy of the results after they have been published in the relevant journal(s). Patients will not be identified in any report/publication. Patients will be able to discover which arm of the study they were in if they so wish, but only after the study has been completed in its entirety.

17. Who is organising and funding the research?

The study is being organised by Dr Aled Rees in the Section of Endocrinology (the Principal Investigator) and Dr Neera Agarwal (Research Registrar). Funding for the study is provided from the Royal College of Physicians UK, and the Section of Endocrinology in the Heath hospital. The doctors conducting the research are not being paid for including and looking after patients in the study.

18. Who has reviewed the study?

The study has been reviewed by the Cardiff and Vale NHS Trust Research and Development Office and by the South Wales Research Ethics Committee.

19. Contact for further information

Should you have any further queries regarding this research study, then please do not hesitate to contact me on 02920 748251 or 07816 888636, or Dr Aled Rees on 02920 742309. You can also contact me via e-mail on neeraagarwal@yahoo.co.uk

Thank you for considering taking part in this study.

Dr Neera Agarwal
Clinical Research Fellow in Endocrinology

You will be given a copy of this Patient Information Sheet and a signed consent form to keep.

Appendix 4

EFFECTS OF METFORMIN ON VASCULAR FUNCTION IN POLYCYSTIC OVARIAN SYNDROME - CONSENT FORM (Version 3 May 2006)

Patient Identification Number for this trial:

Title of Study: A randomised, double-blind, placebo-controlled crossover trial of the effects of metformin therapy on vascular and metabolic risk in young women with polycystic ovary syndrome.

Name of Researchers: Dr Aled Rees, Dr Neera Agarwal

Please initial box

1. I confirm that I have read and understood the information sheet dated May 2006 (version 3) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I consent to my GP being informed of my participation in the Study. ☐
5. I consent to a pregnancy test at each study visit. ☐
6. I agree to take part in the above study. ☐

Name of patient	Date	Signature
Researcher	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature

1 copy for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 5

EFFECTS OF METFORMIN ON VASCULAR FUNCTION IN POLYCYSTIC OVARY SYNDROME – CASE REPORT FORM

PATIENT DETAILS

Initials .....

Date of Birth .....

Trial Enrolment Number .....

Date of Visit .....

INCLUSION CRITERIA

☐ Diagnosis of PCOS (require 2 out of 3)

- Androgen excess (clinical or biochemical)
- Ovulatory dysfunction
- Cysts on ovarian ultrasound

☐ Age 18-30

EXCLUSION CRITERIA

☐ Pregnancy

☐ Breastfeeding

☐ Current/previous use (within last 6 months) of:

- Oral contraceptive pill
- Anti-diabetic drugs
- Anti- hypertensive drugs

☐ Renal impairment

☐ Hepatic impairment

☐ Diabetes

☐ Hypertension

☐ Discuss information sheet & complete consent form.

☐ Discuss contraception.

PREGNANCY TEST ☐ Negative ☐ Positive

MEDICAL HISTORY

.....

.....

..... Birth weight.....

SMOKING STATUS

Current smoker ☐ No ☐ Yes Details .....

Ex-smoker ☐ No ☐ Yes Details .....

FAMILY HISTORY

.....

.....

DRUG THERAPY Previous OCP use .....

Drug	.....	.....	.....	.....	.....
Dose	.....	.....	.....	.....	.....
Duration	.....	.....	.....	.....	.....

Allergies .....

MENSTRUAL HISTORY

Age at menarche .....

☐ Eumenorrhoeic Cycle length..... Day No.....

☐Oligomenorrhoeic LMP.....

☐ Amenorrhoeic LMP.....

CLINICAL EXAMINATION

BMI Height (cm)..... Weight(kg)..... BMI .....

Body Composition Fat mass(kg) ..... Fat % .....

Waist:Hip Waist(cm) ..... Hip(cm)..... Ratio .....

Acanthosis Nigricans ☐ Yes ☐ No Site .....

Ferriman-Gallwey score .....

ADDITIONAL INFORMATION

.....

.....

.....

.....

AUGMENTATION INDEX & PULSE WAVE VELOCITY

Distance:

SSN to carotid pulse.....mm

SSN to radial pulse.....mm

SSN to femoral pulse.....mm

Blood Pressure:

A. .....mmHg

B. .....mmHg

Average .....mmHg

AUGMENTATION INDEX

	A	B	Average
Augemtation index (%)			
Central aortic pressure (mmHg)			
Tr (ms)			

PULSE WAVE VELOCITY

	A	B	Average
Carotid-radial (m/s)			
Carotid-femoral (m/s)			

ASSESSMENT OF ENDOTHELIAL FUNCTION – BASELINE MEASUREMENTS

Time		A	B	Average
5 mins	BP			
	HR			
7 mins	AIx			
15 mins	BP			
	HR			
17 mins	AIx			

ENDOTHELIAL FUNCTION – GTN

Time		A	B	Average
0 min	**Sublingual GTN**			
1 min	BP			
	HR			
3 min	**Remove GTN**			
	AIx			
5 mins	AIx			
7 mins	BP			
	HR			
10 mins	AIx			
12 mins	BP			
	HR			
15 mins	AIx			
17 mins	BP			
	HR			
20 mins	AIx			
25 mins	BP			
	HR			
28 mins	AIx			

ENDOTHELIAL FUNCTION - SALBUTAMOL

Time		A	B	Average
0 min	**2 puffs salbutamol via spacer (x2)**			
2 mins	BP			
	HR			
5 mins	AIx			
7 mins	BP			
	HR			
10 mins	AIx			
12 mins	BP			
	HR			
15 mins	AIx			
17 mins	BP			
	HR			
20 mins	AIx			

LABORATORY RESULTS

Investigation	Result
Testosterone	
SHBG	
HsCRP	
PAI-1	
Total cholesterol	
LDL	
HDL	
TG	
Glucose	
Insulin	
Adiponectin	

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