An Investigation into the Metabolic and Cardiovascular effects of Phytocannabinoids using *in vivo* and *in vitro* techniques

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Abstract

Metabolic syndrome, first described as Syndrome X, includes a cluster of metabolic abnormalities including visceral obesity, insulin resistance, dyslipidaemia and hypertension. The pathophysiology of metabolic syndrome is complex, however, visceral adiposity and insulin resistance are thought to play a central role and both are implicated in the development of type 2 diabetes. Both metabolic syndrome and type 2 diabetes increase the risk of cardiovascular disease, by initiating and accelerating the progression of atherosclerosis, the hallmark of cardiovascular disease. Chronic low-grade inflammation of adipose tissue and vascular endothelium is well documented in type 2 diabetes and associated cardiovascular disease. In addition, stress has also been identified as one of the factors that increases the risk of cardiovascular disease.

The endocannabinoid system is a physiological system that includes cannabinoid (CB_1/CB_2) receptors, their endogenous ligands and the enzymes responsible for their biosynthesis and degradation. Activation of the endocannabinoid system in the central nervous system leads to increased appetite and food intake while in the periphery it causes lipogenesis. Chronic over activation of the endocannabinoid system has been seen in both obesity and type 2 diabetes. Attempts to modulate the endocannabinoid system, by using CB_1 antagonist/inverse agonist, rimonabant, led to positive metabolic and cardiovascular effects, but were associated with adverse psychiatric events. Cannabidiol (CBD) and delta 9 tetrahydrocannabivarin (Δ^9 THCV or THCV), are two phytocannabinoids, obtained from *Cannabis Sativa L.* Both show a distinct pharmacological profile, separate from rimonabant and delta 9 tetrahydrocannabinol (Δ^9 THC or THC), the parent compound obtained from *Cannabis Sativa L*. Both have potent anti-inflammatory and anti-oxidant properties and show multiple desirable cardiovascular and metabolic effects in preclinical studies. The aim of this project was to investigate the metabolic and cardiovascular effects

of these two phytocannabinoids, by employing *in vivo* and *in vitro* techniques. Chapter 2 describes in detail the clinical study in type 2 diabetic subjects, where we used CBD and THCV, alone and in combination and studied their effects on various metabolic, cardiovascular and inflammatory parameters. Chapter 3 presents acute study of the cardiovascular effects of CBD in young healthy volunteers. Chapters 4 and 5 deal with the effects of CBD and THCV on mature human adipocytes and human aortic endothelial cells respectively. Last chapter includes work done on plasma samples and homogenised femoral arteries from Zucker Diabetic Fatty rats and their lean counterparts, treated with CBD. We found that THCV improves glycaemic parameters in type 2 diabetes, while CBD affects resting cardiovascular parameters and cardiovascular response to stress. CBD affects the release of leptin and IL-6 from adipocytes, while both CBD and THCV affect the release of endothelin-1 and vascular cell adhesion molecule-1 from aortic endothelial cells. CBD also shows positive impact on cytokines in diabetic rats.

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Glossary of terms

% CV	%age coefficient of variation
2-AG	2 arachidonoyl glycerol
2-OG	2-Oleoyl glycerol
$5HT_{1A}$	5 hydroxytryptamine 1A
ACS-ACOD	acyl-Coenzyme A synthetase/acyl-Coenzyme A oxidase
ADA	American diabetes association
AE	Adverse event
AEA	Anandamide
AIDS	Acquired immunodeficiency syndrome
AMP	Adinosine monophosphate
AMPK	AMP-activated protein kinase
ANCOVA	Analysis of covariance
ANOVA	Analysis of vairance
Apo A	Apolipoprotein A
Apo B	Apolipoprotein B
ATP	Adinosine tripho
BAS	Bile acid sequestrants
BCA	Bicinchoninic acid
BDI II	Beck's depression inventory
BMI	Body mass index
BNP	Brain natriuretic peptide
BNST	Bed nucleus of stria terminalis
BSA	Bovine serum albumin
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CART	Cocaine-and amphetamine-regulated transcript
CB	Cannabinoid receptor
CBD	Cannabidiol

CBG	Cannabigerol
cDNA	Complementary DNA
CE	Cholesteryl ester
CETP	Cholesteryl ester transport protein
CGIC	Clinician's global impression of change
CHO	Chinese hamster ovary
CIMT	Carotid intima media thickenss
CO	Cardiac output
COX	Cyclooxygenase
CREB	cAMP response element binding protein
CRF	Clinical record form
CVD	Cardiovascular disease
CVS	Cardiovascular system
DAGL	Diacylglycerol lipase
DIO	Diet induced obese
DKA	Diabetic ketoacidosis
DPP-4	Dipeptidyl peptidase 4
ECG	Electrocardiogram
ECS	Endocannabinoid system
EDTA	Ethylenediaminetetraacetic acid
EJT	Ejection time
ELAM	Endothelial leucocyte adhesion molecule
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-related kinase
ET-1	Endothelin 1
ETD	Estimated treatment difference
EU	European Union
FAAH	Fatty acid amide hydrolase

FDA	Food & Drug Administration
FFA	Free fatty acids
FSBF	Forearm skin blood flow
GABA	Gamma amino butyric acid
G-CSF	Granulocyte colony stimulating factor
GDM	Gestational diabetes mellitus
GIP	Glucose-dependent insulinotropic peptide
GLP	Glucagon-like peptide
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G protein coupled receptor
GTPγS	[^{35}S] guanosine 5'-O-[gamma-thio]triphosphate
HAoEC	Human aortic endothelial cell
HbA1c	Glycosylated haemoglobin
HDL	High density lipoprotein
HFD	High fat diet
HMG Co-A	Hydroxymethylglutaryl Coenzyme A
HOMA	Homeostatic model assessment
HR	Heart rate
hs-CRP	High sensitivity C reactive protein
HWP	Human white preadipocytes
ICAM	Intercellular adhesion molecule
IFG	Impaired fasting glycaemia
IFN	Interferon
IGT	Impaired glucose tolerance
IHD	Ischemic heart disease
IL	Interleukin
IMP	Investigational medicinal product
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal

IR	Insulin receptor
IRS	Insulin receptor substrate
ISF	Investigator site file
ITT	Intention to treat
JNK	Jun amino-terminal kinases
LDL	Low density lipoprotein
LPI	Lysophosphatidylinositol
LPS	Lipopolysaccharide
LVEF	Left ventricular ejection fraction
MAGL	Mono acyl glycerol lipase
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinase
MCP1	Monocyte chemoattractant protein
MFI	Median fluorescence intensity
MIP	Macrophage inflammatory protein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRS	Magnetic resonance spectroscopy
MS	Multiple sclerosis
MSNA	Muscle sympathetic nerve activity
MVC	Maximum voluntary contraction
NAcS	Nucleus accumbens shell
NADA	N-arachidonoyl dopamine
NAGly	N-arachidonylglycine
NEFA	Non-esterified fatty acids
NHANES	National Health and Nutrition Examination Survey
NICE	National Institute for Health and Care Excellence
NK	Natural killer
nNOS	Neuronal nitric oxide synthase

NO	Nitric oxide
NPC1L1	Niemann-Pick C1 Like 1
NRS	Numerical rating scale
OEA	Oleoylethanolamide
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PCSK9	Proprotein convertase subtilisin-like kexin type 9
PEA	Palmitoylethanolamide
PGH_2	Prostaglandin H2
PI3-K	Phosphoinositide 3-kinse
PKB	Protein kinase B
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PPY	Pancreatic polypeptide Y
QC	Quality control
RAAS	Renin angiotensin aldosterone system
RBP4	Retinol binding protein 4
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
RVLM	Rostral Ventrolateral Medulla
SAE	Serious adverse event
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of mean
SGIC	Subject's global impression of change
SGLT-2	Sodium glucose co-transporter 2
SOC	System organ class

SST	Serum separator tube
STAT	Signal transducers and activators of transcription
SU	Sulfonylurea
SV	Stroke volume
TG	Triglyceride
TGF	Transforming growth factor
THC	Tetrahydrocannabinol
THCV	Tetrahydrocannabivarin
TIMP-1	Tissue inhibitor of metalloproteinase
TNFα	Tumour necrosis factor
t-PA	Tissue plasminogen activator
tPAI	Tissue plasminogen activator inhibitor
TPR	Total peripheral resistance
TXA_2	Thromboxane A2
TZD	Thiazolidinedione
UKPDS	UK prospective diabetes study
UL	Ultracentrifugation
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
vWF	Von Willebrand factor
ZDF	Zucker diabetic fatty

1. Introduction

1.1. Metabolic Syndrome and Type 2 Diabetes

Metabolic syndrome, first described as syndrome X (Reaven, 1988), includes a cluster of metabolic abnormalities including visceral obesity, insulin resistance, dyslipidaemia and hypertension. According to the new International Diabetes Federation definition of metabolic syndrome, it must have central obesity (defined as waist circumference with ethnicity specific values) as its central feature with two of the following four factors; raised triglycerides (TG), reduced high density lipoprotein cholesterol (HDL), raised blood pressure and raised fasting plasma glucose. Metabolic syndrome increases the risk of type 2 diabetes and cardiovascular disease (CVD) (Alberti *et al.*, 2005). In addition to CVD and type 2 diabetes, individuals with metabolic syndrome are also susceptible to polycystic ovary syndrome, fatty liver, cholesterol gall stones, asthma, sleep disturbance and some forms of cancer (Grundy *et al.*, 2004a). Due to worldwide increased prevalence of the risk factors like obesity, visceral adiposity, physical inactivity and unhealthy diet, both metabolic syndrome and type 2 diabetes are on the rise (Schwarz *et al.*, 2007). It is estimated that by the year 2030, 552 million people will be suffering from type 2 diabetes worldwide, compared to 366 million people in 2011 (Whiting *et al.*, 2011).

1.2. Pathophysiology

1.2.1. Insulin resistance

The pathophysiology of metabolic syndrome is complex, however insulin resistance (Grundy *et al.*, 2004b) and obesity (Reaven, 1995) are thought to play a central role. There is genetic predisposition to insulin resistance (Ahlqvist *et al.*, 2011) but insulin resistance and visceral obesity are closely linked to each other and generally insulin resistance rises with rising body mass index (BMI) (Bogardus *et al.*, 1985). While abdominal obesity exacerbates insulin resistance in muscle and liver through the release of excess amounts of

nonesterified fatty acids from adipose tissue (Grundy *et al.*, 2004b), insulin resistance in muscle, adipose tissue and liver leads to most of the abnormalities seen in metabolic syndrome. It produces hyperglycaemia due to enhanced glucose output form liver and reduced uptake by the muscles. It also leads to increased lipolysis in adipose tissue and increase output of very low density lipoprotein cholesterol (VLDL) from liver (Meshkani and Adeli, 2009).

At a cellular level, defects in insulin signalling cascade lead to insulin resistance. The signalling cascade is quite complex involving multiple genes and proteins isoforms (reviewed by Taniguchi *et al.*, 2006). Briefly interaction of insulin with insulin receptor (IR) leads to phosporylation of insulin receptor substrate (IRS) proteins leading to activation of two main signalling pathways: the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway, responsible for the metabolic actions of insulin and the Ras-mitogen-activated protein kinase (MAPK) pathway, regulating gene expression and interacting with PI3K pathway to control cell growth and differentiation. Increased ligand stimulated internalization and degradation lead to a down-regulation of IR at the protein level, causing insulin resistance as is seen in obesity and type 2 diabetes (Taniguchi *et al.*, 2006). Other mechanisms include altered expression, diminished ligand binding and reduced tyrosine kinase activity of IR, as seen in various forms of severe insulin resistance and impaired coupling of insulin receptor substrates with insulin receptor leading to impaired downstream signalling (Saltiel, 2001).

Proinflammatory cytokines like tumour necrosis factor (TNF) α and interleukin (IL)-6, released from visceral adipose tissue, are known to impair the insulin signalling cascade (Ravussin and Smith, 2002; Rajala and Scherer, 2003). Suppressor of cytokine signalling (SOCS) 3 is a protein that impairs insulin signalling cascade, leading to insulin resistance, and its expression is upregulated by TNFα in white adipose tissue of obese mice

(Emanuelli *et al.*, 2001). SOCS-3 is also induced in human primary myotubes by IL-6 and is related to strong inhibition of insulin signalling. Elevated levels of IL-6 in the plasma of obese, type 2 diabetic subjects are associated with increased expression of SOCS-3 mRNA in the skeletal muscle (Rieusset *et al.*, 2004). SOCS-3 is also mediates IL-6 dependant insulin resistance in hepatocytes (Senn *et al.*, 2003). IL-1β is another proinflammatory cytokine implicated in the pathogenesis of insulin resistance and beta cell dysfunction in type 2 diabetes. It reduces insulin mediated glucose transport into adipocytes by inhibiting IRS-1 expression (Jager *et al.*, 2007). The process of IL-1β secretion requires its activation by caspase-1, through a multiprotein complex, called the "inflammasome", which is composed of NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3), ASC (apoptosis associated speck-like protein containing a CARD) and procaspase-1. Activation of the NLRP3 inflammasome by pathogens leads to the release of IL-1β and IL-18. In type 2 diabetes, NLRP3 inflammasome senses inflammatory changes related to glucotoxicity, islet amyloid polypeptide, lipid intermediates and fatty acids (Grant and Dixit, 2013). There is activation of inflammasome and caspase-1 in adipose tissue, during differentiation and lipid accumulation, leading to increased level of IL-1β and insulin resistance (Stienstra *et al.*, 2010). Various factors have been implicated as a trigger for obesity related adipose tissue inflammation including hypoxia, overactivated (RAAS) in adipose tissue and the type of dietary fat consumed (Kalupahana *et al.*, 2012).

1.2.2. β cell dysfunction

In addition to insulin resistance, individuals with type 2 diabetes have underlying β cell dysfunction, due to a combination of impaired secretory function and reduced cell mass (Rhodes, 2005). Both insulin resistance and β cell dysfunction are present very early in the natural history of type 2 diabetes (Kahn, 2003). Although controversy exists as to the

relative contribution of the two, yet it is estimated that subjects with type 2 diabetes have already lost 50% of their β cell function at the time of diagnosis (UK Prospective Diabetes Study Group, 1995). First degree relatives of subjects with type 2 diabetes, who have normal glucose tolerance, show evidence of impairment of β cell function but not of insulin sensitivity (Pimenta *et al.*, 1995). A recent study reported β cell dysfunction to be the major defect in subjects with low BMI who developed tye 2 diabetes (Kim *et al.*, 2013a). Various factors implicated in the pathogenesis of β cell dysfunction include glucotoxicity, lipotoxicity, insulin resistance within β cells, inflammation and oxidative stress (Bonora, 2008). Studies show improvement in β cell function following Roux-en-Y gastric bypass surgery that is not proportional to the degree of weight loss. On the other hand, improvement in insulin sensitivity occurs in relation to weight loss but is still lower in type 2 diabetic subjects than morbidly obese subjects with normal glucose tolerance (Nannipieri *et al.*, 2011).

1.2.3. The incretin effect

Insulin release in response to oral glucose is higher than when the same amount of glucose is given intravenously (Elrick *et al.*, 1964). This is called the 'incretin effect' and is mediated by two gut hormones, glucose-dependent insulinotropic peptide (GIP) (Brown *et al.*, 1975) and glucagon like peptide (GLP)-1 (Lauritsen *et al.*, 1980). GIP is secreted by K cells, located mainly in the duodenum and upper jejunum (Meier *et al.*, 2002). The precursor for GLP-1 is proglucagon. Its gene is expressed in the pancreatic α cells, L cells of small intestine and some neurons (Eissele *et al.*, 1992; Fehmann *et al.*, 1995). Proglucagon is processed to produce glucagon in the α cells, while in the L cells of the gut it produces GLP-1 and GLP-2 (Drucker, 1998). Native GLP-1 has a very short half-life as it is rapidly inactivated by the enzyme dipeptidyl peptidase (DPP) -4 and is also subject to renal clearance (Kieffer and Habener, 1999). Both GIP and GLP-1 promote β cell

proliferation, but GLP-1 has additional beneficial effects of delaying gastric emptying, increasing satiety and inhibiting glucagon release (Drucker, 2006; Ussher and Drucker, 2012). GLP-1 augments glucose dependent insulin release from pancreatic β cells (Kreymann *et al.*, 1987). Binding of GLP-1 to its receptors on pancreatic β cells leads to insulin release through a mechanism that involves membrane depolarisation, via inhibition of K_{ATP} channels, and intracellular influx of Ca^{+} (Baggio and Drucker, 2007). GLP-1 inhibits glucagon release in a glucose dependent manner (Nauck *et al.*, 2002). The underlying mechanism is not entirely clear. It may be due to direct interaction of GLP-1 with its receptor on pancreatic α cells, with some contribution from pancreatic β and/or δ cells, but is also seen in type 1 diabetics, indicating a β cell independent mechanism (Baggio and Drucker, 2007). GLP-1 slows gastric emptying via vagally mediated pathways (Imeryuz *et al.*, 1997). In addition to the above, GLP-1 induces satiety, presumably through its central effects (Holst, 2007). It has β cell preserving action and improves myocardial and endothelial function (Ahren, 2007). Dysfunctional incretin axis, manifest by deficiency of GLP-1 and resistance to the actions of GIP, is seen in type 2 diabetes (Defronzo, 2009).

1.2.4. Sodium-glucose co-transporter (SGLT)-2

It has been known for more than fifty years that the maximum capacity of renal tubules to reabsorb glucose is higher in diabetes (Farber *et al.*, 1951). Up to 90% of filtered glucose is reabsorbed in proximal renal tubules via sodium-glucose co-transporter (SGLT)-2 (Vallon *et al.*, 2011). Reabsorption of filtered glucose in the kidneys is higher in diabetics than non-diabetic individuals.

In addition to dysfunctional incretin axis and increased reabsorption of glucose from kidneys, hyperglucagonaemia from pancreatic α cells and probable insulin resistance in

brain areas responsible for appetite suppression in response to hyperinsulinaemia, are thought to play their role in the development of type 2 diabetes (Defronzo, 2009).

1.3. Currently Available treatments for type 2 diabetes

Below is a summary of oral and parenteral treatments commonly used for type 2 diabetes.

Oral treatments	Metformin (inhibits hepatic glucose output)
	Sulfonylureas (stimulate insulin release from β cells)
	TZD's (enhance insulin sensitivity)
	DPP-4 inhibitors (inhibit endogenous GLP-1 breakdown)
	SGLT-2 inhibitors (promote renal glucose loss)
Parenteral treatments	Incretin mimetics (act in place of endogenous GLP-1)
	Insulin

1.3.1. Metformin

Metformin (1,1 – dimethylbiguanide) has been in clinical use, in the UK, since 1958. Recent American and European guidelines endorse its use as first line therapy in type 2 diabetes, along with life style modification (Nathan *et al.*, 2009; Adler *et al.*, 2009). The landmark UK Prospective Diabetes Study (UKPDS) showed that intensive glycaemic control, in obese type 2 diabetic subjects, using metformin, was associated with reduced risk of complications and mortality. Metformin caused less weight gain and hypoglycaemia compared to sulfonylureas and insulin (UK Prospective Diabetes Study Group, 1998b).

There has been a lot of research into the exact site and mechanism of action of metformin though it is not entirely understood. It is said to indirectly activate AMP- activated protein kinase (AMPK) through inhibition of complex 1 of mitochondrial respiratory chain. This promotes anaerobic glycolysis and increases adenosine monpphosphate (AMP): adenosine triphosphate (ATP) ratio thereby activating AMPK. The primary effect of metformin is inhibition of hepatic gluconeogenesis, but it also promotes glucose uptake into skeletal muscles and adipose tissue (Coughlan *et al.*, 2014). Metformin also improves fatty liver disease, most likely due to fatty acid oxidation and inhibition of lipogenesis, presumably due to AMPK activation. Some argue that AMPK activation may be responsible for improved insulin sensitivity and reduced hepatic gluconeogenesis.

Recent animal data suggests that metformin ameliorates hyperglycaemia associated tubular injury by decreasing renal oxygen consumption (Takiyama *et al.*, 2011). Since chronic hypoxia is one of the key events in the initiation and progression of diabetic nephropathy, metformin may be useful in this role. Also metformin reduces cardiac ischemia/reperfusion injury, and on its own or in combination with sulfonylurea, reduces mortality and morbidity in type 2 diabetic patients with heart failure, compared to sulfonylurea therapy alone (Viollet *et al.*, 2012). A meta-analysis of 40 clinical trials of oral hypoglycaemics, reporting on macrovascular outcomes, concluded that metformin use was associated with reduced risk of cardiovascular mortality, while none of the other treatments had any significant effect on cardiovascular outcomes (Selvin *et al.*, 2008). The main side effect of metformin is gastrointestinal upset while the common reason to stop metformin therapy is a decline in renal function. Metformin remains the first line treatment for type 2 diabetes in all international guidelines. Current guidelines from ADA also recommend considering the use of metformin for prevention of type 2 diabetes in subjects with impaired glucose tolerance (IGT), impaired fasting glycaemia (IFG) or an HbA1c 5.7

- 6.4% (39- 46mmol/mol). It specifically targets those with BMI >35 kg/m^2 , age <60 years and women with previous history of gestational diabetes mellitus (GDM) (American Diabetes Association, 2015).

1.3.2. Sulfonylureas

Traditionally sulfonylurea (SU) has been the first line, add-on therapy, in type 2 diabetes. It stimulates insulin secretion, by inhibiting ATP-sensitive potassium channel, in the β cell membrane. This causes membrane depolarization and opening of voltage-gated Ca^{2+} channels leading to Ca^{2+} influx and exocytosis of insulin secretory granules (Ashcroft and Rorsman, 1989). The main side effect of SU use is hypoglycaemia, which is a particular problem in the elderly and in those with renal insufficiency (Singh, 2014). There are conflicting results with regards to SU and cardiovascular mortality. In the UKPDS, while early addition of metformin to maximal sulfonylurea therapy resulted in improved glycaemic control, it increased diabetes related mortality compared to sulfonylurea monotherapy (UK Prospective Diabetes Study Group, 1998a; UK Prospective Diabetes Study Group, 1998c). One observational study comparing metformin and sulfonylurea in type 2 diabetes suggested that metformin alone or in combination with sulfonylurea was associated with reduced all-cause and cardiovascular mortality compared with sulfonylurea alone (Johnson *et al.*, 2002). Another observational study comparing metformin and sulfonylurea in newly diagnosed type 2 diabetics concluded that patients treated with sulfonylurea alone or a combination of sulfonylurea and metformin were at higher risk of adverse cardiovascular outcomes than those on metformin alone (Evans *et al.*, 2006). A recent meta-analysis concluded that SU may increase the risk of CV disease in patients with diabetes (Phung *et al.*, 2013). A retrospective study in pregnant women treated with glyburide or insulin within 150 days before delivery showed that infants exposed to SU had increased risk of intensive care unit admission, respiratory distress, hypoglycaemia,

birth injury and being large for gestational age (Camelo Castillo *et al.*, 2015). Another retrospective study showed that treatment with metformin/SU was associated with a higher use of diabetes-related secondary health care resources compared with metformin plus other drugs. There was a 38% increase in admissions and a 10% increase in outpatient visits. Higher admission rate was due to a 77% higher rate of macrovascular complications in patients treated with an SU (Strongman *et al.*, 2015). Due to these observations, some argue that SU should no longer be first line add-on therapy in type 2 diabetes (Genuth, 2015).

1.3.3. Thiazolidinediones (TZDs)

Also known as 'glitazones', these are peroxisome proliferator-activated receptor gamma (PPARγ) agonists. These receptors belong to the superfamily of nuclear hormone receptors, found on adipocytes, but also exist in liver, intestine, vascular smooth muscle, endothelium and spleen. They play an important role in lipid metabolism and glucose homeostasis. Their activation leads to adipogenesis and enhanced insulin sensitivity in liver and muscle. In addition, they have important anti-inflammatory and vascular protective effects (Grygiel-Gorniak, 2014). TZDs also exert some of their anti-diabetic effects through indirect stimulation of AMPK, by inhibiting complex 1 of mitochondrial respiratory chain, and through adiponectin release via PPARγ stimulation (Coughlan *et al.*, 2014). Rosiglitazone was taken off the market because of cardiovascular safety concerns (Nissen and Wolski, 2007), although some believe that there was no evidence that rosiglitazone use was associated with increased cardiovascular risk (Stone *et al.*, 2015). Pioglitazone is the compound currently used, that improves glycaemic control on its own and in combination with other available therapies. It improves β cell function (Gastaldelli *et al.*, 2007), optimizes lipid profile (Charbonnel *et al.*, 2005; Berhanu *et al.*, 2007) and improves carotid intima media thickness (CIMT) (Nakamura *et al.*, 2004), a surrogate

marker of cardiovascular disease, in subjects with type 2 diabetes. It also improves markers of inflammation (Heliovaara *et al.*, 2007) and fatty liver (Yki-Jarvinen, 2009). The side effects associated with TZDs include fluid retention, weight gain and fractures and there have been concerns about the development of bladder cancer. In view of these, some have suggested the need for newer PPARγ modulating drugs (Cariou *et al.*, 2012).

1.3.4. Incretins Mimetics

Insulin release in response to oral glucose is higher than when the same amount of glucose is given intravenously (Elrick *et al.*, 1964). This augmentation of insulin response to oral glucose, the so called 'incretin effect', is mediated by two gut hormones, GIP and GLP-1 (Drucker, 2006; Ussher and Drucker, 2012). The first GLP-1 mimetic to enter clinical practice was exenetide (exendin-4), obtained from the venom of a lizard called 'gila monster' (Heloderma Suspectum), with 50% homology to endogenous GLP-1 (Eng *et al.*, 1992). When compared to insulin and pioglitazone in newly diagnosed type 2 diabetic subjects, all three treatments improved β cell function, but exenatide had the maximum effect. In addition, it improved weight, blood pressure and lipid profile (Xu *et al.*, 2015). Exenatide is normally administered twice daily via subcutaneous injection, however, recently longer acting preparations have been developed. A three year open label study, using once weekly exenatide or insulin glargine, in addition to ongoing oral therapy, in insulin naive patients, showed that improvement in glycaemic profile with exenatide was maintained (Diamant *et al.*, 2014). The main side effects encountered with exenatide were nausea, vomiting and diarrohea, which improved gradually after 26 weeks, but insulin was associated with three times higher hypoglycaemic episodes (Diamant *et al.*, 2014). Exenatide has aslo been trialled as continuous subcutaneous infusion via a miniature osmotic pump system, in metformin treated subjects, where it showed significant improvements in glycaemic control and body weight (Henry *et al.*, 2014). Liraglutide is

the second and longer acting GLP-1 mimetic, with similar effects on glycaemic control and weight to exenetide, but with once daily administration (Tasyurek *et al.*, 2014). Clinical trial data shows that it is superior to exenatide in glycaemic control and has aslo been shown to reduce systolic blood pressure. There is also some indication that it may reduce the risk of non-alcoholic fatty liver disease progression in subjects with poorly controlled type 2 diabetes (Gough, 2012). It improves CIMT, a marker of subclinical atherosclerosis, in patients with type 2 diabetes (Rizzo *et al.*, 2014). Newer, longer acting GLP-1 agonists are being compared to liraglutide in head to head non-inferiority phase 3 trials. Two such compounds are abliglutide and dulaglutide. While once weekly abliglutide was less effective in lowering HbA1c and was associated with more injection site reactions than once daily liraglutide (Pratley *et al.*, 2014), once weekly dulaglutide was non-inferior and had similar safety and tolerability profile to once daily liraglutide (Dungan *et al.*, 2014). The most common side effect associated with the use of GLP-1 agonists is nausea, which in most cases is transient. There have been concerns about the risk of pancreatitis with exenatide (Ahmad and Swann, 2008) and thyroid C-cell hyperplasia, in rodents, with use of liraglutide (Bjerre Knudsen *et al.*, 2010). Currently there is insufficient evidence to support or exclude a causal relationship. The recommendation is to stop therapy if pancreatitis is suspected. In case of C-cell hyperplasia, liraglutide is contraindicated in the USA for individuals with personal or family history of medullary thyroid cancer or personal history of multiple endocrine neoplasia syndrome type 2 (Gough, 2012).

1.3.5. Dipeptidyl-Peptidase 4 (DPP-4) Inhibitors

These act by inhibiting the enzyme dipeptidyl peptidase-4, responsible for the breakdown of endogenous GLP-1, thereby increasing its half-life. Monotherapy with sitagliptin, one of the first DPP-4 inhibitors, improves glycaemic control in fasting and post prandial states and measures of β cell function (Aschner *et al.*, 2006). It has also been trialled as

combination therapy with metformin and pioglitazone, where it improved glycaemic control and was well tolerated (Fonseca *et al.*, 2013). Vildagliptin, another DPP-4 inhibitor, improved insulin sensitivity and β cell function in subjects with impaired fasting glycaemia (Utzschneider *et al.*, 2008). Similar findings have been reported for saxagliptin, which improves glycaemic control on its own and in combination with metformin by modulating α and β cell function (Yang *et al.*, 2011; Sjostrand *et al.*, 2014). While it was suggested that DPP-4 inhibitors may improve heart failure by inhibiting the breakdown of B-type (brain) natriuretic

peptide 1–32 (BNP 1–32) (Angeli and Shannon, 2014), a meta-analysis of randomised controlled trials concluded that DPP-4 inhibitors may be associated with increased risk of heart failure (Monami *et al.*, 2014). Data from SAVOR-TIMI trial showed that although DPP-4 inhibition with saxagliptin did not increased rates of myocardial ischemia, it increased the rate of hospitalisation for heart failure (Scirica *et al.*, 2013). Another study, using alogliptin, in subjects with type 2 diabetes and recent coronary event, did not report increased rate of major cardiovascular events (White *et al.*, 2013). More recent data, from the double-blind Trial Evaluating Cardiovascular Outcomes with Sitagliptin (TECOS), shows that sitagliptin use is not associated with an increased risk of cardiovascular disease, heart failure or all cause mortality (Green *et al.*, 2015). Similar findings have been reported by a new case-control study, showing no increased risk of heart failure with DPP-4 inhibitors (Giorda *et al.*, 2015).

1.3.6. Sodium-Glucose Cotransporter 2 Inhibitors

Reabsorption of filtered glucose in kidneys occurs mainly via sodium glucose cotransporter (SGLT)-2. Phlorizin was first discovered to cause glycosuria and diuresis, though it was only effective when given intravenously (Jadoon and Idris, 2011). SGLT-2 inhibitors work via insulin independent mechanism and along with reducing blood

glucose, also have positive impact on weight and blood pressure (Cefalu and Riddle, 2015). The FDA recently issued a Drug Safety Communication warning of an increased risk of ketoacidosis with the use of SGLT-2 inhibitors in type 2 diabetes. They seem to induce DKA at a relatively normal blood glucose levels and the exact cause remains unclear (Rosenstock and Ferrannini, 2015). It is, however, important to emphasize that rates of euglycaemic DKA with SGLT2 inhibitors are extremely low with just a handful of cases identified globally. It is therefore not clear if this a rare side effect of the SGLT2 inhibitors or a coincidental finding. Furthermore, some of the DKA cases identified actually had type 1 diabetes and were being prescribed SGLT2 inhibitors off licence.

1.3.7. Insulin

Due to the progressive nature of type 2 diabetes, insulin replacement is frequently needed. Usually addition of basal insulin is required with the aim to suppress hepatic glucose output, but in later stages, addtion of meal time insulin may be needed (Inzucchi *et al.*, 2012). Current NICE guidelines recommend the addition of once or twice daily neutral protamine hagedorn (NPH) insulin to ongoing metformin and/or SU and consider once daily insulin analogue if there is risk of recurrent hypoglycaemia or administration of multiple injections is not possible. In cases where there is significant hyperglycaemia (HbA1c $\geq$ 9.0%), NICE recommends starting twice daily pre-mixed (biphasic) human insulin, with consideration being given to pre-mixed analogue insulin under specific circumstances (NICE guidelines [CG87], 2009). While insulin's glucose lowering efficacy is highest compared to non-insulin based therapies, the risk of hypoglycaemia is much higher and insulin therapy is associated with weight gain (Inzucchi *et al.*, 2012). Recent suggestion is therefore to add basal insulin analogues to one of the non-sulfonylurea therapies for effective glycaemic control but with less risk of hypoglycaemia and less weight gain (Donner and Munoz, 2012). Given the complex pathophysiology of type 2

diabetes and its metabolic and cardiovascular consequences, a pathophysiologic approach using initial combination therapy, rather than guideline approach, has been recommended for treatment and this has been incorporated into the updated ADA guidelines (DeFronzo *et al.*, 2013).

1.4. Glycaemic control and risk of complications

Tight glycaemic control reduces the incidence of microvascular complications associated with diabetes. The impact on macrovascular complications is not as clear and may take much longer to become evident (Donner and Munoz, 2012). Review of available data gives conflicitng results about the effect of intensive glycaemic control on cardiovascular outcomes and all cause mortality. A meta-analysis of five big randomised controlled trials suggested that intensive glycaemic control reduced coronary events without increasing risk of death (Ray *et al.*, 2009). A later larger meta-analysis, that included eleven studies, including the five that had been analysed in the earlier meta-analysis, concluded that there were limited benefits of intensive glycaemic control on all cause mortality and deaths from cardiovascular causes (Boussageon *et al.*, 2011). A recent large, multicentre trial showed that insulin glargine when compared to standard care in subjects with IFG, IGT or type 2 diabetes, had a neutral effect on cardiovascular outcomes and cancers (Gerstein *et al.*, 2012).

1.5. Diabetic Dyslipidaemia

Hepatic insulin resistance plays a key role in the dysregulation of fatty acid metabolism and leads to elevated TG (very low density lipoproteins; VLDL) and small dense low density lipoproteins (LDL) and low HDL cholesterol level (Miranda *et al.*, 2005; Meshkani and Adeli, 2009), the characteristic dyslipidaemia seen in metabolic syndrome and type 2 diabetes. The exact mechanism of reduced HDL cholesterol levels is not

known, but is thought to be related to TG enrichment of HDL cholesterol particles making them unstable and prone to degradation (Avramoglu *et al.*, 2006). HDL cholesterol is responsible for transferring excess cholesterol from periphery to liver, to be excreted as bile acids and cholesterol, the so called reverse cholesterol transport (Brewer, 2004; Singh, 2007). It also protects LDL cholesterol from oxidation and causes a selective decrease of endothelial cell adhesion molecules. The cumulative effect of all this is to protect against atherosclerotic plaque formation (Brewer, 2004). Epidemiological studies have shown an inverse relationship between serum HDL cholesterol level and all cause mortality (Okamura *et al.*, 2006). *Post hoc* analysis of the Treating to New Targets (TNT) study showed that HDL cholesterol was predictor of major cardiovascular events, even with optimal LDL cholesterol levels (Barter *et al.*, 2007a).

1.6. Currently available treatments for dyslipidaemia

Below is a summary of currently available treatments used for various types of dyslipidaemia;

Statins	Inhibit HMG Co-A reductase
Fibrates	PPARα receptor ligands
Ezetimibe	Selective inhibitors of NPC1L1 protein
Bile Acid Sequestrants	Interrupt enterohepatic circulation of bile acids
Nicotinic Acid	Inhibit lipolysis in adipose tissue
PCSK9 Inhibitors	Inhibit breakdown of hepatic LDL receptor

1.6.1. Hhydroxyl-methylglutaryl coenzyme-A (HMG Co-A) reductase Inhibitors (Statins)

Clinical trials data show that reducing LDL cholesterol is associated with a 30 - 45% reduction in clinical events (Brewer, 2004). As a result, hypercholesterolaemia has been treated both in the primary and secondary prevention of cardiovascular disease. Statins have been well established in the treatment of hypercholesterolaemia in patients with increased cardiovascular risk. They have proven their efficacy in reducing the incidence of ischemic heart disease (IHD) in a number of clinical trials (Baigent *et al.*, 2005). Statins are inhibitors of HMG Co-A reductase, an enzyme that catalyzes the rate limiting step in the synthesis of cholesterol in liver. In the Scandinavian Simvastatin Survival Study, simvastatin, over a period of 5.4 years of median follow-up, reduced total cholesterol and LDL cholesterol by 25% and 35% respectively and increased HDL cholesterol by 8% (Pedersen *et al.*, 2004). By reducing atherogenic lipoproteins in plasma, statins enhance cholesterol efflux from plaque and reduce macrophage cholesterol content, thereby reducing inflammation and increasing plaque stability (Chapman, 2004). In addition to cholesterol lowering effects, statins have cholesterol-independent effects including improvement in endothelial dysfunction, reducing oxidative stress and inflammation and inhibiting thrombogenic response. Some of the underlying mechanisms include; increasing endothelial nitric oxide (eNO) synthesis in physiological and pathophysiological states, increasing expression of tissue plasminogen activator (t-PA), inhibiting expression of endothelin (ET)-1, inhibiting the production of reactive oxygen species (ROS) and reducing high sensitivity C-reactive protein (hs- CRP) levels (Liao, 2005). The most common side effects related to statins use are myalgia and myositis, that are usually dose dependent and the risk is increased by concomitant use of drugs that inhibit cytochrome P450 (CYP)3A4 system. Other less common side effects include cognitive, pancreatic, hepatic and sexual dysfunction and neuropathy (Golomb and Evans, 2008).

1.6.2. Fibrates

Fibrates are synthetic ligands for PPARα, a member of the superfamily of nuclear hormone receptors that are expressed mainly in heart, kidney, liver and muscle. Activation of these receptors alters the transcription rate of various genes involved in the development of atherosclerosis (Goldenberg *et al.*, 2008). Various mechanisms implicated in the lipid lowering effects of fibrates include; Induction of lipoprotein lipolysis of TG rich particles, increased β oxidation of fatty acids and reduced availability for TG synthesis in the liver, increased removal of LDL cholesterol particles, reduced transfer of neutral lipids between VLDL and HDL cholesterol and increased HDL cholesterol production with stimulation of reverse cholesterol transport (Staels *et al.*, 1998). Fibrates slow down the progression of coronary atherosclerosis and reduce coronary event rate in young patients following myocardial infarction (Ericsson *et al.*, 1996). They are first line treatment for primary hypertriglyceridaemia and type III dysbetalipoproteinaemia. In addition they are useful in the treatment .of mixed hyperlipidaemia and dyslipidaemia associated with type 2 diabetes (Staels *et al.*, 1998). They reduce plasma TG levels by 30 – 50% and increase HDL cholesterol by 5 – 15%. Depending on the underlying lipid abnormality and baseline lipid phenotype, fibrates may reduce LDL cholesterol level by 15 – 20% (Chapman, 2006). Common side effects of fibrate therapy are epigastric distress, flatulence, diarrhoea, constipation, pruritus, urticaria and erythema. Less common side effects include myalgia, headache, haematological changes, abnormal liver function tests and cholelithiasis (Goldenberg *et al.*, 2008).

1.6.3. Ezetimibe

Ezetimibe selectively inhibits cholesterol absorption in the small intestine by blocking Niemann-Pick C1 Like 1 (NPC1L1) protein. NPC1L1, which is expressed in the brush border of enterocytes, is responsible for cholesterol absorption from the gut lumen (Jeu

and Cheng, 2003; Altmann *et al.*, 2004). Blocking NPC1L1 reduces cholesterol absorption from gut thereby reducing its level in the liver and causing an increased expression of LDL receptor on hepatocytes. The net result is a reduction in serum LDL cholesterol (Temel *et al.*, 2007). Monotherapy with ezetimibe reduces LDL cholesterol by 18.5% compared to placebo, in addition to causing an increase of 3% in HDL cholesterol (Pandor *et al.*, 2009). It has an even greater LDL cholesterol lowering effect when used in combination with a statin (Morrone *et al.*, 2012) and produces greater LDL cholesterol lowering effect than atorvastatin, when used as a combination therapy with simvastatin, in subjects with type 2 diabetes (Goldberg *et al.*, 2006). Current NICE guidance recommends the use of ezetimibe as monotherapy for primary hypercholesterolaemia (familial heterozygous and non-familial), when statin is either contraindicated or not tolerated and as a combination therapy with a statin, if adequate control of total and LDL cholesterol is not achieved, despite appropriate dose titration of statin or if dose titration is not possible due to intolerance (NICE, 2007). The safety of ezetimibe as monotherapy and in combination with statins is well documented. In clinical trials, ezetimibe did not cause any significant increase in liver enzymes, when compared to placebo. Similarly when ezetimibe was added to a statin, there was no significant increase in the incidence of myalgia or myositis (Phan *et al.*, 2012).

1.6.4. Bile Acid sequestrants

Bile acid sequestrants (BAS) disrupt enterohepatic circulation of bile acids by causing their excretion in faeces. This leads to increased synthesis of bile acids from cholesterol with secondary upregulation of hepatic LDL cholesterol receptors (Wierzbicki *et al.*, 2012). Depending upon the patient population and underlying lipid abnormality, BAS reduce LDL cholesterol by 12 – 28% and total cholesterol by upto 13% (Staels *et al.*, 2010). They not only improve dyslipidaemia (reducing total and LDL cholesterol) but also

reduce incidence of coronary heart disease (The Lipid Research Clinics Coronary Primary Prevention Trial results, 1984). The use of earlier substances has been limited because of gastrointestinal side effects. Colesevelam is a second generation BAS that not only lowers LDL cholesterol but also improves glycaemic control and has therefore been recommended for use in type 2 diabetes in combination with oral agents (metformin and sulfonylureas) and insulin (Fonseca *et al.*, 2010). A recent study found that colesevelam in combination with pioglitazone with or without other antidiabetic medications, had greater impact on lowering glycated haemoglobin (HbA1c) and improving dyslipidaemia (Rosenstock *et al.*, 2014). BAS are not absorbed from the gut and hence lack systemic toxicity. The main side effect associated with first generation BAS is constipation. In addition they interact with certain treatments like warfarin, digoxin and levothyroxine, impairinng their absorption (Hansen *et al.*, 2014).

1.6.5. Nicotinic Acid

It was in the 1950's that nicotinic acid was first found to have lipid lowering effect in healthy subjects and in patients with hypercholesterolaemia (Altschul *et al.*, 1955) and was the first antidyslipidaemia treatment to be introduced into clinical practice. Nicotinic acid inhibits lipolysis in the adipose tissue and leads to a rapid decline in plasma free fatty acid levels (Carlson and Oro, 1962; Carlson, 1963). This is followed by a slow decline in TG and VLDL cholesterol levels, due to reduced synthesis as a result of limited supply of free fatty acids (Mahboubi *et al.*, 2006). This limits cholesteryl ester transfer protien (CETP) mediated transfer of cholesteryl ester (CE) and TG between various lipoprotein molecules with the net result of a reduction in plasma LDL cholesterol and a rise in HDL cholesterol level (Gille *et al.*, 2008). Nicotinic acid can reduce plasma TG levels by 45% and increase HDL cholesterol by about 35% (Mahboubi *et al.*, 2006). In addition nicotinic acid also reduces lipoprotein LP(a), which is an independent risk factor for coronary artery disease

(Carlson *et al.*, 1989). In the Coronary Drug Project, nicotinic acid reduced mortality by reducing the number of coronary events (Clofibrate and niacin in coronary heart disease, 1975). Recent discovery of two niacin receptors (HM74 and HM74A) has led to a renewed interest in the mechanisms that underlie its antidyslipidaemic actions. These are G protein coupled receptors, with HM74A highly expressed in adipocytes. Its activation leads to reduced adenylate cyclase activity with consequent reduction in intracellular cAMP and inhibition of lipolysis (Vosper, 2009). Flushing is the most common side effect associated with the use of nicotinic acid that affects patients' compliance (Lukasova *et al.*, 2011). NICE recommends the use of nicotinic acid for secondary prevention in subjects with cardiovascular disease who are unable to tolerate statins (NICE, 2010).

1.6.6. PCSK9 inhibitors

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease that causes degradation of hepatic LDL receptor thereby affecting plasma LDL cholesterol levels. Mutations in PCSK9 cause autosomal dominant hypercholesterolaemia (Abifadel *et al.*, 2003) accounting for a very small proportion of cases of dominant hypercholesterolaemia, the majority of cases caused by mutations in genes encoding LDL receptor (familial hypercholesterolaemia) and ApoB-100 (familial defective ApoB-100) (Rader *et al.*, 2003). Alirocumab and evolocumab, which are humanized monoclonal antibodies, were recently approved by the FDA as first-in-class medications, based on their efficacy in reducing LDL cholesterol, as a surrogate marker in the absence of clinical outcome data (Everett *et al.*, 2015). In patients at high risk of cardiovascular disease, already taking maximum tolerated statin dose, with or without other lipid lowering therapy, addition of alirocumab, causes a significant reduction in LDL cholesterol (Robinson *et al.*, 2015). It also shows greater LDL cholesterol lowering efficacy, when compared to ezetimibe in a 24 week phase 3 randomized study (Roth *et al.*, 2014). According to the FDA, alirocumab

(PRALUENT^{TM}) is indicated as adjunct to diet and maximally tolerated statin therapy for the treatment of adults with heterozygous familial hypercholesterolaemia or clinical atherosclerotic CVD requiring additional lowering of LDL cholesterol (Pahon, 2015a). A long-term cardiovascular outcome study is currently underway, aiming to randomize approximately 18,000 patients, following an episode of acute coronary syndrome (Schwartz *et al.*, 2014). Evolocumab has also been tested in homozygous familial hypercholesterolaemia, and causes a significant reduction in LDL cholesterol (Raal *et al.*, 2015). In addition to heterozygous familial hypercholesterolaemia and clinical atherosclerotic CVD, evolocumab (REPATHA) is also indicated in homozygous familial hypercholesterolaemia, as add on therapy to statins, ezetimibe or LDL plasmapheresis (Pahon, 2015b).

1.7. Targeting low HDL cholesterol

Since low HDL cholesterol has been identified as an independent risk factor for cardiovascular disease, researchers have been interested in finding ways to improve low HDL cholesterol levels. Attempts have been made to inhibit CETP, a plasma protein that facilitates transfer of CE and TG between various lipoprotein molecules. Earlier compounds, like trocetrapib, resulted in increased risk of mortality and morbidity, in clinical trials, in patients at high risk of coronary events (Barter *et al.*, 2007b). A new CETP inhibitor K-312, in addition to raising HDL and lowering LDL cholesterol, reduces expression of PCSK9 in various cells lines (Miyosawa *et al.*, 2015). A large phase 3 clinical trial, using a newer CETP inhibitor, is currently underway looking at the cardiovascular outcomes in high risk patients with results expected sometime in 2017 (Gutstein *et al.*, 2012).

1.8. Stress and the Cardiovascular System

Epidemiological studies have shown a positive relationship between long term stress and the development of cardiovascular disease (Everson-Rose and Lewis, 2005; Shen *et al.*, 2008; Figueredo, 2009a). Factors like social isolation, low socio-economic status, depression, stressful family and work life and anxiety have all been associated with increased risk of development and accelerated progression of existing cardiovascular disease. Current European guidelines on the prevention of cardiovascular disease emphasize the importance of tackling these factors (Perk *et al.*, 2012). In general, acute and chronic stress increase cardiovascular mortality through behavioural and direct pathophysiological mechanisms. Behavioural mechanisms include unhealthy lifestyles e.g. smoking (Glassman *et al.*, 1990), reduced patient compliance (Carney *et al.*, 1995a; McDermott *et al.*, 1997), obesity (Clarke *et al.*, 2015) and alcoholism (Kessler *et al.*, 1997). Pathophysiological mechanisms include hypercortisolism (Carroll *et al.*, 1976; Gold *et al.*, 1986), impairment of platelet function, including increased platelet reactivity (Musselman *et al.*, 1996), and release of factors such as platelet factor 4 and β-thromboglobulin (Laghrissi-Thode *et al.*, 1997), reduced heart rate variability (Carney *et al.*, 1995b), impaired vagally mediated baroreflex control of the heart (Watkins and Grossman, 1999) and overactivity of the sympathetic nervous system (Veith *et al.*, 1994). In summary these factors work together to increase the risk of coronary artery disease (by accelerated atherosclerosis), hypertension and sudden death due to arrhythmias. In his review of the effects of acute and chronic psychological stressors (excluding depression and type A behaviour pattern) on the heart, Dimsdale has reported some interesting findings (Dimsdale, 2008). For instance, studies of patients during catastrophic events, like earthquakes, have shown sudden rises in heart rate, a withdrawal of parasympathetic nervous system activity and an increase in sympathetic nervous system activity (increased

sympathetic activity in the long run is associated with cardiovascular disease), increased blood viscosity as demonstrated by a rise in haematocrit, rise in markers of pro-coagulant activity (fibrinogen, von Willibrand factor and D Dimer) and increased incidence of pulmonary embolism due to a combination of psychological stress and relative immobility. Stress induced by public speaking is associated with a 3 fold rise in epinephrine levels (Dimsdale and Moss, 1980). Mental stress induces myocardial ischemia in patients with stable coronary artery disease and this appears to be mediated by adrenal release of catecholamines. There is an increase in heart rate (HR), systolic blood pressure (SBP) and rate pressure product, a measure of stress on cardiac muscle (Goldberg *et al.*, 1996a). Such ischemic changes are predominantly silent and do not cause typical ECG changes (Rozanski *et al.*, 1988b). Young adults, showing an exaggerated heart rate and blood pressure response to application of a cold stimulus, are more likely to develop hypertension later in life (Wood *et al.*, 1984; Menkes *et al.*, 1989).

Baroreceptor reflex or baroreflex is a negative feedback mechanism, which helps to prevent large fluctuations in blood pressure over relatively short periods (seconds to minutes). Recent data suggests that baroreceptors are also implicated in long term blood pressure control (Kougias *et al.*, 2010). A rise in blood pressure activates baroreceptors in the carotid sinus and aortic arch. This reduces sympathetic output to the heart, kidneys and peripheral vasculature and increases parasympathetic output to heart. The end result is slowing down of heart rate in association with reduced vascular tone and a reduction in blood pressure (Kumada *et al.*, 1990). Reduced sensitivity of the baroreceptors (baroreflex) is seen following myocardial infarction in patients with reduced left ventricular ejection fraction (LVEF) and is associated with increased mortality (La Rovere *et al.*, 2001). Studies show that acute stress and chronic depression are also associated with reduced sensitivity of the baroreceptors (Rozanski *et al.*, 1999). This reduces their

ability to respond appropriately to changes in blood pressure and reduced baroreceptor sensitivity, in the long run, has been implicated in the development of hypertension (Kougias *et al.*, 2010).

1.9. Cannabinoids

Cannabinoids are C_{21} terpephenolic compounds that bind to the cannabinoid (CB) receptors or are structurally similar to delta 9 tetrahydrocannabinol (Δ^9 THC or THC). The table below shows various types of cannabinoids. These include endogenously formed compounds called endocannabinoids (e.g. AEA), synthetic compounds (e.g. WIN55212-2) and phytocannabinoids obtained from the *Cannabis Sativa L* plant.

Endocannabinoids	Endogenously formed e.g. AEA, 2-AG
Synthetic cannabinoids	Synthesized in the laboratory e.g. WIN55212-2, AM251
Phytocannabinoids	Obtained from Cannabis Sativa L plant e.g. THC, CBD and THCV

There are over 60 known types of phytocannabinoids (Brenneisen, 2007), and three phytocannabinoids of interest are described below in more detail.

1.9.1. Delta 9 tetrahydrocannabinol (Δ^9THC or THC)

THC is the parent compound present in cannabis or marijuana, responsible for its psychoactive properties (Costa *et al.*, 2007). It was discovered in 1964 (Gaoni and Mechoulam, 1964) and has been the most widely studied phytocannabinoid.

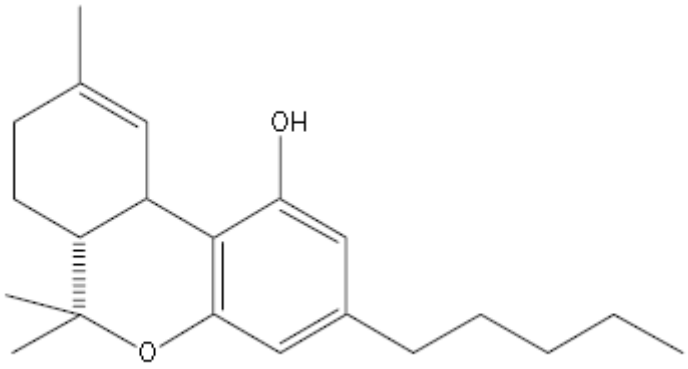


Figure 1.1. Structure of delta 9 tetrahydrocannabinol (THC)

It binds to both CB_1 and CB_2 cannabinoid receptors, with K_i values in the low nanomolar range. It has a higher binding affinity than other phytocannabinoids including cannabinidol (CBD) and delta 9 tetrahydrocannabivarin (Δ^9 THCV or THCV), but its binding affinity is lower than synthetic CB_1/CB_2 agonists. It has a lower efficacy at both CB_1 and CB_2 and therefore acts as a partial agonist (Pertwee, 2008b). THC produces a variety of effects that mimick the endogenous cannabinoids or the endocannabinoids, AEA and 2-AG. THC has the potential to modulate inflammatory response. While it reduces the production of pro-inflammatory cytokines, interferons α and β (IFN-α and IFN-β) from mouse cells after stimulation with lipopolysaccharides (LPS) (Blanchard *et al.*, 1986; Cabral *et al.*, 1986), it can increase the production of other cytokines including TNF, IL-1, 6 and 10 (Klein *et al.*, 1993; Zhu *et al.*, 1994). THC induces antinociception in animal models of pain, mainly through its interaction with CB_1 receprtors (Compton *et al.*, 1996; Ledent *et al.*, 1999; Zimmer *et al.*, 1999). It can also release endogenous opioids interacting with δ- and κ-

opioid receptors, and therefore can have synergistic effects when used in low doses with opiates (Mason *et al.*, 1999; Welch and Eads, 1999; Cichewicz and Welch, 2003). In humans, THC has analgesic effects in chronic cancer pain with similar potency as codeine (Noyes *et al.*, 1975). A 1:1 combination of THC/CBD (Sativex®) is currently licenced for the symptomatic treatment of spasticity in moderate to severe multiple sclerosis and in 2014 it was granted fast track designation by the FDA for use in pain from advanced cancer not responding to optimized chronic opioid therapy. Animal studies also demonstrate the potential of THC in neuroprotection in various neurodegenerative disorders like multiple sclerosis (Fujiwara and Egashira, 2004), amyotrophic lateral sclerosis (Raman *et al.*, 2004) and parkinson's disease (Lastres-Becker *et al.*, 2005). This effect could be related to the activation of presynaptic CB_1 receptors in the brain, that block glutamate and gama amino butyric acid (GABA) neurotransmission (Shen and Thayer, 1999; van der Stelt *et al.*, 2001), but has been observed even after blocking CB_1 receptors (Marsicano *et al.*, 2002a), and may be related to antioxidant properties of THC (Chen *et al.*, 2005). Placebo controlled trials in humans, where THC was given to treat MS related spasticity have produced mixed results (Ben Amar, 2006), while a small study in parkinsons's disease subjects did not produce any objective or subjective improvement in dyskinesias (Carroll *et al.*, 2004).

The unwanted effects of THC limit its clinical use. Studies in rodents show that THC, in a dose-dependant way, produces locomotor suppression, antinociception, hypothermia and catalepsy (impaired ability to initiate movements), the so called 'tetrad effect' (Wiley *et al.*, 2007). This effect is dependent on stimulation of central CB_1 receptors by THC, as it is not seen in mutant mice lacking CB_1 (Monory *et al.*, 2007). In addition to its psychotropic effects, THC also causes tachycarida and orthostatic hypotension (Sidney, 2002). In a recent study in healthy volunteers, with previous exposure to cannabis, a single dose of 10

mg THC was associated with tachycardia, without having any significant effect on blood pressure (Martin-Santos *et al.*, 2012). Despite the preclinical data highlighting the potential benefits of THC, the therapeutic use of THC is limited by the psychotropic side effects due to central CB_1 stimulation and the potential for tolerance and dependence. The search is therefore on to find alternative compounds which can modulate the endocannabinoid system without producing the unwanted effects of THC.

1.9.2. Cannabidiol (CBD)

The other major compound obtained from *Cannabis Sativa L* is CBD, which was discovered in 1940 (Adams *et al.*, 1940) and its chemical structure was elucidated by Mecholaum in 1963 (Mechoulam and Shvo, 1963).

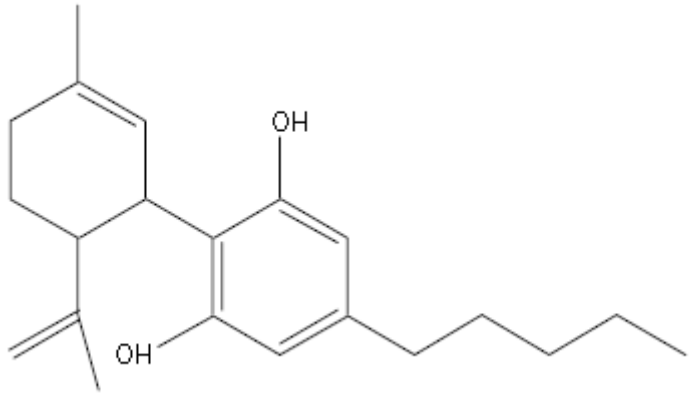


Figure 1.2. Structure of cannabidiol (CBD)

CBD does not bind to either CB_1 or CB_2 receptors with much affinity, but can act as an antagonist of CB_1 and CB_2 receptor agonists, in CB_1 and CB_2 expressing cells or tissues (Thomas *et al.*, 2007). Since CBD does not bind to central CB_1 receptors with great affinity, it does not have any of the psychoactive properties, associated with the use of THC. Sativex®, used as add-on therapy for spasticity in multiple sclerosis, contains a 1:1 combination of CBD and THC, and is available as oro-mucosal spray. The nature of

interaction between CBD and THC is not entirely clear with earlier studies showing that CBD reduced THC induced anxiety in healthy volunteers (Zuardi *et al.*, 1982b), while one recent study showed that CBD potentiated the psychoactive and physiological effects of THC in rats (Klein *et al.*, 2011). This might be explained by the time interval between the administration of CBD and THC and the dose ratios. Pretreatment with CBD may enhance the effects of THC through pharmacokinetic interaction, while simultaneous administration of the two at high dose ratios may lead to pharmacodynamic interaction and may antagonise some of the effects of THC (Zuardi *et al.*, 2012b). In Sativex®, CBD seems to block the central unwanted effects of THC. CBD is currently the focus of enormous research due to its potential beneficial effects in a number of therapeutic areas. Studies have shown the potential of CBD as an anti-inflammatory (Costa *et al.*, 2004a), anti-convulsant (Jones *et al.*, 2012), anti-oxidant (Hampson *et al.*, 1998), anxiolytic (Zuardi *et al.*, 1982a; Crippa *et al.*, 2004), anti-nausea (Parker *et al.*, 2011) and anti-psychotic (Zuardi *et al.*, 2012a) agent. Epidiolex©, which contains CBD, was granted orphan drug designation in Dravet & Lennox-Gastaut syndromes, by the FDA.

The role of CBD in metabolism and cardiovascular system is discussed in more detail in 1.15.1.

1.9.3. Delta 9 tetrahydrocannabivarin (Δ^9 THCV or THCV)

THCV is an *n*-propyl analogue of THC. It was discovered in 1970 by Edward Gill (Gill *et al.*, 1970) and named Δ^9 THCV in 1971 (Merkus, 1971).

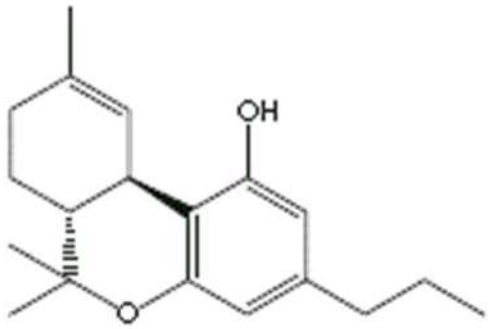


Figure 1.3. Structure of delta 9 tetrahydrocannabivarin (THCV)

THCV differs from THC in that it has a shorter side chain with three carbons (propyl) instead of five (pentyl) (Thomas *et al.*, 2005b). This shortening of the side chain was expected to affect the efficacy of this molecule as a CB_1 receptor agonist, and in line with this, THCV was found to behave either as a CB_1 receptor antagonist or, at higher doses, a CB_1 receptor agonist *in vivo* (Pertwee *et al.*, 2007a). It also acts as a neutral antagonist at CB_2 receptors (Thomas *et al.*, 2005b), however Bolognini and colleagues showed that it acts as CB_2 agonist in [^{35}S] guanosine 5'-O-[gamma-thio]triphosphate (GTPγS) assay, when the expression level of these receptors is increased, concluding that THCV is a partial agonist at CB_2 receptors (Bolognini *et al.*, 2010a). It reduces signs of inflammation and inflammatory pain in mice via CB_1 and/or CB_2 activation (Bolognini *et al.*, 2010b). Also, in animal studies, THCV has antiepileptiform and anticonvulsant properties (Hill *et al.*, 2010a).

Its effects on lipid metabolism are discussed in section 1.15.2.

1.10. Cannabinoid receptors

These are 7-transmembrane G-protein-coupled receptors (GPCR) (Pertwee *et al.*, 2010b). Two types of cannabinoid receptors are known to date; CB_1 and CB_2. Their activation leads to signal transduction via $G_{i/o}$ alpha subunits to inhibit adenylate cyclase. They also

mediate some of their effects via MAPK pathway. In addition CB_1 receptors couple to a number of ion channels, causing cAMP-independent inhibition of N- and Q-type voltage-dependent Ca^{2+} channels and cAMP-dependent stimulation of inwardly rectifying K^+ channels (Marsicano and Lutz, 1999).

1.10.1. CB_1 Receptor

This was first isolated in 1990 from rat cerebral cortex cDNA library (Matsuda *et al.*, 1990). CB_1 receptors cause signal transduction through $G_{i/o}$ proteins, causing inhibition of adenylate cyclase, which is sensitive to pertussis toxin (Pertwee, 1997). In addition to this, CB_1 receptors activate MAPK, inhibit voltage-gated Ca2+ channels, activate K^+ currents (K_{ir}), and influence Nitric Oxide (NO) signalling (Howlett *et al.*, 2010). Some CB_1 receptors can also signal through G_s proteins (Glass and Felder, 1997). CB_1 is expressed mainly in the central and peripheral neurons, where it inhibits the release of various neurotransmitters. By virtue of their distribution in the central nervous system, activation of CB_1 receptors can affect various processes including cognition and memory, motor function and inducing signs of analgesia (Pertwee *et al.*, 2010b). CB_1 receptors are also found in other areas of brain involved in the regulation of appetite and food intake such as hypothalamus, nucleus accumbens shell (NAcS) in limbic forebrain, vagus nerve termination at nodose ganglion and brain stem areas receiving sensory input from duodenum (Matias *et al.*, 2008b). In addition to central and peripheral neurons, CB_1 is found in immune cells (Pertwee, 2008b) and many peripheral tissues including adipose tissue, liver, pancreas and skeletal muscle (Pacher *et al.*, 2006). Functional CB_1 receptors are also present in both α and β cells of the islets in human pancreas, together with enzymes responsible for the synthesis and degradation of 2-AG and FAAH, enzyme responsible for the degradation of AEA, suggesting that endocannabinoids have a role to play in endocrine pancreas (Bermudez-Silva *et al.*, 2008). CB_1 receptors may undergo

upregulation in various tissues under certain conditions. For instance, in obesity, there is evidence of CB_1 upregulation in liver, adipose tissue and skeletal muscle. In human obesity, CB_1 expression is increased in visceral fat but reduced in subcutaneous fat. Also there is significant increase in CB_1 immunoreactivity in hepatic tissue from patients with non-alcoholic fatty liver disease (Kunos and Tam, 2011), a condition associated with obesity and metabolic syndrome (Grundy *et al.*, 2004a). CB_1 upregulation has also been observed in contralateral thalamic regions (Siegling *et al.*, 2001) and ipsilateral spinal cord (Lim *et al.*, 2003) following nerve injury. The differential distribution of CB_1 receptor in different fat depots and its presence in fatty liver, suggests an important role for this receptor in obesity and metabolic syndrome.

1.10.2. CB_2 Receptor

CB_2 receptor was first isolated in 1993 from human promyelocytic leukemic cell line HL60 (Munro *et al.*, 1993). It is mainly expressed in testis, various brain areas, spleen and leukocytes (Liu *et al.*, 2009). In the immune system CB_2 is mainly expressed on T cells, B cells, natural killer (NK) cells, monocytes, neutrophils and microglial cells (Galiegue *et al.*, 1995), where their activation leads to inhibition of release of pro-inflammatory cytokines (Eljaschewitsch *et al.*, 2006). CB_2 activation in the CNS leads to reduced inducible nitric oxide (NO) synthase (Bergamaschi *et al.*, 2011a) production through activation of mitogen-activated protein kinase phosphatase-1 and inhibition of extracellular signal-regulated protein kinase (ERK) 1/2 phosphorylation in the microglia. Expression of CB_2 receptors is up-regulated in the human brain under inflammatory conditions (Rom and Persidsky, 2013). Central CB_2 receptors also play a role in mediating the manifestations of neuropathic pain through modulation of glial activation (Racz *et al.*, 2008). In animal models, overexpression of CB_2 receptors in the central nervous system

leads to decreased food intake and body weight but also causes glucose intolerance (Romero-Zerbo *et al.*, 2012).

Both CB_1 and CB_2 receptors are also present in skeletal muscle, adipose tissue (Cavuoto *et al.*, 2007; Pagano *et al.*, 2007) exocrine pancreas (Linari *et al.*, 2009), islets of Langerhans (Bermudez-Silva *et al.*, 2007b), myocardium and human coronary artery endothelial and smooth muscle cells (Bonz *et al.*, 2003; Mukhopadhyay *et al.*, 2007; Rajesh *et al.*, 2007c; Rajesh *et al.*, 2008). *In vitro* studies, using mice islets, show that CB_2 activation by endocannabinoids affects Ca^{2+} signalling and leads to reduction in insulin secretion (Juan-Pico *et al.*, 2006). In rats, however, activation of CB_2 receptors on beta cells causes an improvement in glucose homeostasis (Bermudez-Silva *et al.*, 2007a). Stimulation of CB_2 receptors present on somatostatin-secreting delta cells in human endocrine pancreas leads to a reduction in insulin secretion (Bermudez-Silva *et al.*, 2008). The role of CB_1 and CB_2 in cardiovascular system is discussed separately.

1.11. Non cannabinoid receptors

Many of the effects of cannabinoids are not explained by their activity at CB_1 and CB_2 and a number of studies have suggested the existence of additional cannabinoid receptors (Begg *et al.*, 2005; Mackie and Stella, 2006). The important ones that interact with endocannabinoids, phytocannabinoids or synthetic CB_1/CB_2 ligands include; transient receptor potential receptors, various G protein coupled receptors, peroxisome proliferator activated recepros and putative endothelial cannabinoid receptors (see figure 1.4).

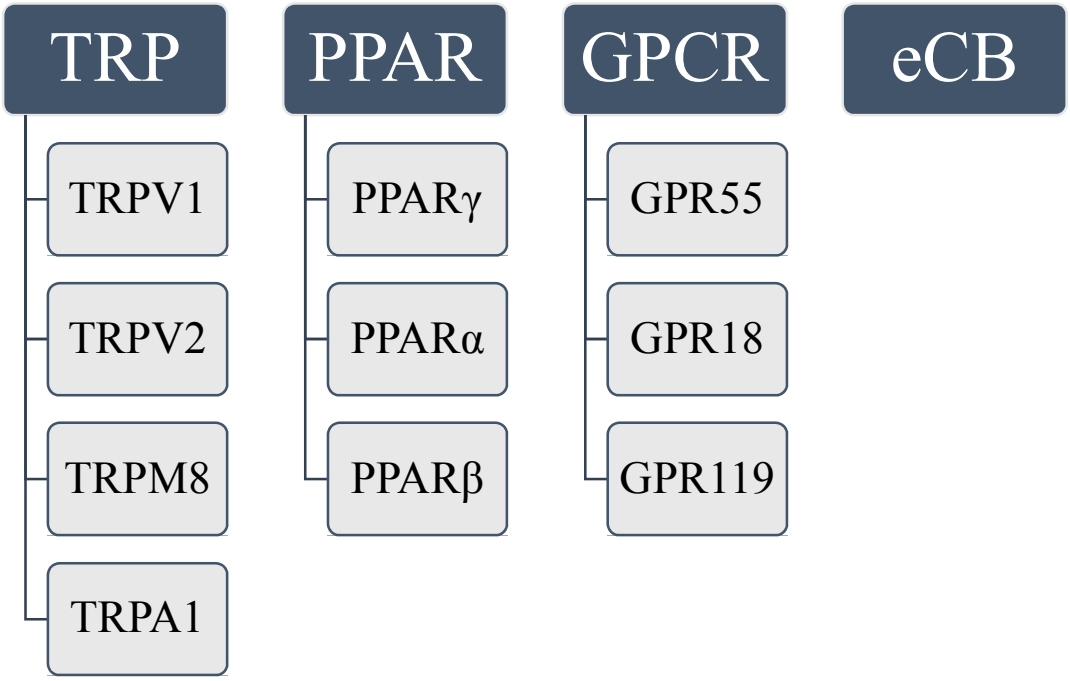


Figure 1.4. Various non-cannabinoid receptors that interact with cannabinoids

1.11.1. Transient Receptor Potential (TRP) channels

TRP channels exhibit six trans-membrane (TM) structure, with cytosolic C- and N-terminal domains and a re-entrant pore-lining loop located between the 5th and 6th TM segments. The region between TMs 5 and 6 acts as a nonselective cation-permeable pore (Benham *et al.*, 2002; Pertwee *et al.*, 2010b). 28 members of the TRP family have been characterised in mammals (Nilius and Voets, 2005) There are 6 subfamilies of the TRP superfamily, and five subtypes belonging to 3 subfamilies have been found to interact with phytocannabinoids, synthetic CB_1 ligands and endocannabinoids: TRPV1, TRPV2, TRPV4, TRPM8 and TRPA1 (Pertwee *et al.*, 2010b).

1.11.1.1. TRPV1

TRPV1 was the first to be cloned from rat cDNA as capsaicin receptor, that is activated by noxious heat (>43°C) and protons (extracellular pH < 6) (Caterina *et al.*, 1997). TRPV1 is predominantly expressed in the sensory neurons of the dorsal root and trigeminal ganglia

and becomes upregulated during nerve injury-induced thermal hyperalgesia and diabetic neuropathy. Recent evidence suggests the presence of TRPV1 in a number of other tissues including brain neurons, endothelial and pancreatic β cells. TRPV1 co-localizes with CB_1 receptors in sensory and brain neurons and with CB_2 receptors in sensory neurons and osteoclasts (Starowicz *et al.*, 2007; Di Marzo and Cristino, 2008; Pertwee *et al.*, 2010b). Endocannabinoids AEA and N-arachidonoyl dopamine (NADA) (Zygmunt *et al.*, 1999b) and phytocannabinoids CBD and cannabigerol (CBG) (Bisogno *et al.*, 2001; Ligresti *et al.*, 2006) act as full agonists at TRPV1.

1.11.1.2. TRPV2

Previously known as growth factor-regulated Ca^{2+} channel or vanilloid receptor-like 1, TRPV2 is activated by high temperature but not by protons. There is strong evidence that CBD, THC and cannabinol interact with TRPV2 (Qin *et al.*, 2008). Initially thought to play a role in mechano and thermoregulation, recent evidence suggests a much wider role for TRPV2 in various systems, including nervous system, endocrine pancreas, immunity, circulation and skeletal muscles (Peralvarez-Marin *et al.*, 2013).

1.11.1.3. TRPM8

TRPM8 was initially cloned from prostate tissue (Tsavaler *et al.*, 2001), and was later found to be present in peripheral sensory neurons. It is a non-selective cation channel, permeable to calcium (McKemy *et al.*, 2002). It is activated by cold temperature and natural and synthetic cooling mimetic compounds like menthol (McKemy, 2005) and icilin (Behrendt *et al.*, 2004). AEA and NADA antagonise TRPM8 agonists, menthol and the synthetic compound icilin, in HEK293 cells (De Petrocellis *et al.*, 2007) and in dorsal root ganglia neurons (De Petrocellis *et al.*, 2008). CBD, cannabigerol and THC exert a similar action at TRPM8, but at a much lower concentration than AEA and NADA (De Petrocellis

et al., 2008). TRPM8 plays an important role in detecting noxius cold stimuli (Fernandez-Pena and Viana, 2013).

1.11.1.4. TRPA1

Like TRPM8, TRPA1 belongs to a subfamily of TRP receptors, different from capsaicin (TRPV1) receptor, but is still involved in thermosensation. TRPA1 is activated by cold stimuli and irritants like mustard oil isothiocyanates and acrolein (McKemy, 2005). Human TRPA1 can sense extracellular acidosis (pH < 7) (de la Roche *et al.*, 2013), a phenomenon that accompanies tissue ischemia, inflammation and cancer growth. Jordt and colleagues first described the activation of TRPA1 by THC and cannabinol in micromolar concentrations (Jordt *et al.*, 2004). It's a nonselective cation channel with six putative transmembrane segments (S1-S6), intracellular N- and C-termini and a pore loop between S5 and S6 (Gaudet, 2008; Nilius *et al.*, 2011).

1.11.2. Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are members of nuclear receptor family that cause signal transduction through gene transcription. The three known isoforms include PPARα, PPARγ and PPARβ. PPARα is the therapeutic target for fibrates, while PPARγ is acted upon by TZD's, treatment used in type 2 diabetes. PPARs are generalized lipid sensors and are activated by fatty acids (for review see Pertwee *et al.*, 2010b). PPARα is highly expressed in liver, heart, skeletal muscle, intestinal mucosa and brown adipose tissue, while PPARγ is expressed in white and brown adipose tissue, large intestine and spleen, with highest expression in adipocytes (Grygiel-Gorniak, 2014). There is evidence of interaction between the PPARs and the ECS. PPARα is stimulated by OEA causing satiety (Fu *et al.*, 2003) and stimulating fat utilization (Guzman *et al.*, 2004). AEA causes 3T3L1-preadipocyte differentiation via PPARγ activation (Bouaboula *et al.*, 2005), while PPARγ agonists inhibit AEA

metabolism in rat brain. Ciglitazone, one of the PPARγ agonists, also interacts with MAGL and CB_1 and CB_2 receptors (Lenman and Fowler, 2007). AEA, 2-AG and ajulemic acid are all known to cause anti-inflammatory effects through PPARγ activation, while THC also acts on PPARγ causing time dependent relaxation in isolated arteries (O'Sullivan, 2007). Given the role of PPARs in metabolism and their interaction with cannabinoids, it is plausible that some of the metabolic effects of phytocannabinoids are the result of their interaction with these nuclear receptors.

1.11.3. GPR55

An orphan GPCR, that belongs to rhodopsin-like (Class A) family of GPCRs, and couples to Gα13 (Ryberg *et al.*, 2007; Henstridge *et al.*, 2009) Gα12, or Gαq proteins (Lauckner *et al.*, 2008). GTPγS functional assay indicates that GPR55 is activated by nanomolar concentrations of the endocannabinoids 2-AG, virodhamine, noladin ether, OEA and PEA and the atypical cannabinoids CBD and abnormal CBD (abn-CBD) (Ryberg *et al.*, 2007). It was initially de-orphanized as a cannabinoid receptor (Brown and Wise, 2001; Drmota *et al.*, 2004), but is now considered an orphan GPCR, as non-cannabinoid lysophosphatidylinositol (LPI) compounds are endogenous ligands for this receptor (Oka *et al.*, 2007), with 2-arachidonoyl-sn-glycero-3-phosphoinositol (2-AGPI) posessing the best LPI activity (Oka *et al.*, 2009). Neither LPI nor 2-AGPI bind to CB_1 or CB_2 receptors. In humans GPR55 has been expressed in the CNS, predominantly in the caudate, putamen and striatum (Sawzdargo *et al.*, 1999), while GPR55 mRNA and protein expression have also been located in liver and in visceral and subcutaneous white adipose tissue (Moreno-Navarrete *et al.*, 2012), suggesting a role for GPR55 in energy homeostasis. Moreno and colleagues reported that GPR55 expression in visceral adipose tissue is positively associated with obesity and type 2 diabetes. Similarly plasma levles of LPI, the endogenous ligand for GPR55, are higher in obese compared to lean patients, suggesting a

positive correlation between LPI/GPR55 and obesity (Moreno-Navarrete *et al.*, 2012). GPR55 is also expressed in pancreatic β cells, suggesting a role for this receptor in endocrine function of the pancreas (Romero-Zerbo *et al.*, 2011). McKillop (McKillop *et al.*, 2013) confirmed GPR55 expression in pancreas and demonstrated that GPR55 was a strong activator of insulin secretion, with *in vivo* glucose lowering effects. Recent evidence shows that GPR55 plays an important role in CB_2 mediated inflammatory response of the neutrophils. It helps with effective recruitment of neutrophils to the site of inflammation while at the same time reducing some of the tissue damaging inflammatory responses (Balenga *et al.*, 2011).

1.11.4. GPR18

GPR18 is a GPCR, that belongs to class A (i.e. rhodopsin like, same as CB_1 and CB_2) of the the GPCRs, but with a structure that is dissimilar to both CB_1 and CB_2 (Pertwee *et al.*, 2010a). It was inadvertently isolated while the researchers were trying to isolate a gastrin-releasing peptide receptor from canine gastric mucosa and human colonic cancer cell line, Colo 320DM (Gantz *et al.*, 1997). They found that it was most abundant in human testis and spleen, while detectable in several other tissues associated with immune system, including thymus, peripheral blood leukocytes and small intestine. Human GPR18 gene is localised in chromosomal region 13q32.3 (Gantz *et al.*, 1997). Vassilatis and colleagues, while using real-time polymerase chain reaction (RT-PCR) tissue profiling, showed strong expression of GPR18 in hypothalamus, thyroid, peripheral blood leucocytes, cerebellum and brain stem of humans and mice (Vassilatis *et al.*, 2003). In 2006, it was cloned from human T-cell leukemia cell line, HUT102 (Kohno *et al.*, 2006b). Because of high expression in peripheral blood mononuclear cells and lymphoid tissues, a role for GPR18 in immune system regulation was suggested. N-arachidonylglycine (NAGly) was found to be an endogenous ligand for GPR18. It caused pertussis toxin-sensitive inhibition of

cAMP production in GPR18 transfected Chinese hamster ovary (CHO) cells, in a dose dependent manner, and at 10μM caused significant elevation of intracellular Ca^{2+} concentration, using GPR18 -expressing L929 cells (Kohno *et al.*, 2006a). NAGly is the carboxylic analogue of AEA, that inhibits its hydrolysis by FAAH, therefore causing an increase in its concentration. It is present in skin, small intestine, kidney, testis and brain (Huang *et al.*, 2001). Recent evidence shows that THC and NAGly both act as agonists at GPR18 while CBD antagonises their effect and also acts as a weak partial agonist (McHugh *et al.*, 2012a).

1.11.5. GPR119

This is another member of the GPCR superfamily that is expressed predominantly in human and rodent pancreas and gastrointestinal-tract and also in rodent brain (Overton *et al.*, 2006b). It is predominantly localized in pancreatic polypeptide (PPY)-producing cells in adult mouse and rat islets (Sakamoto *et al.*, 2006). OEA acts as an agonist at GPR119 (Overton *et al.*, 2006b). It is produced in the small intestine in response to food intake and regulates satiety and body weight. Researchers also found a small-molecule, GPR119 selective agonist, PSN632408, which is similar in efficacy and potency to OEA at GPR119 receptors. Acute administration of PSN632408 to free-feeding male rats reduced food intake for up to 24 hours after the drug, while chronic administration to fat-fed male rats reduced both food intake and body weight (Overton *et al.*, 2006b). Another GPR119 agonist AR231453 has been shown to enhance glucose-dependent insulin release by acting directly on the pancreatic β-cells (Chu *et al.*, 2007). OEA induces GLP-1 release from intestinal L-cells, *in vitro* and *in vivo*, in rats (Lauffer *et al.*, 2009b). Recent evidence shows that 2-Oleoyl glycerol (2-OG) acts as GPR119 agonist and causes increased release of GLP-1 and GIP from human intestine but without having any effects on insulin, C-

peptide or glucose (Hansen *et al.*, 2011). Given above, GPR119 may have a role in regulating body weight and energy balance.

1.11.6. Endothelial Cannabinoid receptor (eCB)

The presence of a novel endothelial cannabinoid receptor was first highlighted in 1999. Abn-CBD that does not bind to CB_1 receptors was shown to cause hypotension and mesenteric vasodilatation in wild type mice and in mice lacking CB_1 receptors or both CB_1 and CB_2 receptors, that was sensitive to SR141716A. CBD, which does not affect AEA or HU-210 induced hypotension, inhibited Abn-CBD induced hypotension. The authors concluded that Abn-CBD and CBD were selective agonist and antagonist, respectively, of this yet-unidentified endothelial receptor, which elicited NO-independent mesenteric vasodilatation (Jarai *et al.*, 1999). Wagner and colleagues showed that AEA caused mesenteric vasodilatation, via an endothelial receptor site that was distinct from CB_1, as CB_1 receptor agonists, WIN55212-2 and HU-210, failed to produce dilatory response. The investigators labelled this site as 'anandamide receptor' (Wagner *et al.*, 1999). Cannabidiol analogue O-1918, inhibits Abn-CBD mediated rat mesenteric vasodilatation, and is therefore considered selective antagonist of the Abn-CBD endothelial receptor (Offertaler *et al.*, 2003). The identity of proposed endothelial cannabinoid receptor remains elusive, as Abn-CBD produces vasodilatation in de-endothelialized rabbit pulmonary artery segments and this effect is blocked by O-1918 (Su and Vo, 2007), suggesting that they both work through non-endothelial sites, possibly through smooth muscle cells.

1.12. The Endocannabinoid System (ECS)

ECS is a natural physiological system that consists of cannabinoid CB_1 and CB_2 receptors, their endogenous ligands (the endocannabinoids) and the enzymes required for endocannabinoid biosynthesis and degradation (Di Marzo *et al.*, 2004). Endoannabinoids

are produced locally, on demand from membrane phospholipids, where they act as signalling molecules. They have a short half life as they are rapidly inactivated by enzymatic hydrolysis and reuptake by the cells (Wang and Ueda, 2009). The two well studied endocannabinoids include Anandamide or N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG). Both are derivatives of arachidonic acid. AEA was first isolated from porcine brain in 1992 (Devane *et al.*, 1992), while 2-AG was identified in 1995 by two groups (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). AEA can be synthesized from its precursor, N-arachidonoyl phosphatidylethanolamine, via multiple pathways, while synthesis of 2-AG, from arachidonic acid-containing inositol phospholipids, involves sequential hydrolysis by phospholipase C (PLC) and diacylglycerol lipase (DAGL). They are inactivated by enzymatic hydrolysis by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) respectively, among other enzymes (Wang and Ueda, 2009).

1.12.1. ECS and food intake

1.12.1.1. Role of AEA, 2-AG and CB_1 receptors

Both AEA and 2-AG stimulate appetite through central CB_1 receptors (Williams and Kirkham, 1999; Jamshidi and Taylor, 2001; Kirkham *et al.*, 2002). Hypothalamic CB_1 is known to co-localize with several neuropeptides involved in the regulation of feeding behaviour (Marsicano and Lutz, 1999). Knock out of CB_1 receptors leads to reduced food intake in animals following temporary food restriction. Also hypothalamic endocannabinoid levels are elevated in defective leptin signalling, while acute leptin treatment reduces these levels (Di Marzo *et al.*, 2001a). Leptin is one of the adipokines that acts centrally to regulate food intake and energy homeostasis (Ahima and Flier, 2000). CB_1 receptors in extrahypothalamic areas like the hippocampus, nucleus accumbens and entopendicular nucleus are down-regulated with intake of palatable food, suggesting an

increased activation of these receptors by endocannabinoids, in areas involved in hedonic aspects of eating (Harrold *et al.*, 2002). The effect of central CB_1 stimulation on appetite has been exploited clinically. Marinol®, that contains the synthetic analogue of THC (Dronabinol), is licenced by the FDA for use in anorexia associated with weight loss in patients with AIDS and nausea and vomiting associated with cancer chemotherapy in patients who have failed to respond adequately to conventional antiemetic treatments. Rimonabant, a selective CB_1 receptor antagonist/inverse agonist was effective at reducing body weight and waist circumference and improving glycaemic control and dyslipidaemia (Christopoulou and Kiortsis, 2011), but was withdrawn from the market due to concerns about its psychiatirc side effects (Le Foll *et al.*, 2009). CB_1 receptors at peripheral sensory nerve terminals and in the nodose ganglion, that contains cell bodies of neurons relaying sensory signals from gut to the hypothalamus, have also been implicated in modulating food intake (Kunos and Tam, 2011). Activation of CB_1 mRNA in peripheral adipose tissue causes lipogenesis, which is blocked by CB_1 selective antagonist SR 141716A (Cota *et al.*, 2003). Similarly AEA has been shown to cause diet induced obesity by stimulating fatty acid synthesis through hepatic CB_1 receptors, an effect that is sensitive to CB_1 blockade (Osei-Hyiaman *et al.*, 2005). Activation of ECS also promotes relaxation, pain reduction, and the extinction of aversive memories (Di Marzo *et al.*, 1998; Marsicano *et al.*, 2002b).

The two other compounds that are related to AEA are oleoylethanolamide (OEA) and palmitoylethanolamide (PEA).

1.12.1.2. Oleoylethanolamide (OEA)

OEA is a monounsaturated analogue of AEA (Bachur and Udenfriend, 1966; Schmid *et al.*, 1996), produced primarily in the small intestine, upon absorption of dietary fat and acts

through mechanisms independent of cannabinoid receptors (Gaetani *et al.*, 2003). In rats. OEA's synthesis in small intestine is markeldly reduced on food deprivation and administration of OEA produces an anorexic effect. The effect of OEA is prevented when peripheral sensory fibres are removed by capsaicin treatment, and is not seen when OEA is injected into the brain, suggesting that OEA is a lipid mediator involved in the peripheral regulation of feeding (Rodriguez de Fonseca *et al.*, 2001). OEA is a potent activator of PPARα, which seems to mediate the hypophagic actions of this compound (Fu *et al.*, 2003). Besides PPARα, OEA is known to target other receptors. These include TRPV1 (Ahern, 2003; Wang *et al.*, 2005) and GPR119 (Overton *et al.*, 2006a). GPR119 is a G-protein coupled receptor (GPCR), expressed predominantly in the human and rodent pancreas and gastrointestinal tract (Overton *et al.*, 2006a). OEA increases GLP-1 release from L- cells of the intestine through activation of GPR119 (Lauffer *et al.*, 2009a). Due to its hypophagic properties and its ability to stimulate the release of endogenous GLP-1, OEA may have a role in future in the treatment of type 2 diabetes.

1.12.1.3. Palmitoylethanolamide (PEA)

PEA is the other compound closely related to AEA and OEA, that activates PPARα and has important analgesic, anti-inflammatory, neuroprotective and antiallergic properties (Alhouayek and Muccioli, 2014).

1.13. ECS in obesity and type 2 diabetes

Recent evidence indicates a chronically elevated tone of the ECS in obesity. Post menopausal women with uncomplicated obesity show increased levels of systemic endocannabinoids with decreased CB_1 receptor and FAAH gene expression in adipose tissue (Engeli *et al.*, 2005). Circulating levels of 2-AG and not AEA, are markedly increased in subjects with visceral obesity (Bluher *et al.*, 2006). Up-regulation of

endocannabinoid signalling has been seen in adipocytes just before differentiation, in β cells kept under conditions of hyperglycaemia, in adipose tissue and pancreas of mice with diet induced obesity, and in visceral adipose tissue of obese human subjects with mild hyperglycaemia (Matias *et al.*, 2006). Studies in mice using standard and high fat diet (HFD) showed higher weight and glucose levels in HFD mice together with dysregulated expression of endocannabinoid metabolic enzymes in pancreatic β cells and subcutaneous fat (Starowicz *et al.*, 2008). Dysregulation of endocannabinoid levels, associated with hyperglycaemia and obesity, as a consequence of high fat diet has also been seen in peripheral tissues including adrenal glands, skeletal muscle, heart, kidney, thyroid and brown adipose tissue (Matias *et al.*, 2008a). Insulin exerts a negative control on plasma endocannabinoid levels, especially AEA, and this control is impaired in obese individuals with insulin resistance and non-obese individuals with fatty liver and dyslipidaemia, leading to post prandial endocannabinoid overactivity (Di Marzo *et al.*, 2009). Increased visceral adiposity is associated with increased CB_1 gene expression in perirenal adipose tissue and higher levels of AEA in the latter. Upregulation of CB_1 receptor expression is also associated with renal microvascular damage (Bordicchia *et al.*, 2010). The differential overactivation of the ECS in visceral than in subcutaneous adipose tissue may lead to accumulation of excessive amounts of fat in the former, thereby contributing towards the development of type 2 diabetes and atherosclerosis (Di Marzo, 2008).

1.14. Rimonabant

SR 141716A or rimonabant, first described in 1994, acts as CB_1 receptor antagonist (Rinaldicarmona *et al.*, 1994). In rodents it reduces caloric intake, body weight (Di Marzo *et al.*, 2001b) and body adiposity (Jbilo *et al.*, 2005). In human studies, rimonabant showed promising results by reducing body weight and waist circumference and also improved glycaemic control in type 2 diabetes and lipid profile in subjects with dyslipidaemia

(Gelfand and Cannon, 2006). Rimonabant was therefore the first in its class to be licensed as an antiobesity drug. However due to increased risk of psychiatric adverse events, European Medicines Agency withdrew marketing authorization for rimonabant in 2008, and this led the manufacturers to stop permanently all clinical research involving rimonabant (Le Foll *et al.*, 2009). In animals reduction in caloric intake induced by rimonabant is transient, although its effect on weight is more prolonged (Ravinet Trillou *et al.*, 2003). Also $CB1^{-/-}$ mice are resistant to diet induced obesity even though their caloric intake is similar to wild-type littermates (Ravinet Trillou *et al.*, 2004). These findings and the above data (Cota *et al.*, 2003; Osei-Hyiaman *et al.*, 2005) suggest the possible role of peripheral CB_1 receptors in energy metabolism and weight regulation.

1.15. Metabolic and Cardiovascular effects of cannabinoids

Cannabis (marijuana) is one of the most commonly used illicit drugs worldwide. There were an estimated 17 million users, only in the USA, in 2010 (Substance Abuse and Mental Health Services Administration, 2011). The main psychoactive component of cannabis is THC (Costa, 2007) , which activates central and peripheral CB_1 receptors (Pertwee, 2008a). Activation of these receptors increases food intake and causes peripheral lipogenesis (Cota *et al.*, 2003). Acute cannabis intake induces glucose intolerance in healthy volunteers (Podolsky *et al.*, 1971; Hollister and Reaven, 1974), while short term marijuana smoking increases appetite, food intake and weight in healthy men (Foltin *et al.*, 1988). Epidemiological data suggests that long-term use of cannabis is not associated with increased incidence of diabetes. Population based data shows lower incidence of diabetes in marijuana users (Rajavashisth *et al.*, 2012). Marijuana users had lower BMI than non-marijuana users, with lowest BMI in heavy users. The prevalence of diabetes was lower in marijuana users even when controlled for low BMI, suggesting a different underlying mechanism. According to the authors, lower incidence of diabetes might be related to the

anti-inflammatory properties of marijuana or the presence of other phytocannabinoids, like CBD and THCV that have a different pharmacological profile than THC (Rajavashisth *et al.*, 2012). Another cross-sectional, case-control study showed that chronic cannabis smoking was associated with visceral adiposity and adipose tissue insulin resistance but with normal hepatic insulin sensitivity, β-cell function and glucose tolerance. Also chronic cannabis smoking was not associated with hepatic steatosis or dyslipidaemia (Muniyappa *et al.*, 2013). Since chronic cannabis use is associated with downregulation of CB_1 receptors (Hirvonen *et al.*, 2012), the authors concluded that this may cause tolerance to the effects of THC and explain discrepant effects of acute and chronic exposure of THC on glucose metabolism. Another possibility is the effect of other phytocannabinoids present in cannabis like CBD, acting through non CB_1 receptors (Muniyappa *et al.*, 2013). Given the role of the endocannabinoid system in energy homeostasis, obesity and diabetes and the fate of CB_1 antagonist/inverse agonist rimonabant, the focus of research is on other phytocannabinoids. The two important members of this group are CBD and THCV. Preclinical studies with both (described below) show promising results and suggest that they have a different pharmacological profile from rimonabant.

1.15.1. CBD

CBD has multiple desirable effects on vasculature in the context of hyperglycaemia and ischemia (Rajesh *et al.*, 2007a; Durst *et al.*, 2007b) and animal studies have also shown its beneficial effects on dyslipidaemia (GW unpublished data). It attenuates high glucose induced inflammatory, oxidative and nitrosative processes and barrier disruption in human coronary artery endothelial cells (Rajesh *et al.*, 2007b). It has *in vivo* protective effect against myocardial ischemic reperfusion injury (Durst *et al.*, 2007b). It reduces infarct size and preserves left ventricular function. The reduction in infarct size is thought to be mediated by reduced inflammatory response to ischemica (Durst *et al.*, 2007a). Early

diabetic retinopathy presents with a breakdown of blood retinal barrier which seems to progress with the progression of diabetes (Engler *et al.*, 1991). Also there is evidence of neuronal cell death in diabetic retinae (Barber *et al.*, 1998; Asnaghi *et al.*, 2003; Martin, 2005) . CBD preserves endothelial integrity through its antioxidant and anti-inflammatory actions in experimental models of diabetes (El-Remessy *et al.*, 2006). In one recent study, the effects of CBD on myocardial dysfunction were investigated, using a mouse model of type 1 diabetic cardiomyopathy and primary human cardiomyocytes exposed to high glucose (Rajesh *et al.*, 2010). CBD attenuated myocardial dysfunction primarily through its anti-inflammatory activity and by reducing the oxidative/nitrosative stress associated with diabetes. To a lesser degree, CBD was able to reverse some of the biochemical and functional changes associated with established diabetic cardiomyopathy. CBD had a similar effect in primary human cardiomyocytes exposed to high glucose (Rajesh *et al.*, 2010). CBD attenuates lipopolysaccharide (LPS)-induced increases in vascular diameter and permeability of cerebral blood vessels through its immunomodulatory effect and reduced expression of iNOS (Ruiz-Valdepenas *et al.*, 2011).

In rodents, CBD modulates the cardiovascular response to stress. Resstel *et al.* (2009) studied the effects of systemic administration of CBD on the acute physiological and behavioural consequences of acute restraint stress in rats and showed that systemic administration of CBD attenuated acute cardiovascular and behavioural consequences of stress via the serotonin-1A ($5HT_{1A}$) receptor. The Bed Nucleus of Stria Terminalis (BNST) is a limbic structure that appears to differentially modulate neuroendocrine responses to acute stress (Choi *et al.*, 2007a). When injected into the BNST of rats, CBD enhanced the reflex bradycardiac response (Alves *et al.*, 2010). The authors concluded that BNST might be the possible site of action for CBD in the modulation of baroreflex and that further studies were needed to investigate the implication of baroreflex modulation by CBD with

regards to development and progression of cardiovascular disease (Alves *et al.*, 2010). A more recent study showed that CBD causes relaxation in isolated human mesenteric arteries through its interaction with CB_1 receptors (Stanley *et al.*, 2015). As discussed in detail in chapter 2, CBD has a favourable outcome in dyslipidaemia in rodents.

In summary CBD has shown promising results through its immunomodulatory and antioxidant properties. It acts as a vasorelaxant, attenuates cardiovascular responses to stress and improves dyslipidaemia. It has a very good safety profile in pre clinical and clinical studies. It is therefore important to explore its cardiovascular and metabolic effects, and the underlying mechanisms, by using appropriate *in vivo* and *in vitro* techniques.

1.15.2. THCV

In two animal models of obesity (diet-induced obese and ob/ob genetically altered mice), low doses of THCV (0.3 mg/kg) reduced body weight gain, increased energy expenditure and reduced fasting plasma insulin and leptin levels (compared to similar effects with rimonabant at a dose of 10 mg/kg), suggesting it could impact positively on a number of symptoms of metabolic risk factors (unpublished). As mentioned above, when used in a 1:1 combination with CBD in ob/ob mice, THCV produced similar positive metabolic effects. THCV also has anti-inflammatory properties. It reduces signs of inflammation and inflammatory pain in mice via CB_1 and/or CB_2 activation (Bolognini *et al.*, 2010b).

It appears therefore that CBD and THCV, alone and in combination, have a positive effect on obesity, dyslipidaemia and CVS. This project, therefore, seeks to investigate the metabolic and cardiovascular effects of these two phytocannabinoids, using various *in vivo* and *in vitro* techniques.

1.16. Hypothesis and Aim of PhD

In view of above we hypothesised that both CBD and THCV should positively modulate dyslipidaemia and hyperglycaemia in type 2 diabetes. Given the anti-inflammatory and immunomodulatory potential of CBD, it should have a positive impact on plasma levels of cytokines, adipokines and markers of vascular dysfunction, in experimental type 2 diabetes, while it should modulate stress response of cardiovascular system in humans, as it does in rodents. We proposed that both CBD and THCV should interact with adipocytes and endothelial cells, by stimulating various post receptor signalling pathways and affect the release of various adipokines and cytokines. The specific questions that we aimed to answer were;

Do CBD and THCV affect lipoprotein and glucose metabolism and cardiovascular parameters in type 2 diabetes?

Does CBD modulate cardiovascular response to stress in humans as it does in rodents?

Do CBD and THCV affect adipokine secretion and if they do, what are the underlying mechanisms?

What is the role of CBD and THCV in vascular endothelium ?

What is the impact of *in vivo* CBD on various cytokines, adipokines and markers of vascualr dysfunction in experimental diabetes?

To answer these questions, we carried out *in vivo* studies in subjects with type 2 diabetes, healthy volunteers and animal models of obesity and diabetes and *in vitro* studies involving mature human adipocytes and human aortic endothelial cells.

2. The effects of Cannabidiol (CBD) and delta 9 tetrahydrocannabivarin (THCV) on glycaemic and lipid parameters in patients with Type 2 diabetes: a randomised, double-blind, placebo-controlled, parallel group pilot study

2.1. Introduction

The ECS modulates food intake and energy homeostasis (Silvestri *et al.*, 2011; Horvath *et al.*, 2012) and chronic over-activation of the ECS has been identified in obesity and type 2 diabetes (Di Marzo, 2008). The ECS exerts some of its actions by activating cannabinoid receptors 1 (CB_1) and 2 (CB_2).

Rimonabant, through its modulation of CB_1 receptors, produces favourable metabolic outcomes, in obesity and type 2 diabetes (Christopoulou and Kiortsis, 2011), but its use is associated with an increased incidence of psychiatric adverse events (AEs) (Le Foll *et al.*, 2009). Rimonabant functions as a CB_1 receptor antagonist/inverse agonist, but it is unclear whether modulation of other cannabinoid receptor activity could have beneficial metabolic effects without significant psychotropic effects.

CBD is one of the major phytocannabinoids obtained from cannabis plants. In rodent studies, CBD has multiple desirable effects in the context of hyperglycaemia, mainly through its anti-inflammatory and anti-oxidant properties (El-Remessy *et al.*, 2006; Rajesh *et al.*, 2007a; Toth *et al.*, 2010; Rajesh *et al.*, 2010; Stanley *et al.*, 2013b). In animal models of obesity (*ob/ob* genetically obese mice), four weeks treatment with CBD, 3mg/kg, produced a 55% increase in HDL cholesterol concentration and reduced total cholesterol by more than 25%. In addition, the same dose reduced liver TG and increased both liver glycogen and adiponectin concentration (unpublished data). There is also evidence from animal studies showing that CBD modulates cardiovascular response to stress (Resstel *et al.*, 2009).

Unlike the related molecule THC, CBD does not activate CB_1 receptors in the brain and therefore lacks the psychotropic actions of THC. Indeed, CBD may mitigate the psychoses associated with cannabis misuse (Schubart *et al.*, 2011). Other receptor sites implicated in the actions of CBD include the orphan GPR55, CB_e, TRPV1 receptor, α1-adrenoceptors, μ opioid receptors and 5-HT_{1A} receptors (Pertwee, 2008a). CBD also activates and has physiological responses mediated by PPARγ (O'Sullivan *et al.*, 2009; Esposito *et al.*, 2011; De Filippis *et al.*, 2011). A CBD/THC combination (Sativex/Nabiximols) is currently licensed, in most EU countries and in New Zealand, Australia and Kuwait, for the symptomatic treatment of spasticity in moderate to severe multiple sclerosis and CBD alone (Epidiolex) was granted orphan drug designation, by the FDA, in Dravet and Lennox-Gastaut syndromes in children.

THCV is a naturally occurring analogue of THC, but with different pharmacological effects. It is suggested to behave as both a CB_1/CB_2 agonist and/or a CB_1/CB_2 neutral antagonist (Thomas *et al.*, 2005a; Pertwee *et al.*, 2007b; Bolognini *et al.*, 2010b; Hill *et al.*, 2010b; Batkai *et al.*, 2012). Other target sites of action include GPR55 (Anavi-Goffer *et al.*, 2012) and transient receptor potential channels (De Petrocellis *et al.*, 2011; De Petrocellis *et al.*, 2012).

Acute intraperitoneal administration of THCV in rodents at 3, 10 and 30 mg/kg body weight, caused hypophagia and weight loss, with food intake and body weight returning to normal on day 2. The effect was similar to that of a CB_1 antagonist AM251, also used in the same study (Riedel *et al.*, 2009). In another study, involving diet-induced obese (DIO) mice, oral THCV (2.5 to 12.5 mg/kg) reduced body fat content, increased energy expenditure, and reduced fasting insulin and 30 min insulin response to OGTT (Wargent *et al.*, 2013). In the same study, in genetically obese *(ob/ob)* mice, a similar increase in 24-hour energy expenditure was observed with 3mg/kg THCV, while 12.5 mg/kg THCV

caused a significant reduction in liver TG. THCV treatment improved insulin-induced phosphorylation of Akt in insulin resistant human hepatocytes and mice myotubes, suggesting improved insulin signalling as one of the mechanisms of action (Wargent *et al.*, 2013).

The findings from these preclinical studies demonstrate a potential beneficial effect of both CBD and THCV in diabetes and lipid metabolism, with very distinct pharmacological profiles, and therefore different side effects, to rimonabant. This prompted the first ever investigation of the effects of CBD and THCV on dyslipidaemia and glycaemic control in subjects with type 2 diabetes.

The aim of this study was to evaluate the effectiveness and safety of CBD and THCV, alone and in combination, in the treatment of dyslipidaemia and hyperglycaemia in type 2 diabetic subjects.

2.2. Methodology

2.2.1. Randomized clinical trial

We proposed to investigate our hypothesis by carrying out a randomised, double blind, placebo controlled, parallel group pilot study, using a 1:1 and 20:1 ratio of formulated CBD and THCV plus CBD and THCV alone in the treatment of dyslipidaemia and diabetes, in subjects with type 2 diabetes. The rationale to use combination treatment came from animal study, where there were positive metabolic effects of CBD and THCV when used together. In animal study, however, only a 1:1 ratio of CBD and THCV was employed. There was no real evidence behind 20:1 ratio that was used in our study. Dr Garry Tan was the principle investigator for this study, who was later replaced by Dr Fran Game, when he moved to take up a position at Oxford.

All the necessary approvals were sought. The study received a favourable opinion from the Regional Ethics Committee and from the Research & Development Department of Royal Derby Hospital NHS Trust. The study was conducted at four sites accross England. With Derby being the leading site, the other three sites included MAC Clinical Research (UK) Manchester, and two GP surgeries in Norfolk.

As a co-investigator based at Derby, I was involved in the study from the very early stages. I participated in the investigators' meeting in September 2010 and presented a review of the study protocol. I was based at Derby where I screened 36 subjects and randomized 22. My involvement in the study can be summarized as below;

- Subject recruitment
    1. Via educational sessions for newly diagnosed type 2 diabetics organized by Derby City Clinical Commissioning Group
    2. From direct contact with GP pracitces
    3. From hospital diabetes clinics
- Obtaining informed consent
- Performing all study related procedures during subjects' visits that included;
    1. General physical examination
    2. Recording ECGs
    3. Anthropometric measurements
    4. OGTT

5. Blood sampling, processing and shipment for analysis or storage at -80°C for future shipment or in house analysis
6. Cardiovascular monitoring during study visits using the finometer
7. Prescribing study medication

- I was responsible to maintain all study related documents including investigator site file (ISF), clinical record forms (CRF) and source documents for each subject.
- I also reviewed safety blood results as they came back with help from the principle investigator as needed.
- At the end of study, I analysed all the plasma and serum samples, from all study participants, for tertiary endpoints, that included;

1. Ketones
2. Orexin A
3. Retinol binding protein (RBP 4)
4. Adiponectin
5. Resistin
6. E- selectin
7. Vascular cell adhesion molecule (VCAM)
8. Von Willebrand factor (vWF)
9. C- reactive protein (CRP)
10. IL-6

11. TNFα

12. GIP

13. Ghrelin

14. GLP-1

All this work was performed under the supervision of Dr Saoirse O'Sullivan, in the clinical sciences laboratory, at University of Nottingham's Graduate Entry Medical School at Derby. At the end of study, I performed an inhouse statistical analysis of the data.

The duration of the study was 15-19 weeks for each subject (1-5 week baseline, 13 weeks treatment period and 1 week post treatment follow-up). After the screening visit (Visit 1), subjects entered a baseline period (-35 to -7 days until Visit 2). For some subjects, who were either not fasting on the day of visit 1 or required a wash out period of 21 days, if they were taking any of the prohibited medications, screening visit was split into 1A and 1B. After screening visit, eligible subjects were invited for randomisation (Visit 2, Day 1). Follow up visits took place at the end of week 4 of treatment (Visit 3, Day 29) and at the end of week 13 of treatment (Visit 5, Day 92). Between visits 3 and 5, subjects were contacted for a telephone assessment (Visit 4, Day 57) and they also had a post study telephone assessment (Visit 6, Day 99). Subjects were required to fast, for a minimum of 8 hours, before visits 1 (or 1B), 2 and 5. At visit 2 and 5, magnetic resonance imaging/spectroscopy (MRI/MRS) was performed to assess visceral adiposity and liver TG content, with rest of the visit procedures completed within 72 hours. Patients were also asked to record their daily diabetic and dyslipidaemic medication usage (where applicable) and their level of appetite, using a Numerical Rating Scale (NRS), from visit 1 until the end of treatment.

2.2.2. Study endpoints

2.2.2.1. Primary endpoint

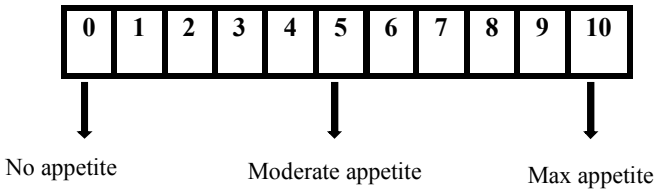
The primary endpoint was the change from baseline in serum HDL cholesterol concentration, after 13 weeks of treatment.

2.2.2.2. Secondary endpoints

Secondary endpoints included changes, from baseline to end of treatment, in following parameters;

Lipid control	Total cholesterol
	LDL cholesterol
	HDL/LDL
	VLDL
	TG
	Apo A
	Apo B
	Apo A/Apo B
	Lipid subfractions using ultracentrifugation
	NEFA
	Proportion of subjects shwoing response (≥10%

	rise in HDL)
Glycaemic control	Fasting glucose
	Glucose response to OGTT
	Fructosamine
	HbA1c
Insulin sensitivity	Fasting insulin
	C- peptide
	Insulin sensitivity using HOMA
	HOMA2 β cell function
	Insulin response to OGTT
Body weight and adipose tissue parmeters	BMI
	Waist circumference
	Hip circumference
	Waist to hip ratio
	Neck circumference
	Skin fold thickness

	Visceral adiposity determined by MRI
	Liver TG content on MRS
	Proportion of subjects showing response (≥ 5% loss in body weight)
Appetite regulation	Changes in appetite on 0-10 NRS 
Subjects global impression of change	
Clinician's global impression of change	

2.2.2.3. Tertiary endpoints

Tertiary endpoints included change from baseline in:

Markers of vascular function	V-CAM, E-Selectin, vWF, *in vivo* vascular assessment bu finometery
Markers of adipocyte function	Leptin, adiponectin and resistin
Markers of inflammation	CRP, TNFα and IL-6
Gut signalling hormones	GLP-1, GIP and ghrelin

Other parameters	Ketone bodies, RBP-4 and Orexin A
Endocannabinoids	AEA, 2-AG, PEA and OEA

Safety assessment was carried out as part of tertiary end points and included vital signs (pulse rate and blood pressure), physical examination, ECG recording, Beck's Depression Inventory II (BDI II) scoring, laboratory findings (full blood count, liver and renal function tests) and AE recording.

2.2.3. Study population

2.2.3.1. Inclusion criteria

Male and female subjects, aged 18 or above, who were willing and able to give informed consent, were eligible to participate in the study. The inclusion criteria included:

- Clinical diagnosis of type 2 diabetes with residual islet function. Islet function was not formally assessed, but it meant subjects did not require insulin to treat their diabetes
- On dietary control or had been receiving a stable dose of oral metformin or a sulfonylurea for at least 3 months prior to enrolment in the study.
- HDL- cholesterol ≤ 1.3mmol/L in females and ≤ 1.2mmol/L in males,
- $HbA_1c \leq 10\%$
- TG ≤ 10mmol/L.

2.2.3.2. Exclusion criteria

The main exclusion criteria included:

- Use of any of the prohibited therapy insluding insulin, fibrates, thiazolidinediones, therapeutic omega-3 fatty acids or alpha-glucosidase inhibitors, nicotinic acid, bile acid sequestrants (e.g. cholestyramine, colesevalam, colestipol) and cholesterol absorption inhibitors (e.g. ezetimibe).
- Current or previous use of recreational or medicinal cannabis or synthetic cannabinoids within 30 days prior to study and unwillingness to abstain.
- History of significant depression or BDI II questionnaire score ≥ 15
- Any underlying genetic dyslipidaemic condition
- Use of a statin but not on a stable dose for at least 4 weeks prior to randomisation
- Pregnancy, lactation or planned pregnancy during the course of the study, or for 3 months after the last dose of study medication
- Female subjects of child bearing age and male subjects whose partners were of child bearing potential, unless they were willing to use appropriate barrier method of contraception
- Any significant cardiac, renal or hepatic impairment.
- Postural blood pressure drop of ≥ 20mmHg
- Planned travel outside the country during the course of the study

Since rimonabant, a CB_1 antagonist/inverse agonist, was withdrawn from market due to psychiatirc adverse events, significant depression or BDI II score of ≥15 was considered a contraindication to participate in study, mainly because of lack of sufficient human data for THCV. Similarly THC is known to cause orthostatic hypotension. Since THCV is an

analogue of THC, it was decided to exclude subjects from the study who had significant postural hypotension.

2.2.4. Study medication and treatment arms

Study medication was provided as hard gelatine capsules containing Cremophor EL and Labrafil M1944CS excipients, plus or minus the active treatments defined below. Patients were required to swallow the capsules twice daily, 30 min before breakfast and 30 min before their evening meal, typically 12 hours apart.

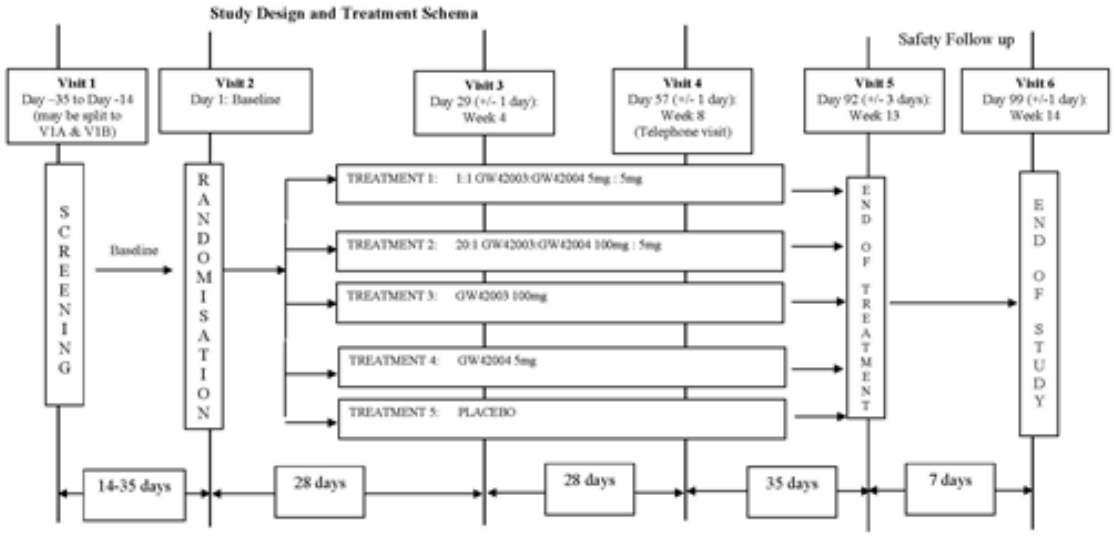
There were 5 treatment arms;

- 5mg CBD/ 5mg THCV twice daily (1:1)
- 100mg CBD/ 5mg THCV twice daily (20:1)
- 100mg CBD twice daily
- 5mg THCV twice daily
- Placebo twice daily (contained excipients only)

Each treatment group was supposed to consist of 10 subjects and therefore the recruitment target was fifty. Sixty two subjects were recruited in total. Th3e study was carried out at 4 sites in the UK, as mentioned above.

2.2.5. Study procedures

Below is a schematic presentation of the study design



Subjects, who were willing to participate, were sent detailed study information. Those who decided to participate were invited for a screening visit or visit 1.

2.2.5.1. Visit 1

Subjects were considered enrolled in the study once they had provided written informed consent (signed and dated). During visit 1, subjects' demographic details and details of relevant ongoing and past medical and surgical problems, including drug treatment, were recorded. A note was made of previous use of cannabis or otherwise. Subjects' pulse rate and blood pressure were recorded once they had been seated for at least 10 minutes and this was followed by complete physical examination. Body weight and height were also recorded. A note was also made of the arm that was used for measuring blood pressure for the sake of consistency. For some subjects, who were either not fasting on the day of visit 1 or required a wash out period of 21 days, if they were taking any of the prohibited medications, screening visit was split into 1A and 1B. During visit 1B subjects were assessed for any adverse events or changes to their concomitant medication since their last visit. Their pulse rate, blood pressure and ECG were recorded and an assessment of postural blood pressure was also made. Subjects were also required to complete BDI II questionnaire. Blood samples were taken for eligibility (HDL cholesterol, HbA_1c and TG)

and safety assessment (full blood count, renal and liver function tests and if applicable, serum pregnancy test). Urine was tested for evidence of infection, blood and protein and a sample was sent for further lab analysis if required. A separate urine sample was taken to screen for drugs of abuse including THC. Subjects were issued with a diary and trained to record, on daily basis, their diabetes and dyslipidaemia treatment and level of appetite using NRS.

2.2.5.2. Visit 2

Subjects, who were deemed eligible following clinical and lab assessments, were invited for visit 2 that took place a minimum of 7 and a maximum of 35 days after visit 1. Subjects were required to fast overnight (minimum 8 hours) for visit 2 assessments and for magnetic resonance imaging of their body adiposity and liver TG content (MRI/MRS), that took place either on the day of visit 2 or with in 72 hours prior to visit 2. During visit 2 subjects were assessed for any adverse events, their vital signs were recorded and concomitant medication were reviewed. Blood samples were sent for assessment of full blood count, renal and liver function tests and pregnancy test (if appropriate) and urine was tested with a dip stick. Body weight and waist, hip and neck circumference were recorded. Skin fold thickness was measured in following seven areas (three readings for each area); axilla, pectoral region, triceps, scapular, abdominal, inguinal and quadriceps area. Height recorded at visit 1 was used for calculating BMI. Subjects were asked to complete food frequency and physical activity questionnaires. In addition to blood samples for safety assessment, fasting blood samples were also taken to analyse primary, secondary and tertiary endpoints followed by an OGTT. *In vivo* assessment of the vascular function was made by finometry. Finometer (shown below) uses a finger-clamp method to detect beat-to-beat changes in digital arterial diameter using an infrared photoplethysmograph (Schutte *et al.*, 2004). This gives a continuous signal of beat-to-beat changes in blood pressure and

blood flow and uses this signal to derive other parameters, including systolic, diastolic and mean blood pressure, interbeat interval, heart rate and left ventricular ejection time, stroke volume, cardiac output, and systemic peripheral resistance.



Finometer PRO
(Image shown courtesy of SMART medical UK)

After completion of all the assessments, subjects were issued investigational medicinal product (IMP) by the study pharmacist, according to the randomisation schedule and enough to last for 4 weeks. Subjects were instructed on how to take IMP and were issued with new study diary to be completed throughout the study.

2.2.5.3. Visit 3

Visit 3 took place at the end of week 4 of treatment. During this visit subject's eligibility to continue in the study was reviewed. Safety assessments were made by enquiring about adverse events, recording vital signs and sending blood samples and urine samples (where appropriate) for laboratory assessments. Body weight parameters including weight, waist, hip and neck circumference and skin fold thickness were measured. Subjects were asked to complete food frequency and physical activity questionnaires. Compliance with the study

medication was also assessed and subjects were dispensed new treatment pack that would last for the remaining study period.

2.2.5.4. Visit 4

At the end of week 8, patients were contacted via telephone to assess their safety and compliance with study medication.

2.2.5.5. Visit 5

At the end of treatment period, subjects were asked to attend after an overnight fast. Subjects underwent body adiposity assessment (MRI/MRS Scan) prior to this visit. Subjects were assessed for their safety and compliance with the study medication. Remaining IMP was retrieved and all the clinical and laboratory assessments that were made at randomisation visit were repeated. In addition subjects were asked to complete BDI II and SGIC questionnaires. An ECG was also recorded. Investigator was also required to make their assessment of global change in patients overall condition, called CGIC.

2.2.5.6. Visit 6

One week after visit 5, subjects were contacted via telephone for post study safety assessment.

2.2.6. Data and sample collection, and experimental techniques

2.2.6.1. Lipid samples for serum analysis

Blood was collected into a 2.5mL gel serum separator tube (SST) and gently inverted 8 times. It was left for 30 min at room temperature to clot. Samples were centrifuged for 10 min at 1500g at room temperature to separate serum. Serum was transferred into a transport tube and shipped at ambient temperature to ACM Global central laboratories

(ACM York, UK). Samples were analysed for HDL, LDL and total cholesterol and TG, using the Roche modular system, using enzymatic colorimetric assays (Roche Diagnostics Ltd, UK) at ACM. NEFAs were quantified on the Roche COBAS 311 system using an acyl-CoA synthetase/acyl-CoA oxidase (ACS-ACOD) method (Roche Diagnostics Ltd, UK), and kits were supplied by Wako Chemicals GmbH (Neuss, Germany). Cholesterol levels were determined enzymatically using cholesterol esterase and cholesterol oxidase. Hydrogen peroxide created a red colour by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase. The colour intensity was directly proportional to the concentration of cholesterol, which was determined photometrically. The enzymatic *in vitro* test for the quantitative determination of triglyceride levels used lipoprotein lipase for the rapid and complete hydrolysis of triglycerides to glycerol followed by oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacted with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase, which produced a red colour. The colour intensity was directly proportional to the concentration of triglycerides and was determined photometrically.

2.2.6.2. Apoliopoprotein markers

Blood was collected into 4mL gel SSTs, gently inverted 8 times and left for 30 min at room temperature to clot. Samples were centrifuged for 10 min at 1500g at room temperature to separate serum. Serum was transferred into a transport tube, stored at -80°C, and later shipped frozen to ACM. Samples were analysed for Apo A and Apo B on the Roche COBAS 311 system (Roche Diagnostics Ltd, UK), using immunoturbidimetric assays based on the principle of immunological agglutination.

2.2.6.3. Lipid samples for ultracentrifugation analysis

VLDL levels were determined by ultracentrifugation. Blood was collected into 6mL K3-EDTA tubes and gently inverted 8 times. Samples were centrifuged for 10 min at 1500g at room temperature to obtain plasma. Plasma was removed to a plain tube, shipped at ambient temperature to ACM, and was processed at Glasgow Western Infirmary (UK). Using the beta-quantification method, a single ultracentrifugation step achieved partial physical separation of lipoprotein classes into VLDL (d<1.006 kg/L Top) and non-VLDL (d>1.006 kg/L Bottom) fractions. The VLDL fraction was calculated by subtraction: total cholesterol minus the Bottom fraction. As a check, measured VLDL had to agree with the calculated VLDL to +/- 0.50mmol/L, or the ultracentrifugation was repeated.

2.2.6.4. Glucose samples

Blood was collected into 2mL Fluoride Oxalate tubes and gently inverted 8 times. Samples were centrifuged for 10 min at 1500g at room temperature to obtain plasma. Plasma was removed to a plain tube and shipped frozen to ACM, where it was analysed using the Roche modular system (Roche Diagnostics Ltd, UK).

2.2.6.5. Insulin samples

Blood was collected into 2.5mL gel SSTs and gently inverted 8 times and left for 30 min at room temperature to clot. Samples were centrifuged for 10 min at 1500g at room temperature to separate serum. Serum was transferred into a transport tube and shipped frozen to ACM, where it was analysed using an Advia Centaur immunoassay analyser. The method was a two-site sandwich assay using direct chemiluminometric technology and labelled anti-insulin antibodies.

2.2.6.6. OGTT

A standard OGTT was performed, measuring the change in plasma glucose levels at 120 min (2 hours) and 180 min (3 hours) from values obtained at 0 min (immediately prior to intake of a drink containing 75g of glucose). If the 0 min value was missing, the -15 min value was used. Blood sample collection and analysis was performed as described above (glucose samples).

2.2.6.7. HOMA2 calculations

HOMA-IR, insulin sensitivity and B-cell function were calculated using the HOMA2 Calculator v2.2© (Diabetes Trials Unit, University of Oxford) by ACM, using the following formula:

HOMA2-IR = [Glucose (mmol/L) x Insulin (pmol/L)] / 156.2.

HOMA2 B cell function = [20 x Insulin (mU/L) / Glucose (mmol/L) - 3.5]

2.2.6.8. Body weight and adipose parameters

Body weight and adipose measurements were carried out by investigators or trained research nurses at individual study centres. MRI scanning took place at three centres (Wolfston Imaging Centre, Manchester; Nuffield, Derby and Addenbrookes, Cambridge), and all collated data was analysed by a single blinded MRI specialist, using SliceOmatic and JMRUI software

2.2.6.9. SGIC and CGIC

At Visit 5 (end of 13 weeks of treatment), patients were asked the following question: "Please assess the change in your diabetes since receiving the first dose of IMP using the 7-point scale below". At Visit 5, clinicians were asked the following question: "Please assess the change in the patient's metabolic function since receiving the first dose of IMP

using the 7-point scale below." The success of treatment was assessed using an ordinal 7-point Likert scale: 1 = very much improved, 2 = much improved, 3 = minimally improved, 4 = no change, 5 = minimally worse, 6 = much worse, 7 = very much worse.

2.2.6.10. Appetite 0-10 NRS

Subjects scored their appetite in a daily diary using an appetite 0-10 NRS, where 0 = no appetite and 10 = maximum appetite. The change from mean baseline score (mean of seven days prior to randomisation) was compared to the mean score from the last seven days on treatment (end of 13 weeks).

2.2.6.11. BDI-II

The BDI-II assessment, performed at study Visits 1 and 5, is a 21 question, multiple choice, self-reported inventory, and is one of the most widely used instruments for measuring the severity of depression. The 21 questions or items each has four possible responses. Each response is assigned a score ranging from zero to three, indicating the severity of the symptom. A higher score is indicative of a worse clinical outcome.

2.2.6.12. Tertiary endpoints

Plasma endocannabinoids were analysed using mass spectrometry. Ketones, orexin A and RBP 4 were analysed using immunoassay, while all other tertiary endpoints including adiponectin, resistin, leptin, E-Selectin, VCAM, vWF, CRP, IL-6, TNFα, GIP, ghrelin and GLP-1 were analysed by multiplex analysis, using commercially available kits (Milliplex™, HMHMAG-34K, HCVD1-67AK, HADK-1-61K-A, HCVD2-67BK, BPHCVD05-6, Merck Millipore®).

Plasma and serum samples for primary and secondary end points were analysed at ACM Global central laboratories, York UK, except for lipid ultracentrifugation that was performed at the Glasgow Western Infirmary. Samples for endocannabinoids were

analysed at University of Nottingham Medical School, while all the tertiray end points were analysed by me under the supervision of Dr Saoirse O'Sullivan at School of Graduate Entry Medicine & Health, University of Nottingham Derby.

2.2.7. Data analysis

Subjects were randomly allocated to treatment groups in a 1:1:1:1:1 ratio, stratified by centre. The final analysis was based on intention to treat (ITT) and therefore included data from all those who were randomised into the study, irrespective of whether they were able to complete it or not. All statistical tests were two-sided at 5% significance level with 95% confidence interval. The primary endpoint and the majority of secondary endpoints were analysed using analysis of covariance (ANCOVA) of the changes from baseline to the end of treatment in the associated parameter, with the exception of the SGIC and CGIC, which used a pairwise Fisher's Exact test. The parameter's baseline values were included as a covariate, and treatment was included as a factor. The null hypothesis was one of no difference in the effects of any of the four active treatments compared individually with placebo. If the p-value for the comparison of any one of the treatment groups was less than 0.05 then the difference was deemed statistically significant and the alternative hypothesis, of a difference between at least one of the active treatments and placebo, was accepted. Changes from baseline in resistin and GIP levels, were analysed post hoc using a paired t-test, while 3 hour glucose response to OGTT was analysed using 2 way ANOVA.

2.2.8. Sample size

As this study was a proof of concept Phase II pilot study, no formal sample size calculation was performed

2.3. Results

In total, 125 patients screened and 62 were randomised and analysed at four study centres in the UK. Of these, 11 received a 1:1 ratio (5mg: 5mg) combination of CBD and THCV, 12 received a 20:1 ratio (100mg: 5mg) combination of CBD and THCV, 13 received CBD alone (100mg), 12 received THCV alone (5mg), and 14 received placebo. Majority of screen failures did not meet inclusion or exclusion criteria. HDL cholesterol levels were outside the required range in most cases. A total of 56 patients completed the study and six withdrew. Four (6.5%) patients withdrew due to an AE (subject 1: bilateral painful swollen knees, subject 2: worsening glycaemic control and dyslipidaemia, subject 3: light-headedness and fatigue and subject 4: reduced appetite, diarrhoea and lethargy), one (1.6%) met the withdrawal criteria and one (1.6%) was withdrawn by the investigator.

Patient demographics are presented in Table 2.1 (Appendix 1). The mean age of patients in the study was 59 years with little variation between treatment groups. The majority of patients (95%) were White/Caucasian, and the majority (92%) had not previously used cannabis. The distribution of males and females varied a little between treatment groups, with a greater proportion of male (68%) to female (32%) patients overall in this study. The remaining demographic profiles of the patients were similar between treatment groups. The mean duration of Type 2 diabetes was 4.2 years, with a minimum mean of 2.8 years in the CBD alone treatment group and a maximum mean of 5.1 years in the 20:1 CBD:THCV treatment group. The mean duration since most recent episode of dyslipidaemia, which referred to documented dyslipidaemia on a blood test, was 2.9 years overall with a minimum mean of 1.7 years in the 20:1 CBD:THCV treatment group and a maximum mean of 4.0 years in the THCV alone treatment group. There did not appear to be any correlation between the duration since most recent episode of dyslipidaemia and the duration of Type 2 diabetes.

2.3.1. Concomitant medication

A summary of lipid-lowering and anti-diabetic medications taken while on randomised treatment is presented in Table 2.2 (Appendix 1), with a similar proportion of patients in each group taking each therapeutic class of treatment. The most common anti-diabetic treatment taken was metformin, taken by 51 (82%) of patients. Simvastatin was the most common lipid-lowering agent used, taken by 34 (55%) of patients.

2.3.2. Primary endpoint

2.3.2.1. Serum HDL Cholesterol

There were no significant effects of any active treatment on HDL cholesterol levels at the concentrations investigated, with similar baseline and end of treatment values in all treatment groups, presented in Table 2.3 (Appendix 1). This finding was supported by the per-protocol (PP) analysis set, which also failed to show a significant effect of any active treatment on HDL cholesterol levels (Table 2.3).

2.3.3. Secondary endpoints

2.3.3.1. Lipids

There was a small increase in serum Apo A levels from baseline to the end of treatment in the THCV alone treatment group, that was not apparent in any other treatment group. In the placebo group, Apo A levels decreased from baseline, giving a treatment difference that was in favour of THCV (ETD = 6.02μmol/L, $p<0.05$; Figure 2.2). In line with this, compared to placebo, there was a trend towards improvement in Apo B: Apo A, in the THCV alone group, but it did not reach statistical significance ($p=0.075$; Table 2.3). CBD on its own or in combination with THCV had no effect on Apo A levels (Figure 2.2).

2.3.3.2. Glycaemic Control

Compared with baseline rather than placebo, there was no significant difference in glucose response to OGTT at 2 hours, but 3 hours glucose level was significantly lower after 13 weeks treatment with THCV (Figure 2.3A). There was no significant effect on insulin response to OGTT (Figure 2.3B). In the placebo treatment group, fasting glucose levels increased from baseline, while in the THCV alone treatment group, there was a decline in fasting glucose levels. This gave a treatment difference that was in favour of THCV (ETD = -1.24mmol/L, $p<0.05$; Figure 2.3C). In keeping with this, a rise in β cell function was noted in THCV alone treatment group, while a decline in β cell function was seen with placebo. This was again in favour of THCV (ETD = 44.68 points, $p<0.01$; Figure 2.3D).

No other glucose control or insulin sensitivity parameters were significantly affected by any of the treatments (Figures 2.4, 2.5, 2.6).

2.3.3.3. Vascular Function

Neither THCV nor CBD, on their own or in combination, significantly affected any cardiovascular parameter or plasma markers of vascular function (Table 2.3, Appendix 1).

2.3.3.4. Adipokines

There was an increase from baseline in adiponectin concentration in the THCV group and a reduction in placebo group; the treatment difference was statistically significant in favour of THCV treatment (ETD = -5.9 x 10^6pg/mL, $p<0.01$, Figure 2.7). Plasma concentrations of leptin and resistin remained unchanged with THCV treatment (Table 2.3, Appendix 1). CBD on its own and in combination with THCV did not significantly affect plasma concentrations of adiponectin (Figure 2.7). Compared with baseline rather than placebo, CBD caused a significant reduction in the concentration of resistin (-898 pg/mL, $p<0.05$, Figure 2.8A), but had no effect on leptin or adiponectin (Table 2.3, Appendix 1). The same

effect on plasma concentrations of resistin was not seen with THCV (Figure 2.8 B) or a combination of CBD with THCV (Figure 2.8 C, D).

2.3.3.5. Markers of Inflammation

Both THCV and CBD had no significant effect on plasma markers of inflammation (CRP, TNF α and IL-6; (Table 2.3, Appendix 1), on their own and in combination.

2.3.3.6. Gut Hormones

Compared with baseline rather than placebo, CBD caused a significant increase in the concentration of GIP (21.2pg/mL, $p<0.05$, Figure 2.9 A), without any effect on GLP-1 or ghrelin concentrations (Table 2.3, Appendix 1). THCV on its own or in combination with CBD had no significant impact on the levels of GIP (Figure 2.9 B, C, D), GLP-1 and Ghrelin (Table 2.3).

2.3.3.7. Body weight

There were no statistically significant changes in anthropometric parameters including weight, waist circumference, waist to hip ratio and skin fold thickness in any of the treatment groups (Table 2.3, Appendix 1).

2.3.3.8. Visceral adiposity and liver triglycerides

There were no changes in visceral adiposity (Table 2.3, Appendix 1) or liver TG (Table 2.3, Appendix 1) as assessed by MRI/MRS in any of the treatment groups.

2.3.3.9. Appetite 0-10 NRS scores

None of the treatments affected appetite as assessed by 0-10 NRS scores (Table 2.3, Appendix 1).

2.3.3.10. SGIC and CGIC

A full summary of the SGIC and CGIC assessment responses is presented in Figure 2.10. Analysis of these responses showed a treatment difference in favour of all the active treatments, to varying degrees, but most notably between the 1:1 CBD:THCV and placebo treatment groups on CGIC (Figure 2.10 B). There were reported improvements in seven out of 11 (63.6%) patients in the CGIC on 1:1 CBD:THCV treatment, compared to only two of the 14 (14.3%) placebo patients with a recorded improvement on CGIG. This translated to a statistically significant treatment effect of 1:1 CBD:THCV treatment compared to placebo, with an odds ratio of 9.43 ($p<0.05$) in the CGIC. No other statistically significant effects were calculated for any other active treatment in either assessment.

2.3.4. Post hoc analysis in THCV and CBD groups

An improvement in 3 hours glucose response to OGTT was noted in the THCV group, when subjects on any form of diabetes treatment other than diet/metformin were excluded from analysis ($p<0.01$, Figure 2.11 A). In the same subgroup, there was a significant deterioration in HbA1c, in the placebo group and minimal improvement in THCV group. The difference was statistically significant in favour of THCV ($p<0.05$, Figure 2.12A). CBD showed no improvement in glucose response to OGGT (Figure 2.11B, $p>0.05$) or HbA1c (Figure 2.12B, $p>0.05$), in this subgroup of patients.

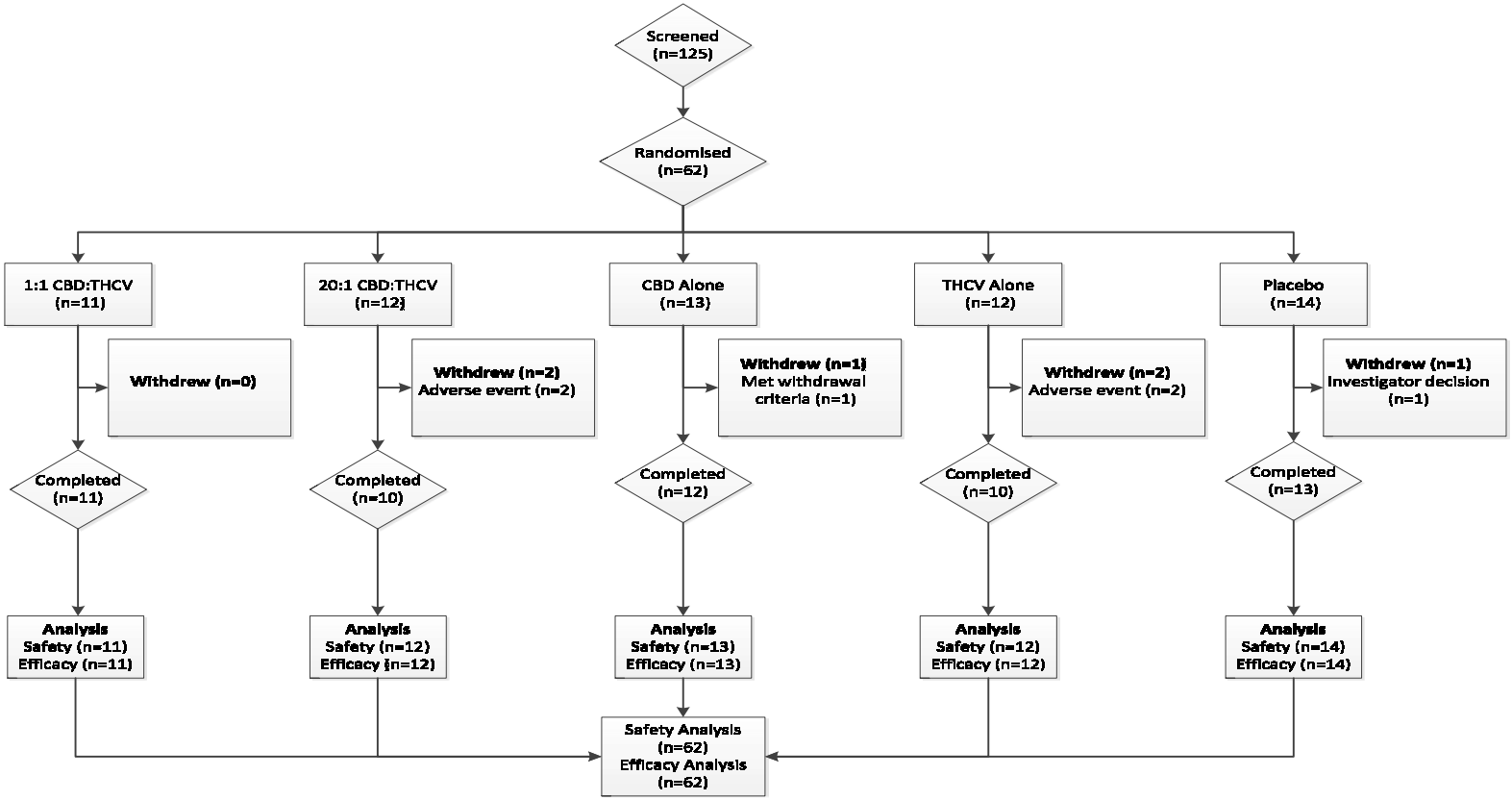


Figure 2.1. Summary of breakdown of patients enrolled in the study

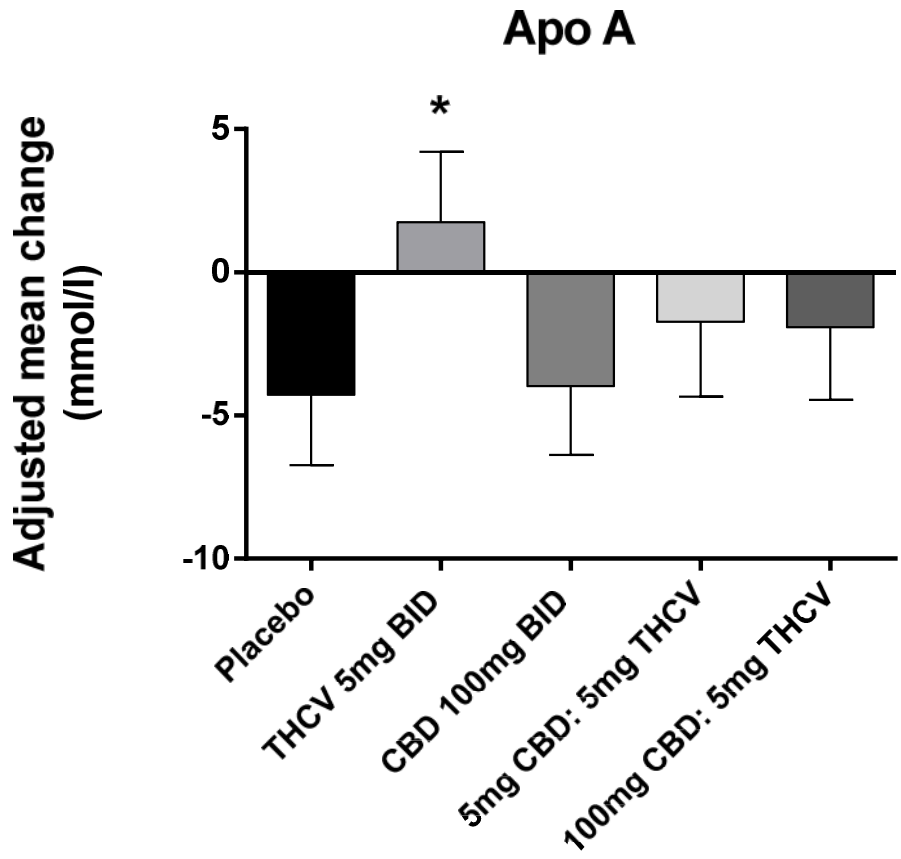


Figure 2.2. Compared with placebo (n=14), THCV (n=12) caused a significant improvement in the concentration of Apo A (p<0.05). CBD (n=13) on its own or in a 1:1 (n=9) or 20:1 (n=11) ratio combination with THCV had no impact on Apo A levels. Data was analysed by analysis of covariance and presented as mean ± SEM.

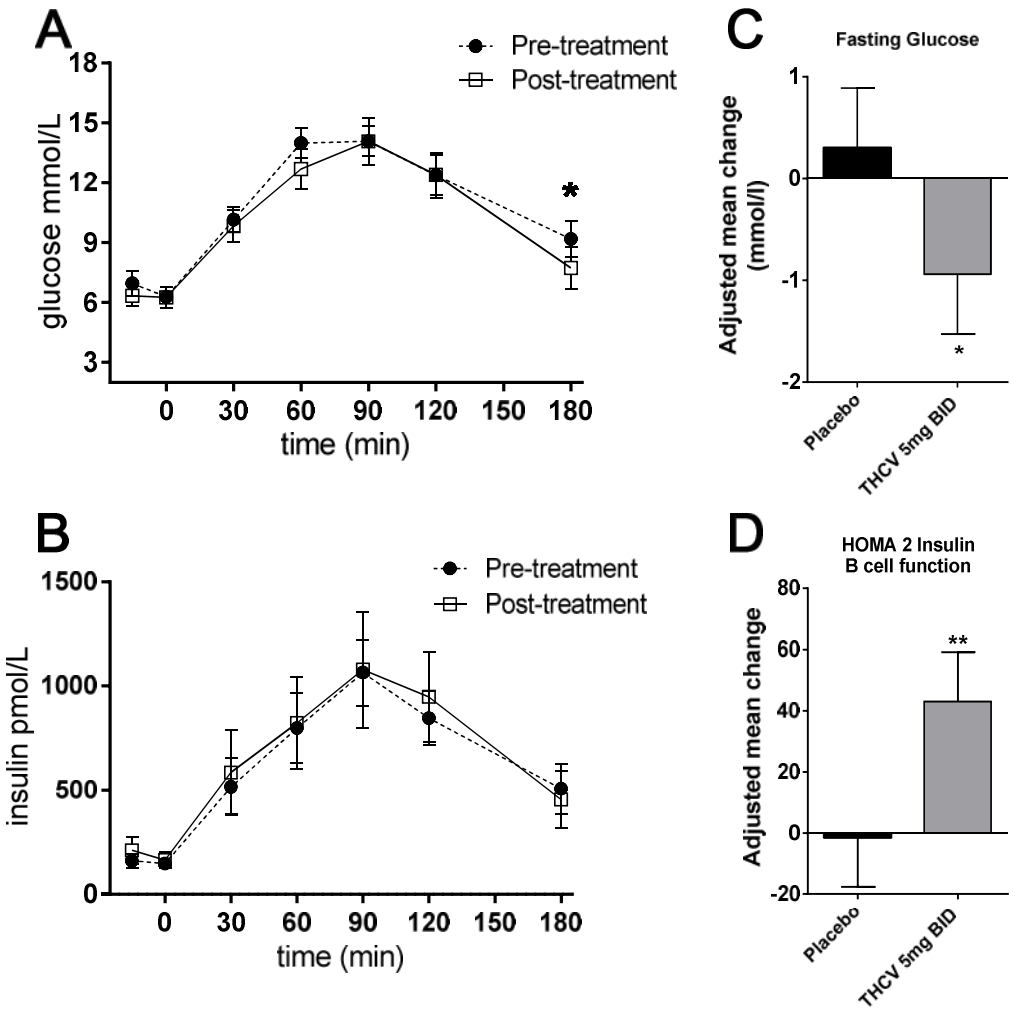


Figure 2.3. When compared to pre-treatment values, THCV caused significant improvement in 3 hour glucose response during OGTT ($p<0.05$; A, n=12). No significant change was observed in insulin response (B). Compared with placebo (n=14), THCV caused significant improvement in fasting glucose ($p<0.05$; C, n=12)) and in keeping with this there was a highly significant improvement in β cell function measured by HOMA2 ($p<0.01$; D). Data in figures 2.3A and 2.3B were analysed using repeated measures 2 way ANOVA and presented as mean ± SEM. Data presented in figures 2.3C and 2.3D were analysed using analysis of covariance and presented as mean ± SEM.

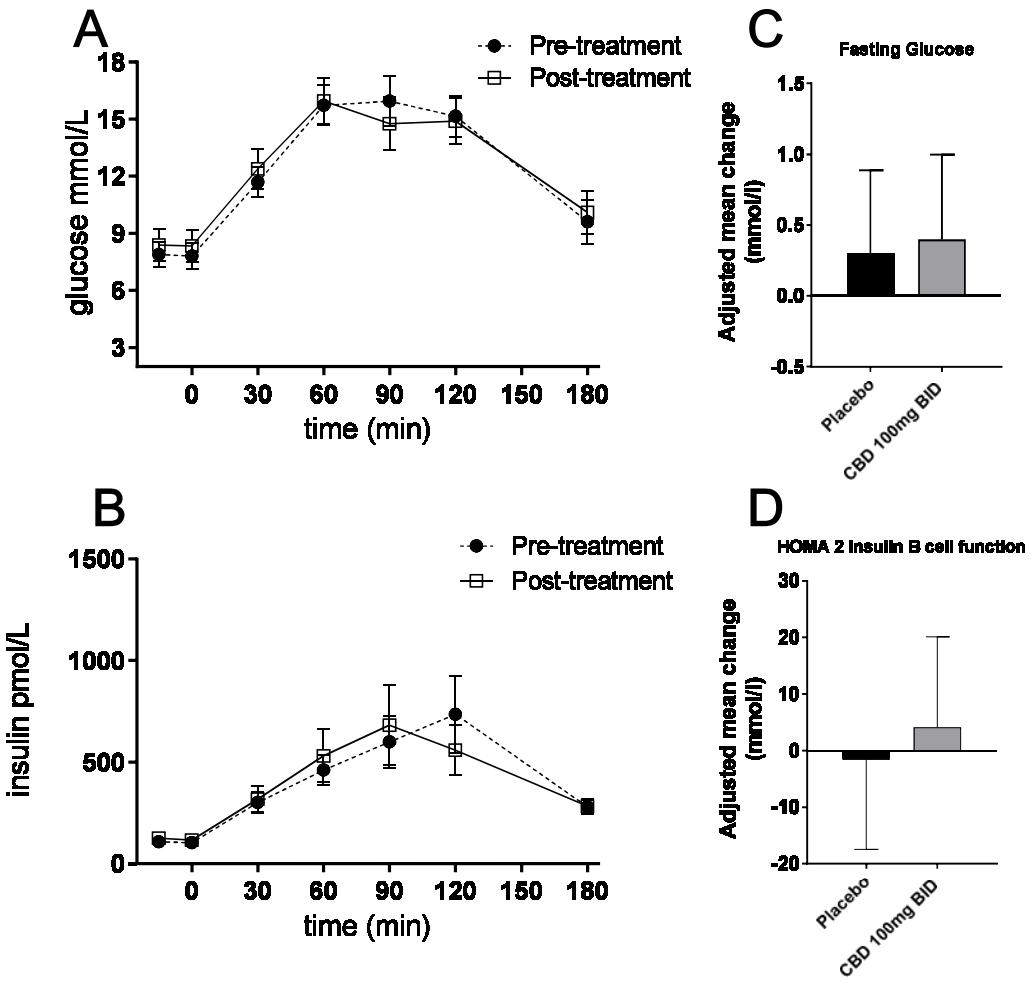


Figure 2.4. CBD (n=13) had no effect on glucose (A) and insulin (B) response to OGTT, when pre and post treatment levels were compared. Compared with placebo (n=14), CBD made no difference to fasting glucose (C) and HOMA 2 β cell function (D). Data in figures 2.4A and 2.4B were analysed using repeated measures 2 way ANOVA and presented as mean ± SEM. Data presented in figures 2.4C and 2.4D were analysed using analysis of covariance and presented as mean ± SEM.

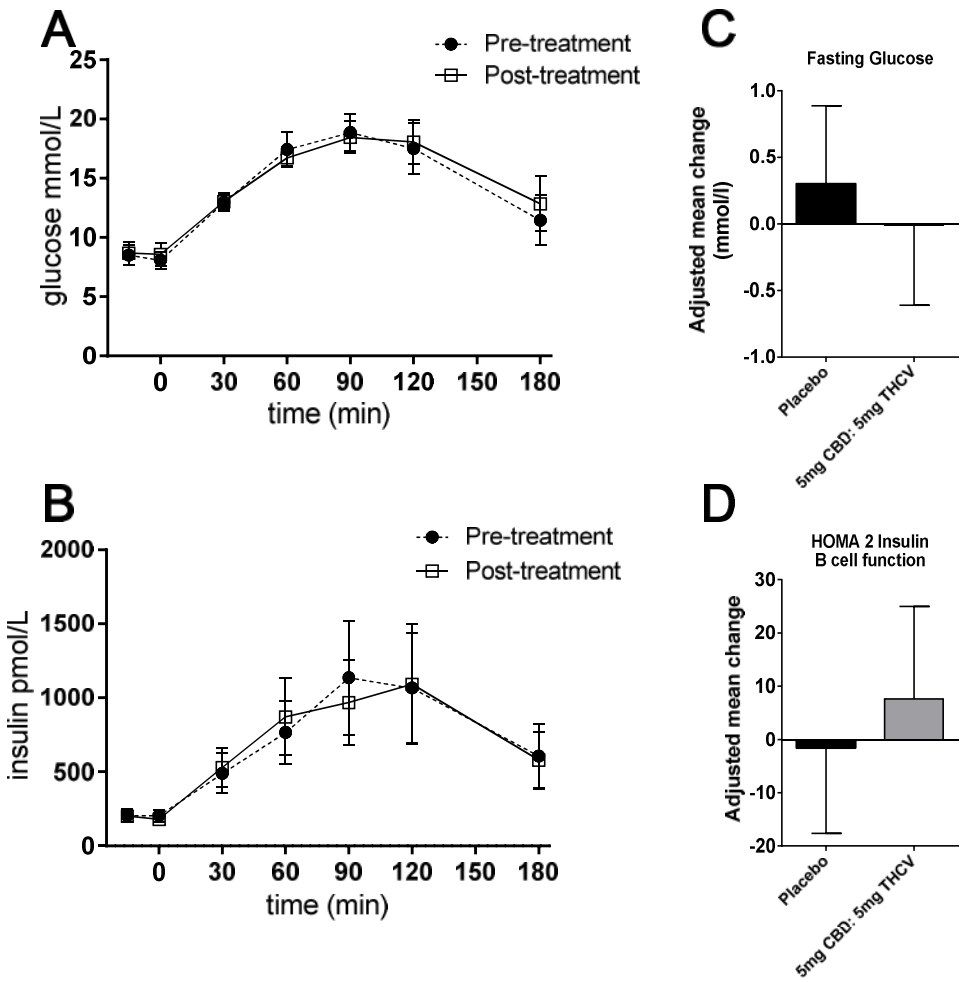


Figure 2.5. 1:1 combination of CBD and THCV (n=9) had no effect on glucose (A) and insulin (B) response to OGTT, when pre and post treatment levels were compared. Compared with placebo (n=14), there was no significant change in fasting glucose (C) and HOMA 2 β cell function (D). Data in figures 2.5A and 2.5B were analysed using repeated measures 2 way ANOVA and presented as mean ± SEM. Data presented in figures 2.5C and 2.5D were analysed using analysis of covariance and presented as mean ± SEM.

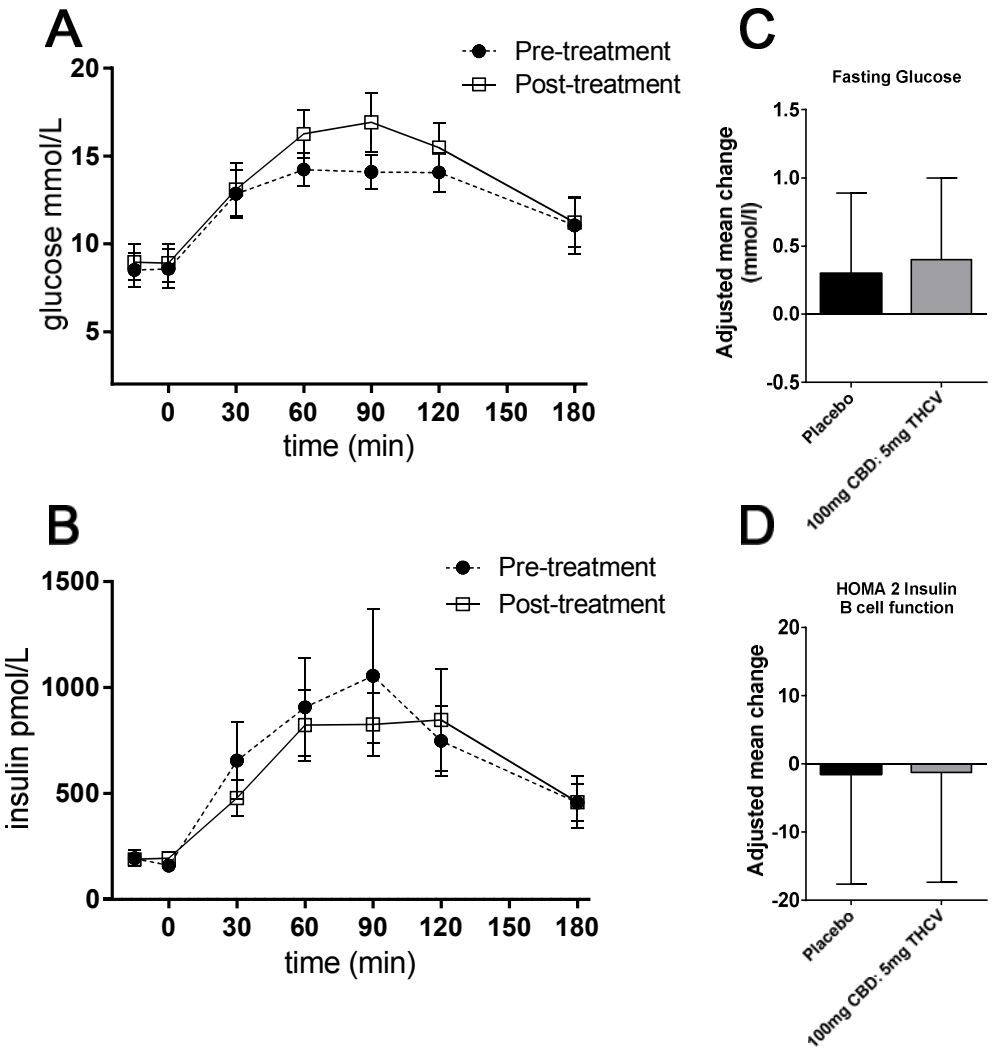


Figure 2.6. 20:1 combination of CBD and THCV (n=11) had no effect on glucose (A) and insulin (B) response to OGTT, when pre and post treatment levels were compared. Compared with placebo, there was no significant change in fasting glucose (C) and HOMA 2 β cell function (D). Data in figures 2.6A and 2.6B were analysed using repeated measures 2 way ANOVA and presented as mean ± SEM. Data presented in figures 2.6C and 2.6D were analysed using analysis of covariance and presented as mean ± SEM.

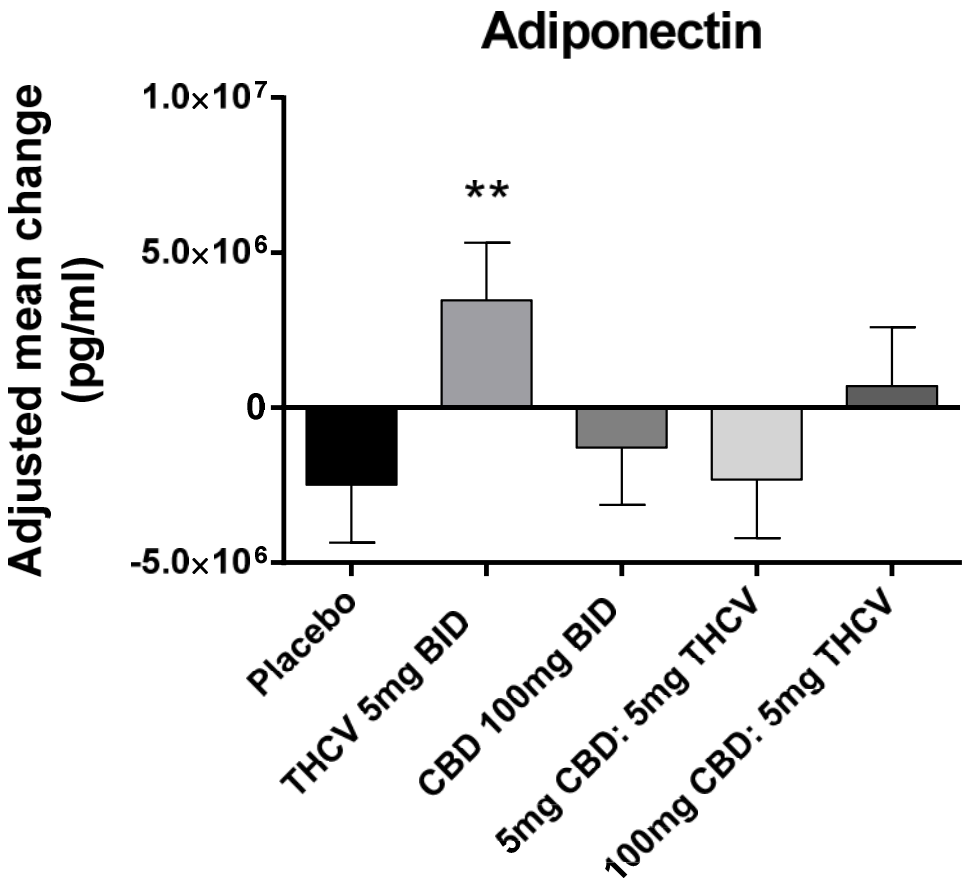


Figure 2.7. Compared with placebo (n=14), THCV (n=12) caused a significant improvement in the concentration of Adiponectin (p<0.01). CBD (n=13) on its own or in a 1:1 (n=9) or 20:1 (n=11) ratio combination with THCV had no impact on adiponectin levels. Data was analysed by analysis of covariance and presented as mean ± SEM.

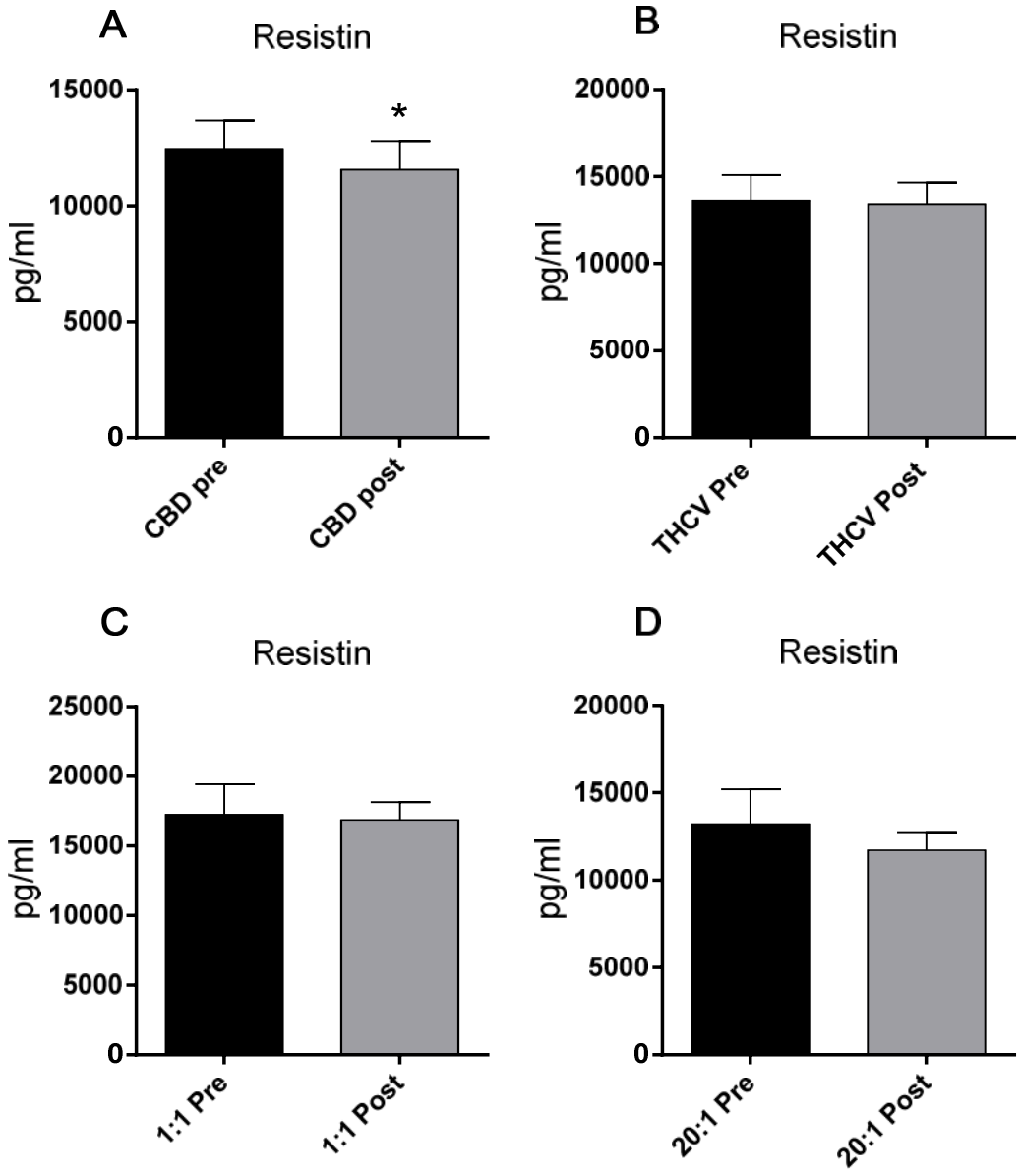


Figure 2.8. CBD (n=13) caused a significant reduction in the levels of Resistin ($p<0.05$; Figure 2.8A) when compared with pre-treatment values. THCV on its own (n=12, Figure 2.8B) or in combination with CBD (1:1, Figure 2.8C, n=9 and 20:1, Figure 2.8D, n=11) had no impact on the levels of resistin. Data were analysed post hoc using paired t-test and presented as mean ± SEM

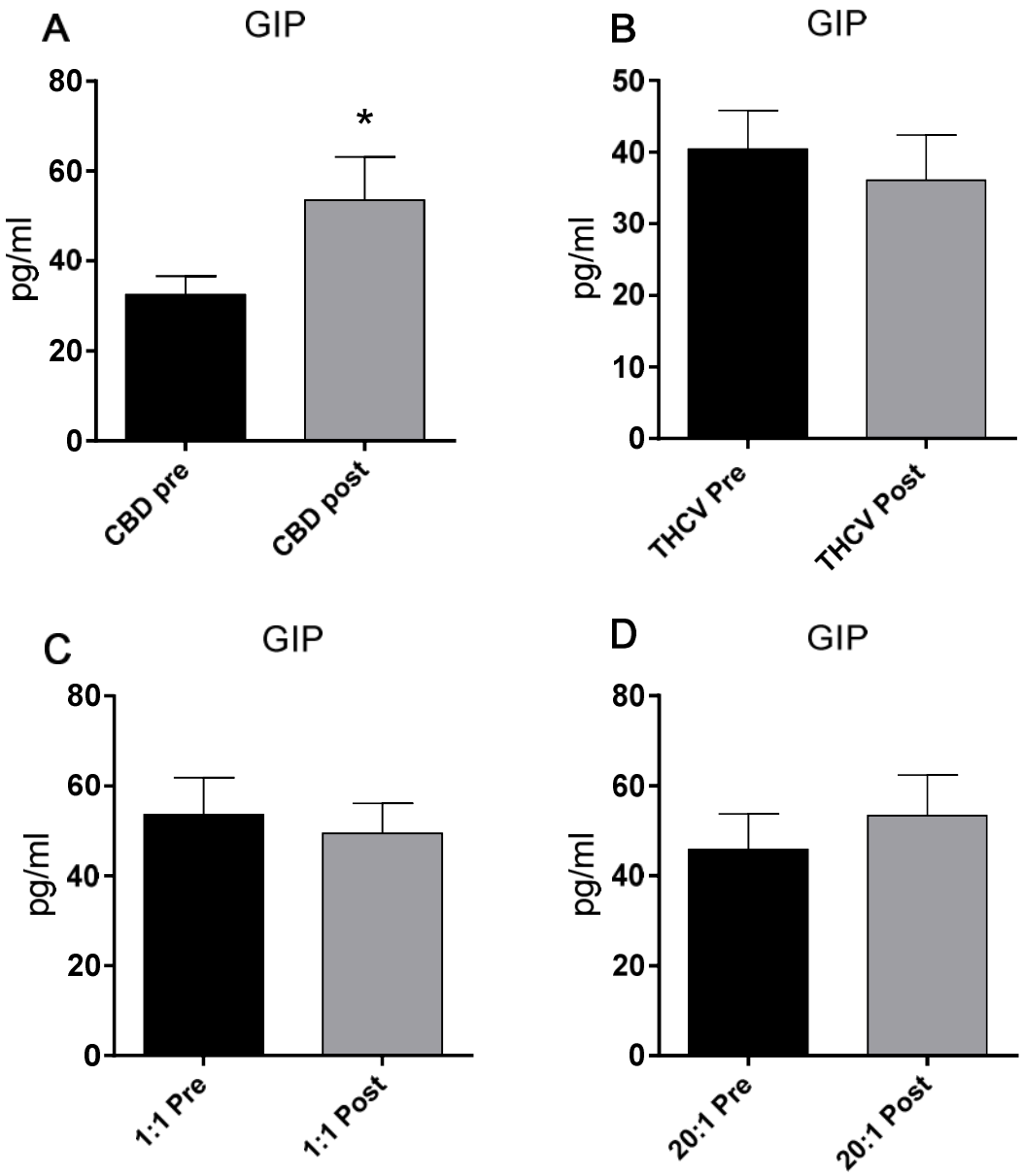
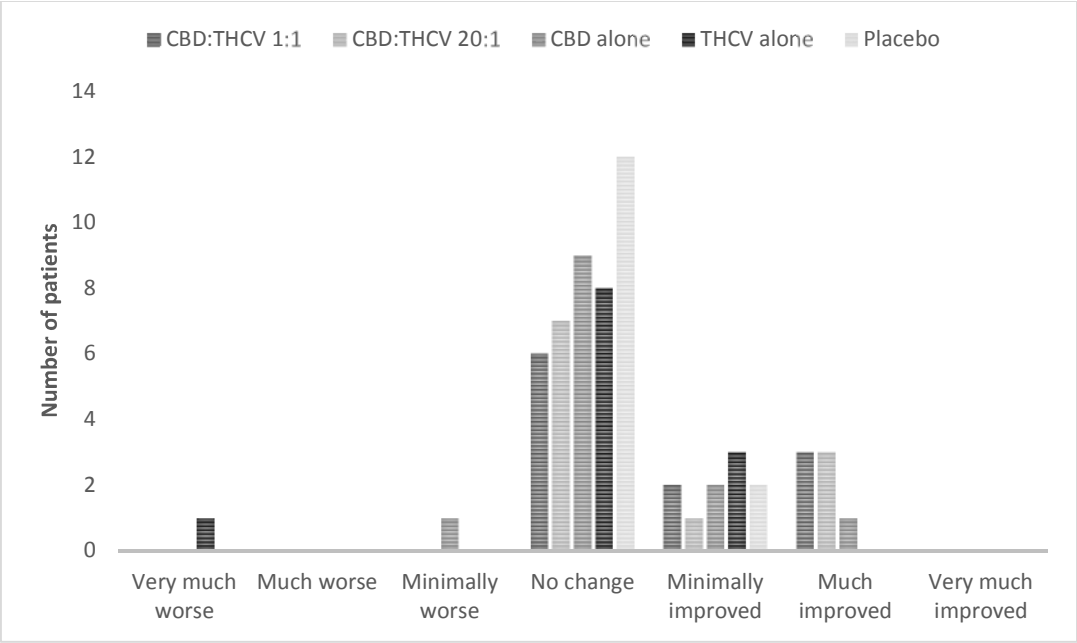


Figure 2.9. CBD (n=13) caused a significant increase in the levels of GIP ($p<0.05$; Figure 2.9A) when compared with pre-treatment values. THCV on its own (n=12, Figure 2.9B) or in combination with CBD (1:1, Figure 2.9C, n=9 and 20:1, Figure 2.9D, n=11) had no impact on the levels of GIP. Data were analysed post hoc using paired t-test and presented as mean ± SEM

A

SGIC



B

CGIC

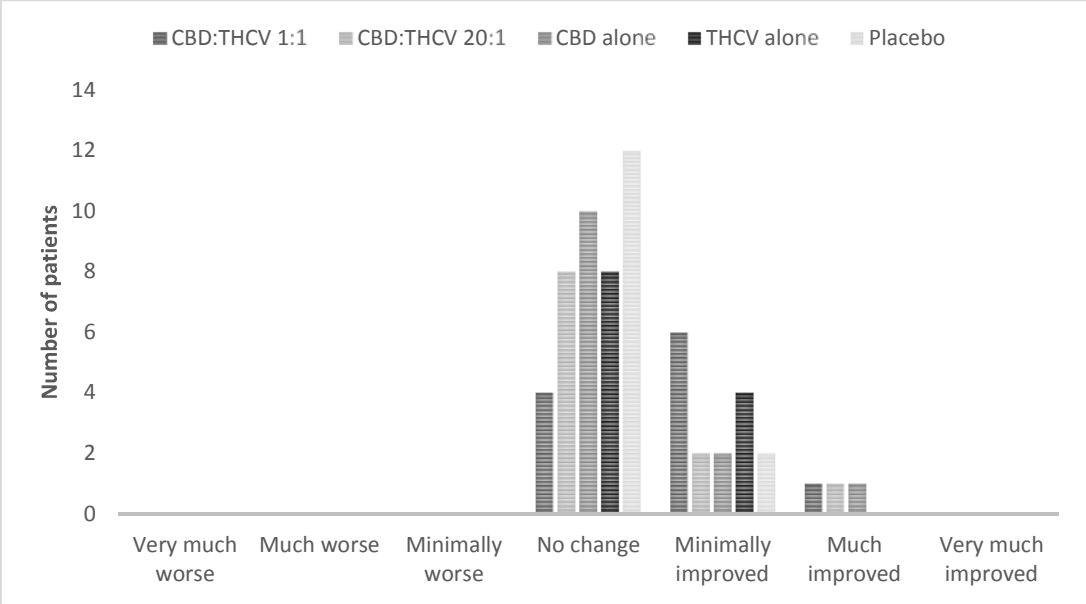


Figure 2.10. Summary of SGIC (A) and CGIC (B) assessment responses. There was a statistically significant treatment effect of 1:1 CBD:THCV treatment compared to placebo, with an odds ratio of 9.43 (p<0.05) in the CGIC.

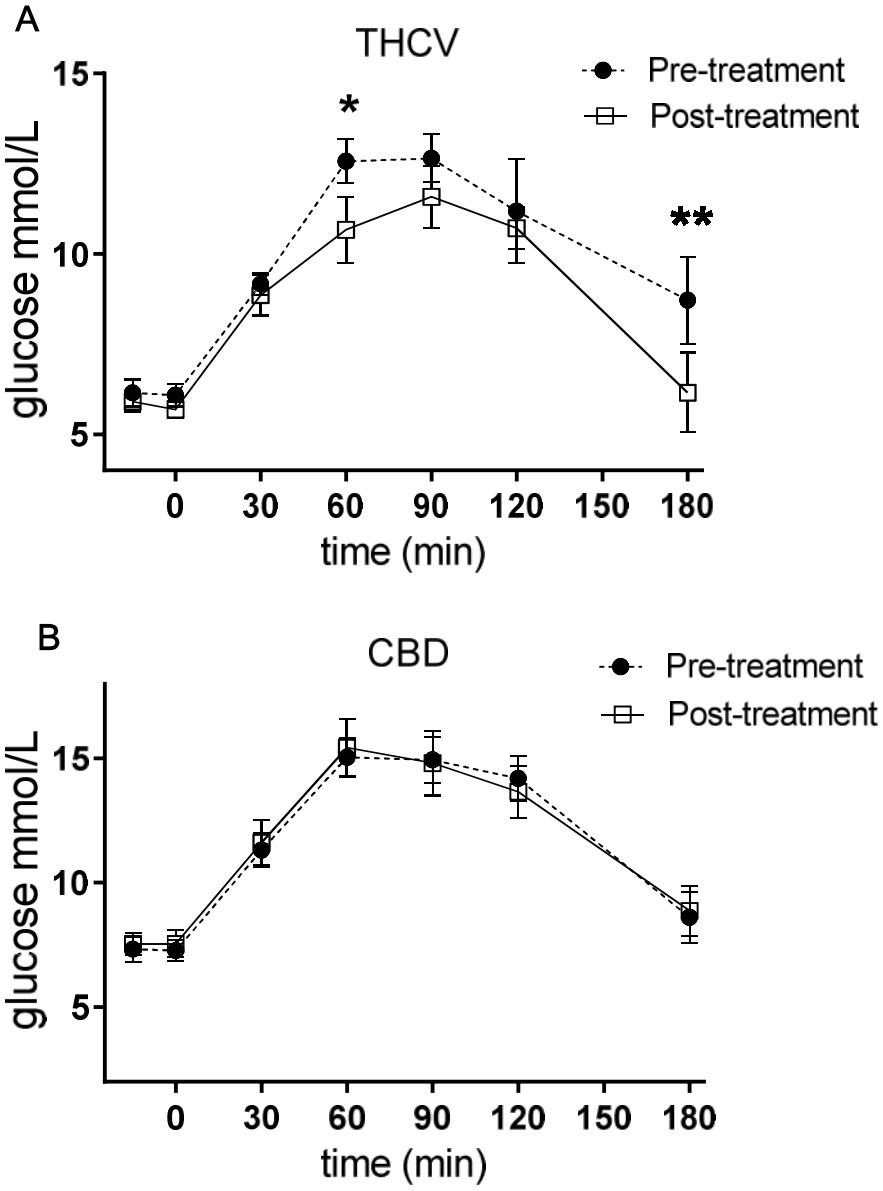


Figure 2.11. Compared with pre-treatment values, there was a highly significant improvement in 3 hour glucose response to OGTT with THCV, when subjects on any oral hypoglycaemic therapy other than diet and/or metformin were excluded from analysis (Figure 2.11A, $P < 0.01$, n=6). CBD had no effect on glucose tolerance (Figure 2.11B) in this subgroup of patients (n=9). OGTT data were analysed using repeated measures 2 way ANOVA and presented as mean ± SEM

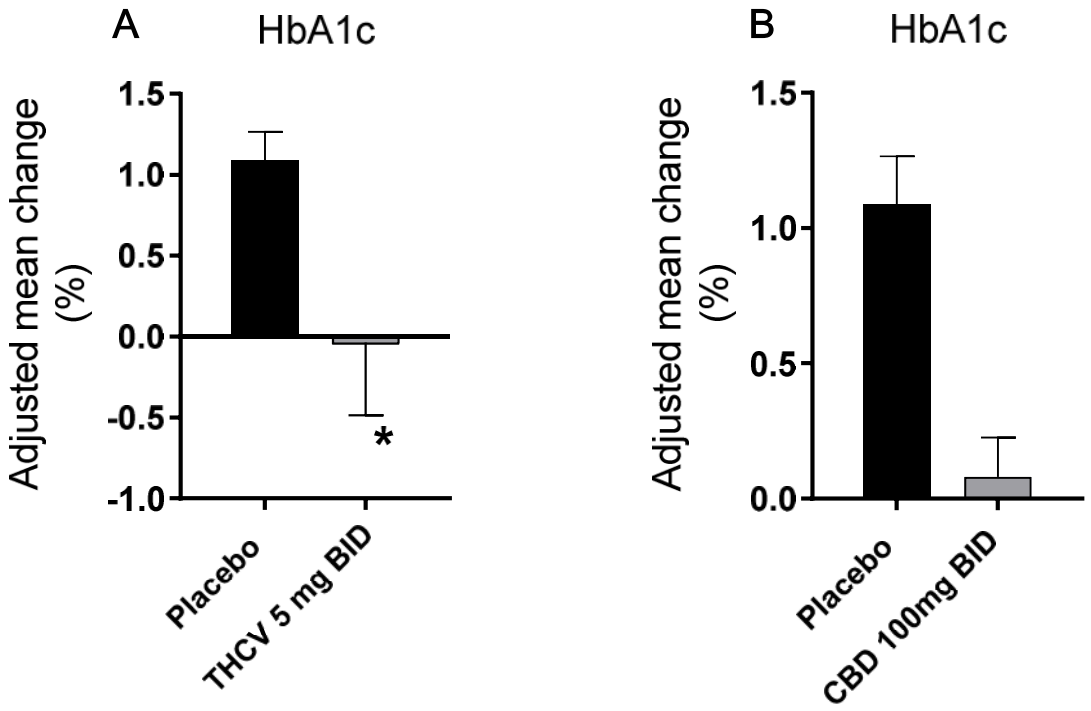


Figure 2.12. In the subgroup of patients on diet and/or metformin, there was a significant deterioration in HbA1c, in the placebo group and minimal improvement in THCV group. The difference was statistically significant in favour of THCV (p<0.05, Figure 2.12A). CBD had no effect on HbA1c (Figure 2.12B) in this subgroup of patients (n=9). Data were analysed using paired t-test and presented as mean ± SEM

2.4. Discussion

The aim of this pilot study was to establish the efficacy and tolerability of two phytocannabinoids, THCV and CBD, on their own and in combination, in subjects with poor glycaemic control and dyslipidaemia. The main finding of the study was an improvement in glycaemic profile and β cell function and a rise in the levels of adiponectin and Apo A caused by THCV. These findings suggest that THCV may represent a new therapeutic agent for glycaemic control in subjects with type 2 diabetes.

The ECS plays an important role in modulating energy intake and expenditure [for reviews, see (Silvestri *et al.*, 2011; Horvath *et al.*, 2012)], and a chronically over active ECS may have a role in diabetes and its various complications (Horvath *et al.*, 2012). A recent cross-sectional study showed that marijuana use was associated with lower concentrations of fasting insulin, insulin resistance and waist circumference (Penner *et al.*, 2013). Some of the favourable metabolic effects seen with smoking cannabis may be due to partial CB_1 agonists like THC, which may act as a functional antagonist in conditions of increased endocannabinoid tone like obesity (Le Foll *et al.*, 2013). Rimonabant, a CB_1 receptor antagonist, was the first in its class to be used as anti-obesity drug, but led to significant psychiatric adverse events (Le Foll *et al.*, 2009). Pre-clinical studies with the plant-derived compound THCV have shown that it produces hypophagia and weight reduction in lean mice (Riedel *et al.*, 2009) and improves glucose tolerance and insulin sensitivity in DIO mice (Wargent *et al.*, 2013). Similar results have been seen with CBD in *ob/ob* mice (Unpublished data). Given the positive metabolic effects of both THCV and CBD in preclinical studies and their potent anti-inflammatory and antioxidant properties (Costa *et al.*, 2004b; Lastres-Becker *et al.*, 2005; Bolognini *et al.*, 2010b), we decided to investigate, for the first time, their efficacy and tolerability in subjects with type 2 diabetes.

2.4.1. THCV

THCV treatment had no effect on HDL cholesterol concentration. It did, however, produce a significant rise in serum Apo A, when compared with placebo. Apo A makes 90% of HDL protein and constitutes an important structural component of the HDL particle. Apo AI, which accounts for 70% of the Apo A (the remaining 20% accounted for by Apo AII), plays an important role in reverse cholesterol transport (Barter, 2002). The significance of this result is, however, unclear.

THCV significantly reduced fasting blood glucose concentrations, improved HOMA2 B and improved 3 hour blood glucose response to OGTT, without any significant difference in insulin response. These findings are in keeping with the recent animal data, where THCV improved fasting glucose and 30 min glucose response to OGTT, and reduced fasting and post glucose insulin levels, suggesting improvement in insulin sensitivity (Wargent *et al.*, 2013). In the same study, THCV treatment improved insulin-induced phosphorylation of Akt in insulin-resistant human hepatocytes and mice myotubes, suggesting improved insulin signalling as one of the possible mechanisms of action.

Although there was an improvement in fasting and 3 hour post OGTT blood glucose, there were no changes in body weight and gut hormone concentrations. In fact, a rise in the concentration of RBP-4 was observed with THCV, an adipokine that has been associated with obesity and insulin resistance (Christou *et al.*, 2012). Therefore, the mechanism by which THCV improves glycaemic control remains unclear.

THCV significantly increased adiponectin concentrations. Adiponectin enhances hepatic insulin sensitivity, increases fatty acid oxidation and has important anti-atherogenic properties. Its concentrations are reduced in obesity and type 2 diabetes (Whitehead *et al.*, 2006).

Positive metabolic effects of THCV on glycaemic control and adiponectin concentrations were also seen with rimonabant, the first CB_1 antagonist to be licenced as anti-obesity medication that was later withdrawn from market due to increased incidence of psychiatric adverse events (Le Foll *et al.*, 2009). It is, however, important to emphasize that while rimonabant consistently reduced body weight in all the reported randomised clinical trials, no such change was seen with THCV, suggesting clear differences in the mechanisms of action of these compounds. Recent animal data with THCV similarly showed no effect on body weight (Wargent *et al.*, 2013). Moreover, rimonabant improved the lipid profile (increased HDL cholesterol and reduced TG levels) (Christopoulou and Kiortsis, 2011), while THCV had no effect on lipid parameters in our study. We therefore believe that THCV and rimonabant have different pharmacological profiles. At micromolar concentrations, THCV inhibits the activity of both fatty acid amide hydrolase (FAAH) and monoacyl glycerol lipase (MAGL), thereby inhibiting the hydrolysis of AEA and 2-AG respectively (McPartland *et al.*, 2015). THCV, therefore, can act as an indirect agonist at the cannabinoid receptors, by enhancing the activity of the endocannabinoid system. Since such a change was not seen in our study, it is reasonable to believe that, at the dose tested, THCV was unable to modulate the endocannabinoid system. Recent animal data from Wargent and colleagues (Wargent *et al.*, 2013) showed that most of the positive metabolic effects of THCV were seen with 5 and 12.5 mg/kg doses given orally. In comparison to this, the dose used on our study (10 mg/day) was much lower.

2.4.2. CBD

Although CBD did not produce any effects on the primary and secondary efficacy outcomes, it reduced circulating resistin concentrations, while increasing the concentration of GIP. Increased concentrations of resistin are associated with obesity and insulin resistance (Steppan *et al.*, 2001). GIP is one of the incretin hormones, produced by K cells

in proximal duodenum, which is known to have insulinotropic and pancreatic β-cell preserving properties (Irwin and Flatt, 2009a). Despite having positive effects on resistin and GIP, CBD did not produce any improvement in glycaemic control.

CBD is known for its indirect agonism at the CB_1 receptors, by either increasing CB_1 constitutional activity or the endocannabinoid tone. CBD inhibits hydrolysis of AEA by FAAH and also increases 2-AG levels (McPartland *et al.*, 2015). In a recent clinical study, in subjects with schizophrenia, 800 mg per day of CBD treatment significantly increased serum AEA levels and was associated with an improvement in clinical profile of these subjects (Leweke *et al.*, 2012). In our study, CBD had no effect on the plasma levels of endocannabinoids, suggesting that it had no positive or negative interaction with the endocannabinoid system.

Studies in rodents have used intraperitoneal CBD in a dose ranging from 1mg/kg/day to 20mg/kg/day, with positive effects on the metabolism seen only with higher dose ranges (El-Remessy *et al.*, 2006; Toth *et al.*, 2010; Rajesh *et al.*, 2010). In a 70 kg individual, a 20mg/kg/day dose equates to 1400mg/day. The dose used in our study was 200mg/day, which could possibly explain lack of therapeutic effects seen with CBD.

2.4.3. Combination of CBD and THCV

Except for an improvement in CGIC assessments with 1:1 CBD:THCV treatment, none of the efficacy parameters were affected by 1:1 or 20:1 combination of CBD and THCV. There was a trend towards an improvement in most lipid parameters and the overall incidence of all-causality treatment related AEs was lowest with 1:1 CBD:THCV, and these factors could have led to an impression of improvement in subjects' overall condition with this treatment. Currently there is no published human data on the combination of CBD with THCV. CBD has been combined with THC. Sativex® contains a 1:1

combination of THC and CBD and has been licenced for use to treat spasticity in multiple sclerosis. The nature of interaction between CBD and THC is not entirely clear with earlier studies showing that CBD reduced THC induced anxiety in healthy volunteers (Zuardi *et al.*, 1982b), while one recent study showed that CBD potentiated the psychoactive and physiological effects of THC in rats (Klein *et al.*, 2011). This might be explained by the time interval between the administration of CBD and THC and the dose ratios. In Sativex®, CBD seems to block the central unwanted effects of THC. The lack of effect of combination treatment may suggest that either the dose of CBD and THCV or the ratio of the combination was not right to positively modulate symptoms of type 2 diabetes. While the combination of CBD and THCV did not produce any favourable effects on any of the parameters, the favourable effects of THCV were also lost in the combination treatment. Similarly, the positive effects of CBD on GIP and resistin were not seen in any of the combination treatments. This means that somehow both CBD and THCV counteract the therapeutic effects of each other when given as a combination. This is against the findings of GW Pharma (GW Pharmaceuticals plc, Cambridge UK) in animal study, though this data has not been published yet. The interaction between CBD and THCV can be at the receptor level or by interfering with each other's metabolism or therapeutic half-life.

2.5. Safety

A full summary of treatment-emergent (all-causality) and treatment related AEs experienced by one or more randomised patients are presented in Table 3.4. Treatment-emergent (all-causality) AEs were reported by seven patients (63.6%) receiving 1:1 CBD:THCV, eight (66.7%) receiving 20:1 CBD:THCV, 11 (84.6%) receiving CBD alone and 11 (91.7%) receiving THCV alone, compared with 13 patients (92.9%) receiving placebo. Treatment-emergent, treatment related AEs were reported by seven patients (63.6%) receiving 1:1 CBD:THCV, four (41.7%) receiving 20:1 CBD:THCV, seven

(53.8%) receiving CBD alone and eight (66.7%) receiving THCV alone, compared with seven patients (50.0%) receiving placebo (Table 3.4).

Four patients (6.5% overall) withdrew from the study due to AEs, two each (16.7% of patients in both groups) in the 20:1 CBD:THCV and THCV alone treatment groups. The three most common treatment related AEs developed by patients in the different treatment groups were; decreased appetite (1:1 CBD:THCV = 9.1%; 20:1 CBD:THCV = 0%; CBD alone = 15.4%; THCV alone = 33.3%, compared to placebo = 14.3%), headache (1:1 CBD:THCV = 9.1%; 20:1 CBD:THCV = 8.3%; CBD alone = 7.7%; THCV alone = 8.3%, compared to placebo = 7.1%) and dizziness (1:1 CBD:THCV = 9.1%; 20:1 CBD:THCV = 0%; CBD alone = 0%; THCV alone = 8.3%, compared to placebo = 14.3%). The majority of AEs experienced in the study were mild in severity.

There were two serious AEs (SAEs) in this study. One patient taking 20:1 CBD:THCV treatment experienced an SAE of myocardial infarction that was considered moderate in severity, had recovered by the end of study, and was not considered to be treatment related. One patient taking placebo experienced an SAE of myocardial ischaemia that was considered mild in severity, was continuing at the end of the study, but was not considered to be treatment related.

There were no fatalities in the study. Additionally, there were no clinically significant abnormalities observed in the laboratory results. There were clinically significant findings in the parameters of SBP, diastolic BP (DBP) and pulse rate in all treatment groups. However, there were no apparent trends between active treatment groups and any of these findings.

In the BDI-II assessment, all baseline and end of treatment scores for all treatments remained within the 0-9 range, indicating the best possible outcome of ‘minimal

depression' (Table 3). The largest change from baseline to the end of treatment in BDI-II score was in the 20:1 CBD:THCV treatment group, where the score increased from 2.75 at baseline to 7.91 at the end of treatment (a change from baseline of 4.91 points). While this remained within 'minimal depression' bracket, statistical analysis of the data demonstrated that this was a significant increase in BDI-II score, with an estimated treatment difference of 4.77 compared to placebo ($p<0.01$). No other treatments produced a statistically significant effect on the BDI-II compared to placebo.

2.6. Study Limitations

There are some important limitations to this study. It was regarded as a proof of concept study and therefore no formal calculations were carried for sample size. This meant that it was potentially underpowered to detect some of the small changes, such as in the primary endpoint of HDL cholesterol. It would have been very useful to, at least, determine the plasma levels of CBD and THCV during the course of the study. This is especially relevant because most human studies with CBD have used a higher dose than the one used in our study, and CBD did not produce any desirable effects described in the literature. Another important point is that there is no data looking at the interaction of CBD and THCV. In our study, THCV lost its positive effects when it was combined with CBD. Could it be due to their interaction at pharmacokinetic level, where one drug increases the clearance of other and reduces its half-life and therefore its therapeutic potential, or due to opposing cellular effects of the two molecules, given the plethora of receptor variants acted upon? Further research is needed in this area to determine the true nature of interaction between these two phytocannabinoids and the right dose ratio, before another clinical study.

2.7. Conclusion

In this clinical study, the first to study the effects of CBD and THCV, CBD failed to show any positive metabolic effects despite producing desirable changes in some adipokines and gut hormone concentrations. THCV, on the other hand, improved glycaemic control, and increased the levels of adiponectin and Apo A. THCV therefore warrants further investigation as a possible treatment for glycaemic control in type 2 diabetes.

3. Effect of cannabidiol (CBD) on cardiovascular response to stress in healthy young volunteers

3.1. Introduction

Epidemiological studies have shown a positive relationship between long term stress and the development of cardiovascular disease (Figueredo, 2009b). Factors like social isolation, low socio-economic status, depression, stressful family and work life and anxiety are associated with an increased risk of the development and accelerated progression of existing cardiovascular disease (Perk *et al.*, 2012). Mental stress induces myocardial ischaemia in patients with stable coronary artery disease and this appears to be mediated by adrenal release of catecholamines (Goldberg *et al.*, 1996b). Such ischemic changes are predominantly silent and do not cause typical ECG changes (Rozanski *et al.*, 1988a).

CBD is one of the phytocannabinoids that lacks psychotropic effects and is currently the focus of much research due to its potential in a number of therapeutic areas. It has anti-inflammatory, anti-convulsant, anti-oxidant, anxiolytic, anti-nausea and anti-psychotic properties (Mechoulam *et al.*, 2002). A CBD/THC combination (Sativex/Nabiximols, GW Pharmaceuticals, Cambridge, UK) is licensed for the treatment of spasticity in multiple sclerosis, and CBD alone (Epidiolex, GW Pharmaceuticals, Cambridge, UK) has entered an expanded access programme in children with intractable epilepsies (Dravet Syndrome and Lennox-Gastaut Syndrome). CBD alone has also received orphan designation status for the treatment of neonatal hypoxia-ischaemic encephalopathy.

A number of preclinical studies also show beneficial effects of CBD in a range of disorders of the cardiovascular system (Stanley *et al.*, 2013a). There is evidence from animal studies that CBD modulates the cardiovascular response to stress. Resstel and colleagues (Resstel *et al.*, 2009) showed in rats that intraperitoneal (IP) injection of CBD (10 & 20 mg/kg, -30 min) reduced restraint stress-induced cardiovascular response and behaviour. Both these

effects were blocked by pre-administration of WAY100635 (0.1 mg/kg), a $5HT_{1A}$ antagonist. These effects appear to be mediated centrally and involve BNST, a limbic structure that modulates neuroendocrine responses to acute stress (Choi *et al.*, 2007b).

To date, there are no dedicated studies in humans looking at the effect of CBD on either resting cardiovascular measurement or on the responses to stress, with continuous monitoring of CV parameters. Therefore, the aim of the present study was to investigate whether CBD decreases the cardiovascular response to stress after the administration of a single dose of CBD (600 mg) in healthy volunteers. Non-invasive cardiovascular measurements were used along with stress tests in the form of mental arithmetic, isometric exercise and the cold pressor test.

3.2. Methods

9 healthy male volunteers, mean age 24 years (range 19-29), with no underlying cardiovascular or metabolic disorders, were recruited for this study, which was approved in advance by the University of Nottingham Faculty of Medicine Ethics Committee. Written informed consent was obtained according to the Declaration of Helsinki. Main exclusion criteria included any significant cardiovascular or metabolic disorder or use of any medication.

3.2.1. Study design

The study was a crossover design with each subject given CBD (BN: K12067A) or placebo in a double blind fashion, with a minimum time interval of at least 48 hours. The actual interval between the two visits was between 3 to 16 days. 2 hours after CBD/placebo was administered, subjects performed various stress tests (O'Sullivan and Bell, 2001). Non-invasive cardiovascular monitoring using Finometer and Laser Doppler flowmetry was carried out during the 2 hours to assess changes in baseline parameters and

during the stress test periods. During an initial visit, subjects were familiarized with the stress tests and with non-invasive CVS monitoring, and an ECG was done to rule out any pre-existing cardiac condition. Subjects were advised to fast overnight, to avoid beverages containing caffeine or alcohol and to avoid strenuous exercise for 24 hours before each of the two study visits. All the volunteers were non-smokers and had taken no prescribed or over the counter medication within a week prior to randomisation. None had ever used cannabis. I carried out all the study procedures on my own including the use of laser doppler and finometer.

3.2.2. Study Procedures

Upon arrival, subjects were rested for 10-15 minutes and their baseline blood pressure and heart rate were recorded using a digital blood pressure monitor. Participants were given a standardised breakfast and 15 minutes later they were given either oral CBD (600 mg) or placebo in a double blind fashion. 2 hours afterwards, subjects were asked to perform the stress tests (O'Sullivan and Bell, 2001). Timing of the tests was chosen to coincide with peak plasma levels for CBD (Fusar-Poli *et al.*, 2009; Fusar-Poli *et al.*, 2010). All the experiments were performed in a sitting position under ambient temperature conditions. Maximum voluntary contraction for the isometric hand grip test, was assessed for each subject prior to administering study medication.

After administration of CBD or placebo, subjects remained seated, doing nothing, reading or using a computer. During this time, subjects were connected to a Finometer, which uses a finger-clamp method to detect beat-to-beat changes in digital arterial diameter using an infrared photoplethysmograph (Schutte *et al.*, 2004). The Finometer gives a continuous signal of beat-to-beat changes in blood pressure and blood flow and uses this signal to derive other parameters, including systolic, diastolic and mean blood pressure, interbeat

interval, heart rate and left ventricular ejection time, stroke volume, cardiac output, and systemic peripheral resistance. Baseline cardiovascular data was recorded for 2 hours following administration of CBD or placebo, with two 10-15 min rest intervals in between. Forearm blood flow was measured using a Laser Doppler flowmeter, which has been used to measure blood flow in microcirculation of various tissues including skin (Johnson *et al.*, 1984). For each recording, five images of microcirculation were taken, over an area 19mm x 19mm, using the upper one third of the left forearm under high resolution. After 2 hours, subjects underwent the cardiovascular stress tests in the following order; mental arithmetic, isometric exercise and the cold pressor test.

3.2.2.1. Mental Arithmetic Test

The mental arithmetic test consisted of calculating a sum every 2 second for 2 minutes. Subjects were seated in front of a computer screen and a PowerPoint presentation delivered a slide with a simple mathematical sum of a three digit number minus a smaller number (e.g. 317-9, 212-11, 185-7) every two seconds; the subject had to give the answer verbally.

3.2.2.2. Isometric Exercise Test

In the isometric exercise stress test, using a dynamometer, handgrip was maintained at 30% of maximum voluntary contraction (MVC) for 2 min.

3.2.2.3. Cold Pressor Test

For the cold pressor test, subjects immersed their left foot (up to ankle) in ice slush (temperature 4-6°C) for 2 min.

Cardiovascular parameters were measured continuously using the Finometer, while skin blood flow measurements were taken just before, during and 5 minutes after each test.

Each stress test lasted for 2 min and there was recovery period of at least 10 min to allow cardiovascular parameters to return to baseline.

3.2.3. Statistics

Data were analysed using repeated measures ANOVA to determine the effect of treatment and time on different variables using GraphPad PRISM® version 6.02. Level of significance was set at $\alpha = 0.05$ and values presented as mean±SEM. Sidak's post-hoc test was used to see treatment affect at various time points. Data were not unblinded until after statistical analysis.

3.2.4. Drugs

Study medication consisted of capsules containing either 100mg of CBD or excipients, which were a gift from GW Pharmaceuticals. There was no difference between the two formulations in colour, taste or smell.

3.3. Results

10 male subjects were recruited, but one withdrew for personal reasons. The mean age, weight and height of the volunteers were 23.7±3.2years, 77.5±6.4kg and 178.6±4.5cm (mean±SD).

3.3.1. Effect of CBD on resting cardiovascular parameters

CBD treatment reduced resting SBP (mean difference -6 mmHg; 95% CI: -1 to -12; $P<0.05$, Figure 3.1A). Although there was no overall difference in diastolic blood pressure (DBP) and mean arterial pressure (MAP) between the two groups, post-hoc analysis showed that both DBP and MAP were lower with CBD treatment (Figure 3.1B and 3.1C respectively; $P<0.01$), particularly in the latter time points (120-160 min).

CBD treatment reduced resting stroke volume (SV) (mean difference -8 ml; 95 CI: -2 to -14; P<0.05; Figure 3.1E), and increased heart rate (HR), with significant difference in HR between CBD and placebo from an hour after ingestion of drug (Figure 3.1D, P<0.05). There was a decline in left ventricular ejection time (EJT) with both treatments (P<0.05; Figure 3.1G), while cardiac output (CO) remained unchanged.

There was a trend towards reduction in total peripheral resistance (TPR) (Figure 3.1H) with CBD in the latter half of the resting period, and a significant reduction in forearm skin blood flow before the start of the stress tests (Figure 3.1I; P<0.01).

3.3.2. Effect of CBD on cardiovascular parameters in response to mental stress

Mental stress caused a rise in HR (P<0.05; Figure 3.2D) and a decline in SV (P<0.01; Figure 3.2E), which was seen in both the CBD and placebo groups. There was a rise in DBP (P<0.05; Figure 3.2B) and a decline in EJT (P<0.05; Figure 3.2G), seen only in those who had taken CBD.

Although overall, there was no difference in the cardiovascular parameters between the two treatments, post-hoc analysis, showed that SBP (Figure 3.2A; P<0.05 to <0.0001), DBP (Figure 3.2B; P<0.05 to <0.01) and MAP (Figure 3.2C; P<0.05 to <0.0001) were significantly lower in volunteers who had taken CBD, especially immediately after the stress test.

CBD increased HR during the latter part of mental stress and in the post stress period (Figure 3.2D; P<0.05 to <0.0001), with a corresponding decline in EJT (Figure 3.2G; P<0.05 to <0.0001). Although CBD reduced SV (Figure 3.2E; P<0.05 to <0.0001), there was no difference in CO between CBD and placebo.

Compared to placebo, volunteers who had taken CBD had a lower TPR throughout and especially in the post mental stress period (Figure 3.2H; P<0.05 to <0.0001). Volunteers who had taken CBD also had reduced forearm skin blood flow, before and during latter part of mental stress (Figure 3.2I; P<0.05 to <0.01).

3.3.3. Effect of CBD on cardiovascular parameters in response to exercise stress

Isometric exercise stress caused a significant rise in following parameters in both treatment groups; SBP (Placebo P<0.01, CBD P<0.001; Figure 3.3A), DBP (Placebo P<0.01, CBD P<0.0001; Figure 3.3B), MAP (Placebo P<0.01, CBD P<0.0001; Figure 3.3C) and HR (Placebo P<0.05, CBD P<0.001; Figure 3.3D).

Overall, CBD treatment reduced SBP (mean difference -5 mmHg; 95% CI: -1 to -10; P<0.05; Figure 3.3A) and MAP (mean difference -5 mmHg, 95% CI: -2 to -9, P<0.05, Figure 3.3C) during the exercise stress. Those who taken taken CBD also had increased HR (mean difference 10 bpm; 95% CI: 5 to 14; P<0.01; Figure 3.3D) and decreased SV (mean difference -13 ml; 95% CI: -4 to -22; P<0.01; Figure 3.3E) and EJT (mean difference -0.01 sec, 95% CI: -0.001 to -0.03; P<0.05, Figure 3.3G). There was no difference in CO.

A rise in FSBF in response to exercise (as would be expected) was only seen in volunteers who had taken placebo (P<0.05; Figure 3.3I). Post-hoc analysis showed significantly lower forearm skin blood flow in those who had taken CBD (Figure 3.3I; P<0.001 to <0.0001), during early and latter part of stress test. This was associated with reduced TPR (Figure 3.3H; P<0.05 to <0.001)) before, after and in the latter half of exercise stress.

3.3.4. Effect of CBD on cardiovascular parameters in response to cold stress

The cold pressor test caused a rise in SBP (Placebo P<0.01, CBD P<0.05; Figure 3.4A) and MAP (Placebo P<0.001, CBD P<0.05; Figure 3.4C), in both groups and a rise in DBP

only with placebo (P<0.01; Figure 3.4B). An equal rise in SBP and MAP was seen with both CBD and placebo in the first half of this stress test. However, while the blood pressure (SBP and MAP) continued to rise in the placebo group, it plateaued in volunteers who had taken CBD, and therefore both SBP and MAP were significantly lower in volunteers after CBD (mean difference -8 mmHg; 95% CI: -4 to -12; P<0.01 and -6 mmHg; 95% CI: -2 to -11; P<0.01 respectively). Post-hoc analysis showed that DBP was also significantly lower in those who had taken CBD in the latter half of stress period (Figure 3.4B; P<0.001).

As before, HR was higher in volunteers who had taken CBD (mean difference 7 bpm; 95% CI: 2 to 13; P<0.05; Figure 3.4D) and EJT was lower (mean difference -0.01 sec; 95% CI: 0 to -0.02; P<0.05; Figure 3.4G). Sidak post-hoc analysis showed that SV was significantly lower with CBD throughout (Figure 3.4E; P<0.05 to <0.0001), but there was no decline in cardiac output.

The cold pressor test caused a significant rise in TPR (as expected) in the placebo group only (Figure 3.4H; P<0.01) and rise in forearm skin blood flow with both CBD and placebo (Figure 3.4I; P<0.05). The overall trend was for lower TPR and FSBF in those who had taken CBD, with post-hoc analysis showing a reduction in both just before and in the latter half of cold stress (Figure 3.4H; P<0.05 to <0.0001 and Figure 3.4I; P<0.001 respectively).

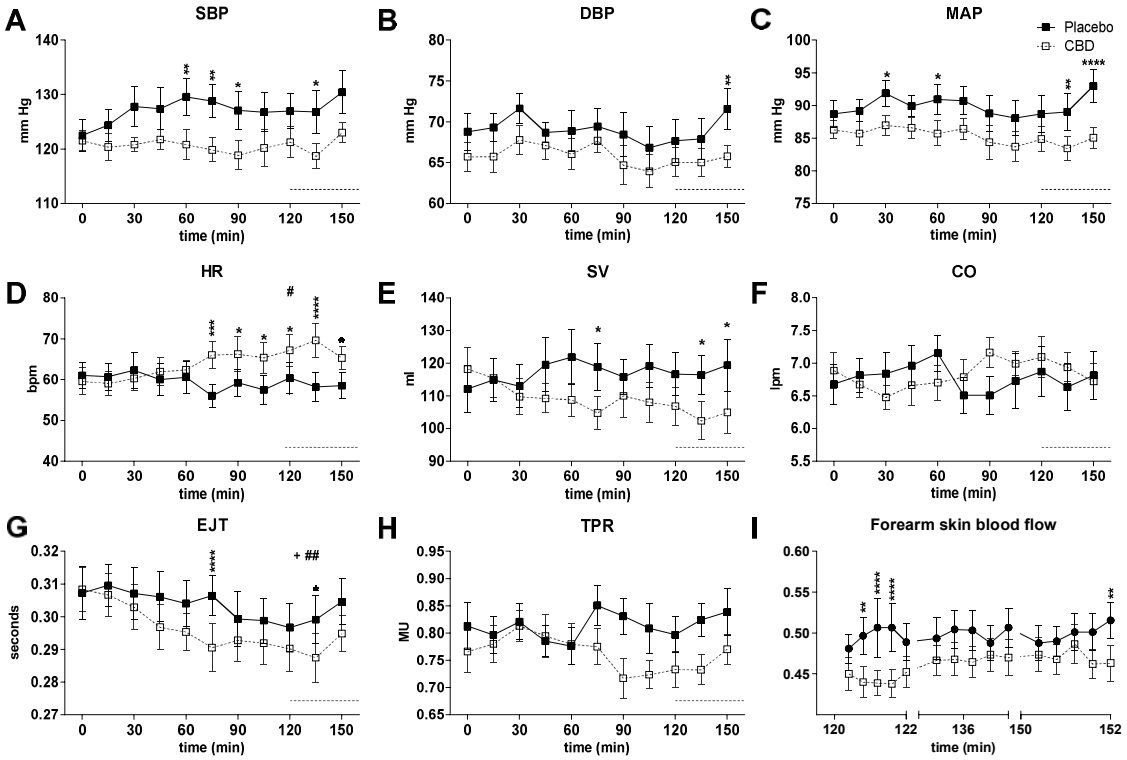


Figure 3.1. Changes in resting cardiovascular parameters after a single dose (600mg) of CBD in healthy volunteers. The effects of placebo (closed square) and CBD (open square) on SBP (A), DBP (B), MAP (C), HR (D), SV (E), CO (F), EJT (G), TPR (H) and Forearm skin blood flow (I), measured continuously over 2 hours post drug ingestion, except for forearm blood flow. Forearm blood was measured over a time period of 2 minutes just before the start and in between the stress tests. Dotted line denotes baseline values between the stress tests. Repeated measures 2 way ANOVA; mean ± SEM (* P<0.05, **P<0.01; * denotes significant difference at different time points using Sidak's post hoc analysis. + and # represent significant change in any parameter over time seen with placebo and CBD respectively).

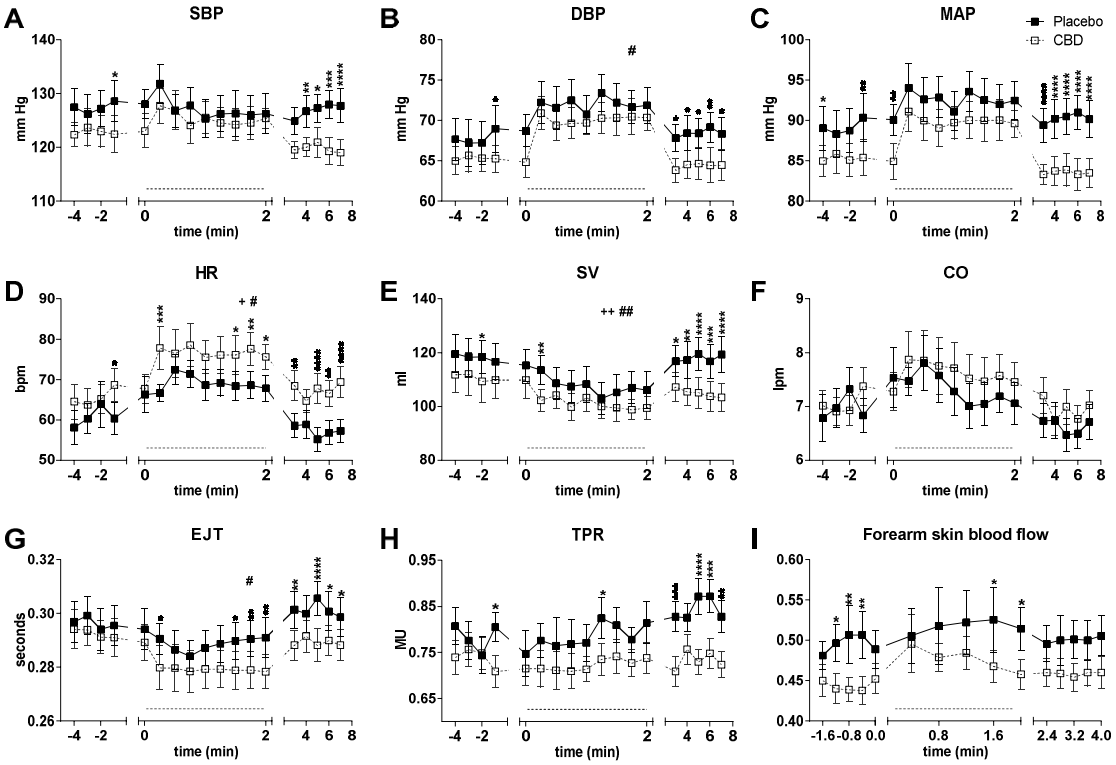


Figure 3.2. Cardiovascular response to mental stress after a single dose (600mg) of CBD in healthy volunteers. The effects of placebo (closed square) and CBD (open square) on SBP (A), DBP (B), MAP (C), HR (D), SV (E), CO (F), EJT (G), TPR (H) and Forearm skin blood flow (I), measured continuously just before, during and after mental arithmetic test (Blue line denotes stress test period), except for forearm blood flow. Measurements for forearm blood flow were made over a 2 minute window just before, during and after the stress test. Repeated measures 2 way ANOVA; mean ± SEM (+ and # denote significant change in a parameter during the stress period seen with placebo and CBD respectively).

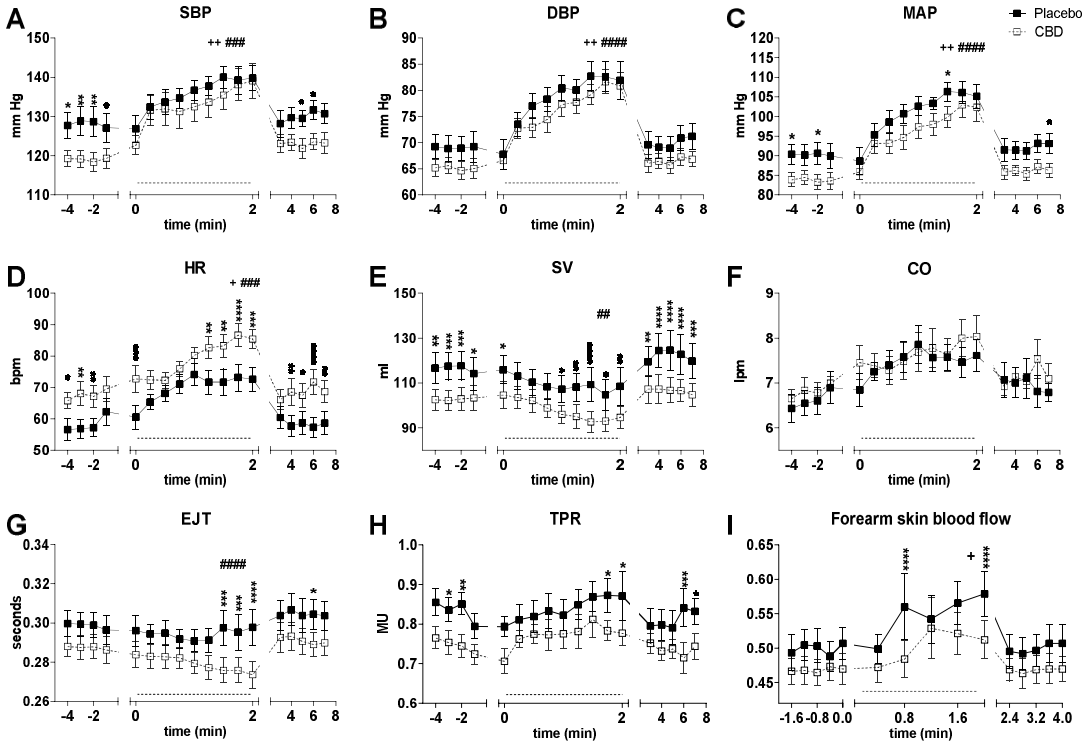


Figure 3.3. Cardiovascular parameters in response to exercise stress after a single dose (600mg) of CBD in healthy volunteers. The effects of placebo (closed square) and CBD (open square) on SBP (A), DBP (B), MAP (C), HR (D), SV (E), CO (F), EJT (G), TPR (H) and Forearm skin blood flow (I), measured continuously just before, during and after isometric exercise test (Blue line denotes stress test period), except for forearm blood flow. Measurements for forearm blood flow were made over a 2 minute window just before, during and after the stress test. Repeated measures 2 way ANOVA; mean ±SEM (* P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001; * denotes significant difference at different time points as per Sidak post hoc analysis. + and # denote significant change in a parameter during the stress period seen with placebo and CBD respectively).

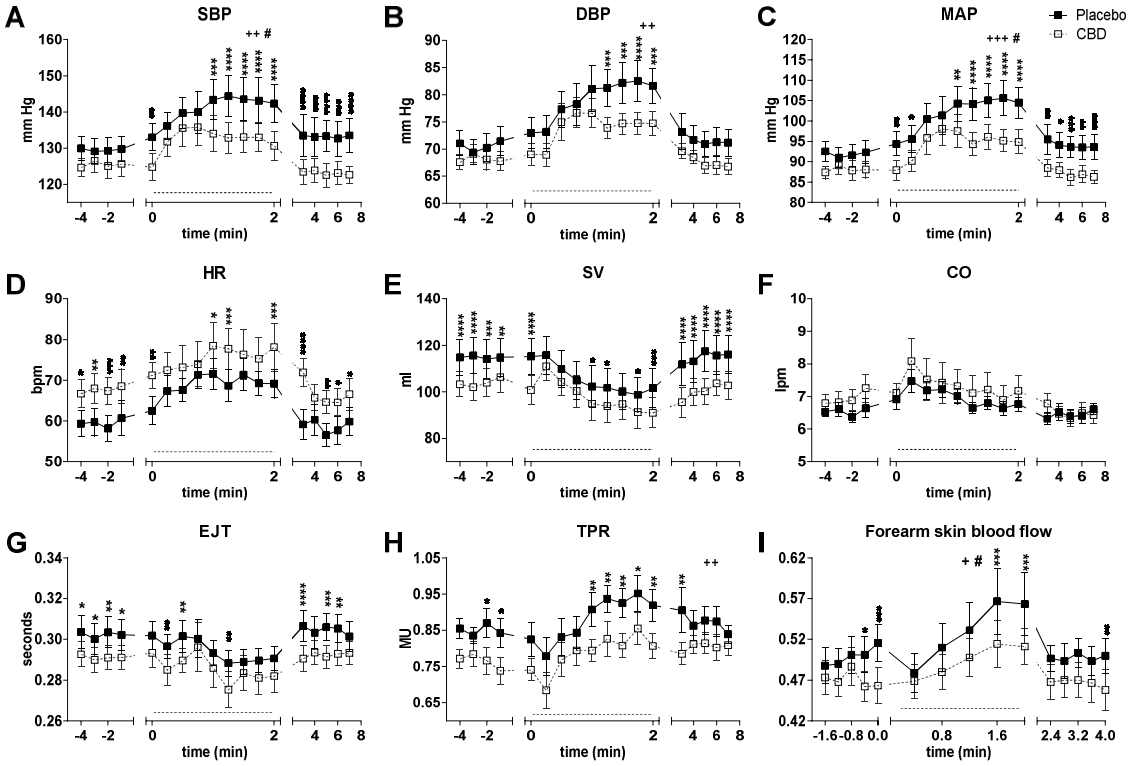


Figure 3.4. Cardiovascular response to cold stress after a single dose (600mg) of CBD in healthy volunteers. The effects of placebo (closed square) and CBD (open square) on SBP (A), DBP (B), MAP (C), HR (D), SV (E), CO (F), EJT (G), TPR (H) and Forearm blood flow (I), measured continuously just before, during and after cold pressor test (Blue line denotes stress test period), except for forearm blood flow. Measurements for forearm blood flow were made over a 2 minute window just before, during and after the stress test. Repeated measures 2 way ANOVA; mean ± SEM (* P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001; * denotes significant difference at different time points as per Sidak post hoc analysis. + and # denote significant change in a parameter during the stress period seen with placebo and CBD respectively).

3.4. Discussion

Based on preclinical evidence, the aim of this study was to test the hypothesis that CBD would reduce the cardiovascular response to stress in healthy volunteers. We found that resting blood pressure was lower after subjects had taken CBD and that CBD blunted the blood pressure response to stress, particularly in the pre and post stress periods. Post-hoc analysis showed an overall trend of lower SBP, MAP, DBP, SV, TPR, forearm skin blood flow and left ventricular EJT and a higher HR in subjects who had taken CBD. These haemodynamic changes should be considered for people taking CBD, and suggest further research is warranted to establish whether CBD has any role in the treatment of cardiovascular disorders.

3.4.1. Effect of CBD on resting cardiovascular parameters

We have shown for the first time that, in humans, acute administration of CBD reduces resting blood pressure, with a lower stroke volume and a higher heart rate. This response may be secondary to the known anxiolytic properties of CBD (Zuardi *et al.*, 1982b), and may account for the lack of anticipatory rise in blood pressure seen after placebo. These findings are in contrast to animal data, where CBD did not affect baseline cardiovascular parameters (Resstel *et al.*, 2009). While the dose of CBD used in the animal study by Resstel and colleagues was comparable to that used in the present study (10 mg/kg body weight), CBD was administered intraperitoneally and CV monitoring was performed only during the 30 minute interval between drug delivery and restraint stress. In our study, the difference in blood pressure only became apparent after 60 min. In another study (Gomes *et al.*, 2013) where CBD had no effect on baseline HR and MAP, but augmented a stress induced rise in HR, CBD was administered locally into BNST and the baseline period was only 10 minutes (between drug delivery and restraint stress) during which CV monitoring

was performed. Thus, potentially these studies did not measure resting cardiovascular parameters for long enough.

THC, the major psychoactive component of cannabis, is known to cause tachycardia and orthostatic hypotension in humans (Sidney, 2002). In a recent study in healthy volunteers, a single dose of 10mg THC was associated with an increase in heart rate, with no significant effect on blood pressure, over a three hour period after administration. The same group of subjects showed no changes in HR and blood pressure when given 600mg CBD or placebo (Martin-Santos *et al.*, 2012). We used the same dose of CBD, but all our subjects were cannabis naive, while subjects in above-mentioned study had used cannabis in the past. Since tolerance develops to the haemodynamic response to cannabinoids in humans, this may explain the differences between studies. Another important difference was the method of CV monitoring. In the present study, CV parameters were measured continuously, while in the above study baseline monitoring for SBP, DBP and HR was only performed at 1, 2 and 3 hours after drug delivery.

THC is a partial agonist at both CB_1 and CB_2 receptors (Pertwee, 2008a), and the effects of THC on heart rate are mediated through CB_1 receptors (Sidney, 2002). CBD does not bind with any great affinity to CB_1, but can interact indirectly by augmenting CB_1 receptor's constitutional activity or endocannabinoid tone, the so called 'indirect agonism' (McPartland *et al.*, 2015). We recently showed that CBD also causes endothelium-dependent vasorelaxation in isolated human mesenteric arteries through CB_1 activation (Stanley *et al.*, 2015). Therefore, it is possible that the changes in haemodynamics brought about by CBD are mediated through CB_1.

CBD may cause sympathoinhibition (through CB_1 or some other mechanism) thereby preventing an increase in blood pressure and cardiac output, and causing a compensatory

rise in HR to maintain cardiac output. Indeed, the changes in SBP preceded any changes in HR, which strengthens this argument. Another possibility is that CBD inhibits cardiac vagal tone, thereby increasing HR. A recent study in male Sprague-Dawley rats showed that GPR18 activation in the Rostral Ventrolateral Medulla (RVLM) by Abn-CBD resulted in reduced blood pressure and increased HR (Penumarti and Abdel-Rahman, 2014) (similar to that observed in the present study). The same study showed that pre-treatment with atropine and propranolol, fully abrogated the HR response, suggesting a role for the autonomic nervous system. CBD is also a weak partial agonist at GPR18 (McHugh *et al.*, 2012b), which is another potential mechanism of action.

3.4.2. Effect of CBD on cardiovascular parameters in response to mental stress

Mental arithmetic has been shown to cause a rise in MAP and muscle sympathetic nerve activity (Schwartz *et al.*, 2011) and vasodilatation in forearm skeletal muscle (Barcroft *et al.*, 1960). In our study, none of the cardiovascular parameters other than HR, DBP and SV were affected, suggesting that the level of stress to this test was minimal. This could be because of added visual stimulus of computer screen, displaying the sums, which would have helped volunteers perform the task. Removing this could have increased the level of stress resulting in greater sympathetic response. Overall, there was trend for lower SBP, DBP, MAP, SV, TPR and forearm skin blood flow in subjects who had taken CBD, particularly in the pre and post stress test periods. Like resting cardiovascular parameters, these changes may indicate anxiolytic effects of CBD and/or generalised sympathoinhibition.

3.4.3. Effect of CBD on cardiovascular parameters in response to exercise stress

Isometric exercise produces a pressor response, via sympathoexcitaion, originating in the contracting muscle and relayed to the RVLM via nucleus of solitary tract. The result is a

rise in HR and CO and vasoconstriction in nonexercising organs (Lind *et al.*, 1964; Delius *et al.*, 1972; Sander *et al.*, 2010). There is increased skeletal muscle blood flow in the non-exercising limb, sensitive to atropine and propranolol (Ishii *et al.*, 2014). A similar response was seen in our study, where isometric exercise caused a significant rise in SBP, DBP, MAP and HR, and an increase in forearm blood flow, although this was significant in the placebo group only. Subject who had taken CBD had reduced blood pressure during the exercise stress test and this was most pronounced in the pre and post-test period. Before the exercise stress, HR was higher and SV lower in volunteers when they had taken CBD, and this trend continued throughout exercise stress and in the post-stress period. There was also a significant reduction in EJT with CBD, which represents a reciprocal change to increased HR. The rise in cutaneous blood flow was only seen with placebo and not with CBD, possibly suggesting reduced β_2- adrenergic mediated vasodilatation which could be a result of general sympathoinhibition or a specific effect at the β_2-adrenoceptors. The tissue distribution of β_2-adrenoceptors and CB_1 receptors overlaps in many tissues, including cardiovascular system (Hudson *et al.*, 2010). At the cellular level, a complex physical and functional interaction between these two receptors has been demonstrated. There is evidence of co-internalisation of β2-adrenoceptors, with CB_1 receptors, mediated by CB_1 agonist WIN 55,212-2, leading to desensitisation of β_2-adrenoceptors, in human embryonic kidney 293H cells (Hudson *et al.*, 2010).

3.4.4. Effect of CBD on cardiovascular parameters in response to cold stress

Cold stress causes intense sympathoexcitation, producing a tachycardic and pressor response, and an increase in muscle sympathetic nerve activity (MSNA) (Victor *et al.*, 1987; Mathias and Bannister, 1992). The pressor response is due to an initial rise in CO, in response to increased HR and a later increase in MSNA, causing vasoconstriction. Both MAP and TPR show a linear correlation with MSNA during cold stress (Yamamoto *et al.*,

1992). The tachycardic response reflects reciprocal changes in sympathetic and parasympathetic activity and can be variable in healthy individuals (Mourot *et al.*, 2009). In our study, cold stress produced a pressor response in both groups but, interestingly, while SBP and MAP continued to rise with placebo throughout the test period, the pressor response to cold was blunted in subjects who had taken CBD, and SBP and MAP were significantly lower. In keeping with this, TPR was much lower with CBD than placebo, suggesting a possible inhibition of sympathetic outflow. This could also be due to analgesic properties of CBD (Russo, 2008), reducing cold stress and therefore minimizing the sympathetic response (explaining also why the cold pressor test was affected more by CBD than the exercise test). In the animal study of Resstel and colleagues (Resstel *et al.*, 2009), the authors suggested that the modulation of cardiovascular response was most likely secondary to attenuation of emotional response to stress. However, given our findings that CBD produced similar changes in cardiovascular parameters, though to a variable degree, during rest and stress, this may indicate that CBD also has direct cardiovascular effects.

3.5. Safety and tolerance

CBD was well tolerated and there were no adverse events on the day of stress tests. None of the subjects reported any adverse events over the following week.

3.6. Study Limitations

It was an acute study, looking at the cardiovascular effects of a single dose of CBD in young healthy volunteers. One of the study limitations was that it only consisted of male volunteers. Female volunteers were not included in the study for logistic reasons (the need to exclude pregnancy in this young age group) and to eliminate the effects of oestrogen, which attenuates sympathoadrenal responsiveness (Kajantie and Phillips, 2006). Since the

study examined acute effects of CBD on cardiovascular response to stress, drawing conclusions about the chronic cardiovascular effects of CBD from our data may not be very accurate. There is a theoretical possibility that, like THC, tolerance may develop to the cardiovascular effects of CBD with chronic use. It will be interesting to see the impact on cardiovascular parameters in patient population, where there are multiple pathophysiological mechanisms at work. These include increased sympathetic drive, over activation of renin-angiotensin-aldosterone system, left ventricular hypertrophy and chronic changes that take place in vascular endothelium and vascular smooth muscle cells, in response to chronic low grade inflammation and the release of various vasoactive molecules.

3.7. Conclusion

With all its limitations, our data show that a single dose of CBD reduces resting blood pressure and the blood pressure response to stress, particularly cold stress, and especially in the pre and post-test periods. This may reflect the anxiolytic and analgesic effects of CBD, as well as any direct cardiovascular effects such as sympathoinhibition. CBD also affected cardiac parameters, but without affecting cardiac output. Given the increasing use of CBD as a medicinal product, these haemodynamic changes should be considered for people taking CBD. Further research is also required to establish whether CBD has any role in the treatment of cardiovascular disorders such as a hypertension.

4. The effects of CBD and THCV on adipokine secretion from human adipocytes

4.1. Introduction

White adipose tissue, the main site of energy storage, is a dynamic organ with well recognised endocrine function. It responds to both central and peripheral metabolic signals and has the unlimited capacity to grow (Rajala and Scherer, 2003). Adipocytes secrete several important hormones, alongside many other proteins, collectively called as adipokines. There is an ever-growing list of adipokines (e.g. TNFα, IL-6, IL-8, transforming growth factor (TGF)-β, adipsin, acylation-stimulating protein, plasminogen activator inhibitor (PAI)-1, angiotensinogen, retinol binding protein (RBP), cholesteryl transport protein (CETP), adiponectin, leptin, vascular endothelial growth factor (VEGF), haptoglobin and metallothionein etc) that play important roles in many physiological and pathophysiological processes including appetite and energy balance, lipid metabolism, insulin sensitivity, blood pressure, angiogenesis, immunity, haemostasis and inflammation and acute phase response (for review Trayhurn and Wood, 2004; Trayhurn and Wood, 2007). Conditions like lipodystrophy, where there is reduced proportion of functional adipose tissue, are associated with insulin resistance, due to impaired secretion of adipokines and reduced capacity of adipose triglyceride stores. On the other hand, obesity related expansion in adipose tissue mass is associated with the release of various pro-inflammatory cytokines, that cause lipolysis, aggravating the problem of high circulating free fatty acids (FFA), that impair insulin sensitivity (Guilherme *et al.*, 2008).

Leptin, adiponectin and IL-6 are three well known adipokines playing important roles in energy homeostasis, weight regulation and insulin resistance respectively.

4.1.1. Leptin

Leptin is a product of the *obese (ob/ob)* gene that was discovered in 1994 (Zhang *et al.*, 1994). It acts centrally to regulate energy homeostasis (Ahima and Flier, 2000), by upregulating anorexigenic neuropeptides (Schwartz *et al.*, 1997; Seeley *et al.*, 1997) and downregulating orexigenic factors (Stephens *et al.*, 1995), thereby causing reduced food intake. Leptin reduces food intake through its effects on the pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons in the hypothalamus (Cowley *et al.*, 2001). Mutations in *obese (ob)* gene and *diabetes (db)* gene (that encodes leptin receptor) result in morbid obesity and diabetes (Friedman and Halaas, 1998). Leptin administration to *ob/ob* mice improves plasma glucose (Schwartz *et al.*, 1996), independent of its actions on weight, and lowers plasma insulin levels, by a direct inhibitory effect (Emilsson *et al.*, 1997). Working through its receptors on panreatic β-cells, leptin activates the ATP-sensitive K^+ channels, causing hyperpolarization of of β-cells and inhibiting insulin release (Kieffer *et al.*, 1997) . Leptin is secreted mainly by adipose tissue (Ahima and Flier, 2000) and its levels are directly proportional to the degree of adiposity. Factors like fasting and cold exposure reduce leptin secretion by adipose tissue via sympathetic activation (Trayhurn and Beattie, 2001), while insulin promotes adipogenesis and leptin secretion (Seufert *et al.*, 1999). Sensitivity to leptin is reduced in obese individuals (LealCerro *et al.*, 1996), due to changes at cellular level that attenuate leptin signalling and amplify weight gain (Myers *et al.*, 2010). Leptin suppresses expression of proinsuln mRNA within islets and stimulation of proinsulin mRNA in human islets mediated by GLP-1, suggesting an antagonism between the effects of leptin and GLP-1 (Seufert *et al.*, 1999). It has been suggested that, in obesity, there is desensitization of β-cells to the effects of leptin, causing hyperinsulinaemia, which in turn

increases adipogenesis, insulin resistance and leads to the development of diabetes (Seufert *et al.*, 1999).

4.1.2. Adiponectin

Adiponectin, also known as adipocyte complement-related protein of 30 kDa (Arcp30), is produced specifically by the adipose tissue (Nigro *et al.*, 2014) that has important anti-atherogenic and vascular protective properties and plays a role in weight homeostasis and insulin sensitivity (Lihn *et al.*, 2005). It stimulates NO production (Chen *et al.*, 2003b) and attenuates TNFα induced expression of adhesion molecules in vascular endothelial cells (Ouchi *et al.*, 1999a). It enhances hepatic insulin sensitivity and reduces endogenous glucose production (Berg *et al.*, 2001; Combs *et al.*, 2001). It also stimulates fatty acid oxidation and decreases body weight. Its levels are reduced in obesity (Fruebis *et al.*, 2001) and in type 2 diabetes (Hotta *et al.*, 2000). Acute treatment of mice with adiponectin reduces plasma FFA and TG content of muscle and liver and enhances insulin sensitivity and energy expenditure (Yamauchi *et al.*, 2001).

4.1.3. IL-6

Adipocytes secrete a number of cytokines including IL-6. Plasma levels of IL-6 are elevated in obesity (Mohamed-Ali *et al.*, 1997) and are inversely related to insulin sensitivity (Kern *et al.*, 2001). Acute treatment with IL-6, however, increases insulin stimulated glucose uptake in humans and glucose uptake and fatty acid oxidation *in vitro* via AMP-activated protein kinase (Carey *et al.*, 2006). Similarly post-exercise rise in IL-6 enhances insulin sensitivity, increasing insulin mediated glucose uptake and fatty acid oxidation in skeletal muscles. It has been suggested that sustained IL-6 release in obesity and its interaction with other adipokines like TNFα and CRP, may result in sympathetic

nervous system activation, and therefore cause insulin resistance, which is not seen with transient and isolated rises in IL-6, seen post exercise (Smith and Minson, 2012).

THC, the parent compound present in *cannabis sativa* increases adiponectin and TGF-β in the 3T3-L1 adipocytes (Teixeira *et al.*, 2010). There is currently no data on the direct effects of CBD and THCV on adipokine secretion from human adipocytes. Our study, in subjects with diabetes and dyslipidaemia, showed that THCV increased adiponectin while CBD reduced resistin and increased GIP concentrations. In a more recent study, both CBD and THCV reduced lipid levels in hepatocytes and 3T3-L1 adipocytes through increased lipolysis (Silvestri *et al.*, 2015). These data and evidence that both CBD and THCV have prominent anti-inflammatory properties suggest that these phytocannabinoids can modulate certain aspects of obesity and insulin resistance and some of their actions might be mediated through affecting adipokine secretion from the adipose tissue. We therefore hypothesized that both CBD and THCV will interact with mature human adipocytes and this interaction may have an impact on the release of leptin, adiponectin and IL-6.

4.2. Aims

The aim of this study was to establish whether THCV and CBD had a direct effect on adipocytes and wether this interaction translated into changes in the way adipocytes released various adipokines. We selected leptin, adiponectin and IL-6, because of their role in weight homeostasis and insulin reistance, and because of the findings of our clinical study.

4.3. Materials and Methods

4.3.1. Cell culture and Harvesting

Cryopreserved Subcutaneous Human White Preadipocytes (HWP) (passages 4 to 5) from PromoCell® were grown using Dulbecco's Modified Eagle Medium (D-MEM), containing

1000 mg/L D-glucose and sodium pyruvate and by adding a physiological concentration (1ng/mL) of insulin and 10% foetal bovine serum along with penicillin and streptomycin and incubating them in a humidified environment at 37ºC with 5% CO_2. Once 70%-80% confluent, the cells were seeded into 12 and 24 well cell culture dishes. Differentiation was triggered by treating cells with D-MEM® containing 1µM each of dexamethasone, insulin and PPARγ ligand (pioglitazone) for 48 to 72 hours. Once the cells were fully differentiated (Figures 4.1), they were treated with 33.3nM, 0.33µM and 3.33µM concentrations of either CBD or THCV for 2 or 24 hours and medium was collected and cells were harvested separately, using cell lysis buffer (RIPA, Sigma-Aldrich®-UK) with protease and phosphatase inhibitors and stored at -80ºC until analysed for adipokines using ELISA. The protocol was to use 100nM, 1µM and 10µM concentration of CBD and THCV, however by omission, the volume of medium already in each well (1mL) was not taken into consideration when adding 500µL of drug, which meant that the final concentration was one third of the anticipated concentration. As this had already happened in a few experiments before it was realized, we decided to use the same concentrations for all the remaining experiments.

4.3.2. Measurement of protein concentration of cell lysate

Protein concentration of the cell lysates was measured using bicinchoninic acid (BCA) assay (BCA-1 Sigma-Aldrich®-UK), based on the principle of reduction of Cu^{2+} to Cu^{1+}, after Cu^{2+} forms complex with proteins under alkaline conditions. The amount of reduction is proportional to the protein content, and forms a purple solution with peak absorbance of 562nm, read via a plate reader. Frozen cell lysates were taken out of -80°C freezer and allowed to thaw. 1mg of bovine serum albumin (BSA, Sigma-Aldrich®-UK) was mixed with 1mL of radio-immunoprecipitation assay (RIPA, Sigma-Aldrich®-UK) buffer to give 1mg/mL concentration. This stock was used to construct a standard curve by serial

dilutions. 1mL of pure RIPA was used as a blank control. 10µL of each standard was transferred into a 96-well plate, in duplicate. 10µL of each sample was then added into the same 96-well plate. BCA working reagent was made my mixing BCA solution with copper (II) sulfate penthydrate 4% solution (Sigma-Aldrich®-UK), at a 50:1 ratio. 100µL of this was added to each well. The plate was incubated for 30 mins at 37°C with moderate shake and read at 562 nm maximum absorbance. Data was extrapolated by creating a standard curve, using non linear regression and 2nd order polynomial quadritc model, using the GraphPad® software.

4.3.3. Measurement of changes in posphorylated cell signalling proteins after acute exposure of human adipocytes to vehicle and 10µM CBD and THCV

The levels of phosphorylated CREB (Ser133), JNK (Thr183/Tyr185), NF-κB (Ser536), p38 (Thr180/Tyr182), ERK/MAP kinase 1/2 (Thr185/Tyr187), Akt (Ser473), p70 S6 Kinase (Thr412), STAT3 (Ser727) and STAT5A/B (Tyr694/699) were measured in cell lysates using the Luminex® xMAP® technology, using commercially available panel (Milliplex™, 48-680MAG, Merck Millipore). Human preadipocytes were grown as described earlier. Once fully differentiated, they were treated with 10µM each of CBD and THCV or vehicle, for 10 mins. Medium was discarded and cells were collected in cell lysis buffer (RIPA, Sigma-Aldrich®-UK), with phosphatase and protease inhibitors (Roche®) and stored at -80°C for later analysis. The assay was performed according to manufacturer's instructions. 50µL of assay buffer was added to each well of a 96 well plate and left to shake for 10 min at room temperature. Assay buffer was then decanted and 25µL of bead suspension was added to each well. This was followed by adding 25µL of assay buffer to blank wells and 25µL of control and samples to appropriate wells. The plate was then left to incubate overnight on a plate shaker (600-800 rpm), in the dark at 4°C. The next morning samples and controls were decanted and plate washed twice with

100µL of wash buffer, while holding the plate on top of a magnet to prevent loss of beads, followed by addition of 25µL of detection antibody to each well. It was left to incubate, in the dark, at room temperature for 1 hour. The detection antibody was removed and 25µL of streptavadin-PE (SAPE) was added to each well and the plate was left on the shaker for 15 min in the dark, at room temperature. After this 25µL of amplification buffer was added to each well and left for 15 min. The final step was to remove streptavidin-PE/amplification buffer and resuspend beads in 150µL of assay buffer. The plate was then analyzed using the Luminex® system. Data was presented as percentage change in median fluorescence intensity (MFI) of phosphorylated signalling proteins, in response to CBD and THCV, compared to vehicle.

4.3.4. Measurement of adipokines in medium and cells

Duoset enzyme-linked immunosorbent assay (ELISA) kits from R&D systems® were used to measure adiponectin, leptin and IL-6, in the medium and cells. All the kits had required components for developing sandwich ELISA, that included capture and detection antibodies, standard and streptavidin-HRP and they all followed the same principle. Since the kit did not come with quality control reagents (QC's), standards 2 and 6 were used as QC1 and QC2 respectively. The solutions required for the ELISA were developed in the lab or were bought separately. These included; Phosphate buffered saline (PBS, 137mM NaCl, 2.7mM KCL, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2µm filtered), wash buffer (0.05% Tween® 20 in PBS, pH 7.2-7.4), reagent diluent (1% bovine serum albumin in PBS, pH 7.2-7.4, 0.2µm filtered), substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) and stop solution (2 N H_2SO_4).

Capture antibody was diluted to working concentration, using PBS without carrier protein and a 96-well microplate was coated using 100µL per well of diluted capture antibody.

The plate was then left for overnight incubation at room temperature. Next day the plate was aspirated and washed 3 times using 250µL per well of wash buffer and blocked by adding 250µL of reagent diluent to each well and left for 1 hour incubation. This was followed by another aspiration and wash cycle followed by the addition of 100µL of standards and samples to each well. The plate was then left for 2 hours incubation at room temperature followed by aspiration and wash as described above. 100µL of detection antibody was added to each well, covering the plate with a new adhesive strip and leaving it to incubate for 2 hours at room temperature. The plate was aspirated and washed as above and 100µL of working dilution of streptavidin was added to each well and the plate was left for 20 minutes incubation in a dark room. After another wash, 100µL of substrate solution was added to each well and left for 20 minutes incubation in a dark room. The reaction was stopped by adding 50µL of stop solution to each well and optical density of each well was determined immediately using a microplate reader set to 450 nm with wavelength correction for background absorbance. Data was extrapolated by creating standard curve using nonlinear regression. There was significant intra-experiment variability in adiponectin and IL-6 data with coefficient of varation (% CV) ranging from 1-60%. There was less variability in the leptin data, with % CV less than 15% for all except four experiments. The inter-experiment variability was even greater, showing significant difference in the level of adipokines produced by different batches of cells, with % CV of up to 150%. The difference in adipokine secretion was not related to the passage of the cells. %CV for QC1 and QC2, for all the three adipokines, was between 4 and 10%.

4.3.5. Statistical Analyses

All the statistical analyses were performed using GraphPad Prism 6 Software. Data for adipokines and protein content of cell lysates was extrapolated by creating standard curves, using nonlinear regression model. The data for phosphorylated cell signalling proteins was

generated automatically by using Luminex software, and presented as changes in MFI. All data were analysed using one way ANOVA except for cell signalling proteins, where unpaired t-test was used, and presented as mean ± SEM

4.4. Results

4.4.1. Protein content of Human adipocytes incubated with increasing concentrations of CBD and THCV for 2 and 24 hours

There was no significant difference in the protein content of human adipocytes after they were incubated with increasing concentrations of CBD (Figure 4.2; A,B) and THCV (Figure 4.2; C,D) for 2 or 24 hours (n=12, based on 4 separate experiments performed in triplicates).

4.4.2. Changes in the level of phosphorylated signalling proteins after acute exposure of human adipocytes to vehicle and 10μM CBD

We measured the level of phosphorylated signalling proteins in order to see if CBD affected down stream signalling and found that acute exposure of human adipocytes to 10μM CBD, led to a significant increase in the level of phosphorylated ERK 1/2 ($p<0.01$; Figure 4.3E), Akt ($p<0.01$; Figure 4.3F) and p70s6K ($p<0.01$; Figure 4.3G) (n=6, based on 2 separate experiments performed in triplicate).

4.4.3. Changes in the level of phosphorylated signalling proteins after acute exposure of human adipocytes to vehicle and 10μM THCV

Acute exposure of human adipocytes to 10μM THCV, led to a significant increase in the level of NFκB ($p<0.05$; Figure 4.4C), p38 ($p<0.01$; Figure 4.4D), ERK 1/2 ($p<0.0001$; Figure 4.4E), AKT ($p<0.01$; Figure 4.4F) and p70s6K ($p<0.01$; Figure 4.4G) (n=6, based on 6 separate experiments performed in triplicate).

4.4.4. Effect of CBD on adipokine secretion from mature human adipocytes

4.4.4.1. Adiponectin

Compared to vehicle, when measured in medium (Figure 4.5; A,B) and cells (Figure 4.5; C,D), 2 and 24 hours incubation of human adipocytes with CBD had no effect on adiponectin secretion (n=9-12, based on 4 separate experiments performed in triplicate).

4.4.4.2. Leptin

When measured in medium (Figure 4.6A), 2 hours incubation of human adipocytes with 3.33μM CBD caused a significant increase in leptin secretion ($p<0.05$), while 24 hours' incubation had no effect (Figure 4.6B). When measured in cell lysates, 2 or 24 hours incubation, with increasing concentrations of CBD, had no effect on leptin secretion (Figure 4.6;C,D) (n=11-12, based on 4 separate experiments performed in triplicate).

4.4.4.3. IL-6

The amount of IL-6 in cell lysates, though detectable, was negligible (data not shown). When measured in medium, there was a significant decrease in the concentration of IL-6 ($p<0.001$), after 24 hours' incubation with 0.33μM of CBD (Figure 4.7B). 2 hours' incubation with CBD had no effect on IL-6 secretion (Figure 4.7A) (n=10-12, based on 4 separate experiments performed in triplicate).

4.4.5. Effect of THCV on adipokline secretion from mature human adipocytes

4.4.5.1. Adiponectin

2 or 24 hours' incubation of mature human adipocytes with THCV had no effect on adiponecitn secretion, when measured in medium (Figure 4.8; A,B), or cell lysates (Figure 4.8; C,D) (n=9-12, based on 4 separate experiments performed in triplicate).

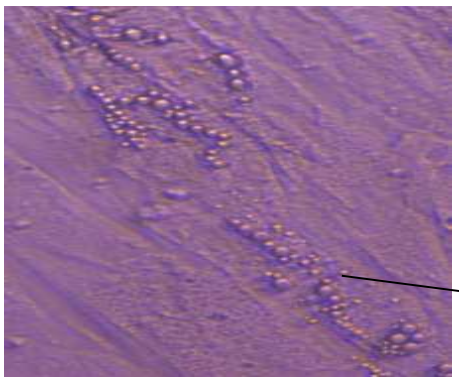
4.4.5.2. Leptin

THCV did not affect leptin secretion from mature human adipocytes after 2 or 24 hours incubation (Figure 4.9; A,B and C,D) (n=11-12, based on 4 separate experiments performed in triplicate).

4.4.5.3. IL-6

There was no significant change in IL-6 concentration, in medium, when human adipocytes were incubated with increasing concentrations of THCV, for 2 (Figure 4.10C) or 24 (Figure 4.10D). The amount of IL-6 in cell lysates was negligible, though detectable (data not shown) (n=10-12, based on 4 separate experiments performed in triplicate).

A



Fat droplets starting to accumulate, while the cells are still spindly (elongated)

B



Fat droplets increasing in size while the cell is becoming more round shaped

C

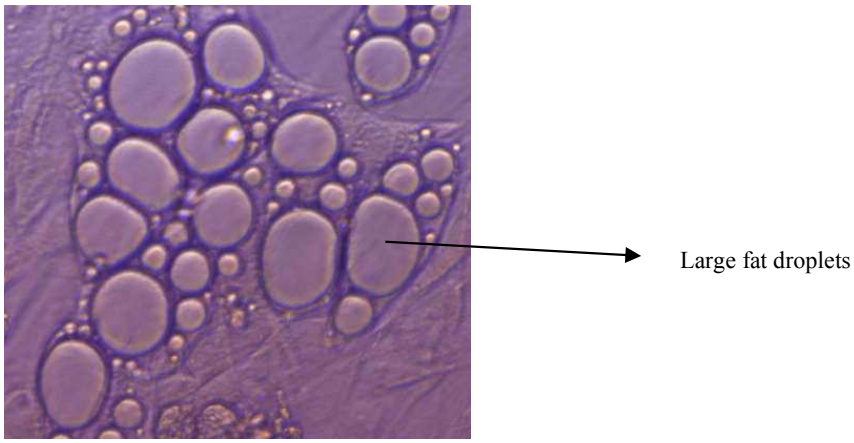


Figure 4.1. Cryopreserved Subcutaneous Human White Preadipocytes (HWP) (passages 4 to 5) from PromoCell® were grown using Dulbecco's Modified Eagle Medium (D-MEM), containing 1000 mg/L D-glucose and sodium pyruvate and by adding a physiological concentration (1ng/mL) of insulin and 10% foetal bovine serum along with penicillin and streptomycin and incubating them in a humidified environment at 37ºC with 5% CO_2. Once 70%-80% confluent, the cells were seeded into 12 and 24 well cell culture dishes. Differentiation was triggered by treating cells with D-MEM® containing 1µM each of dexamethasone, insulin and PPARγ ligand (pioglitazone) for 48 to 72 hours. Panels A, B and C show various stages of cell proliferation

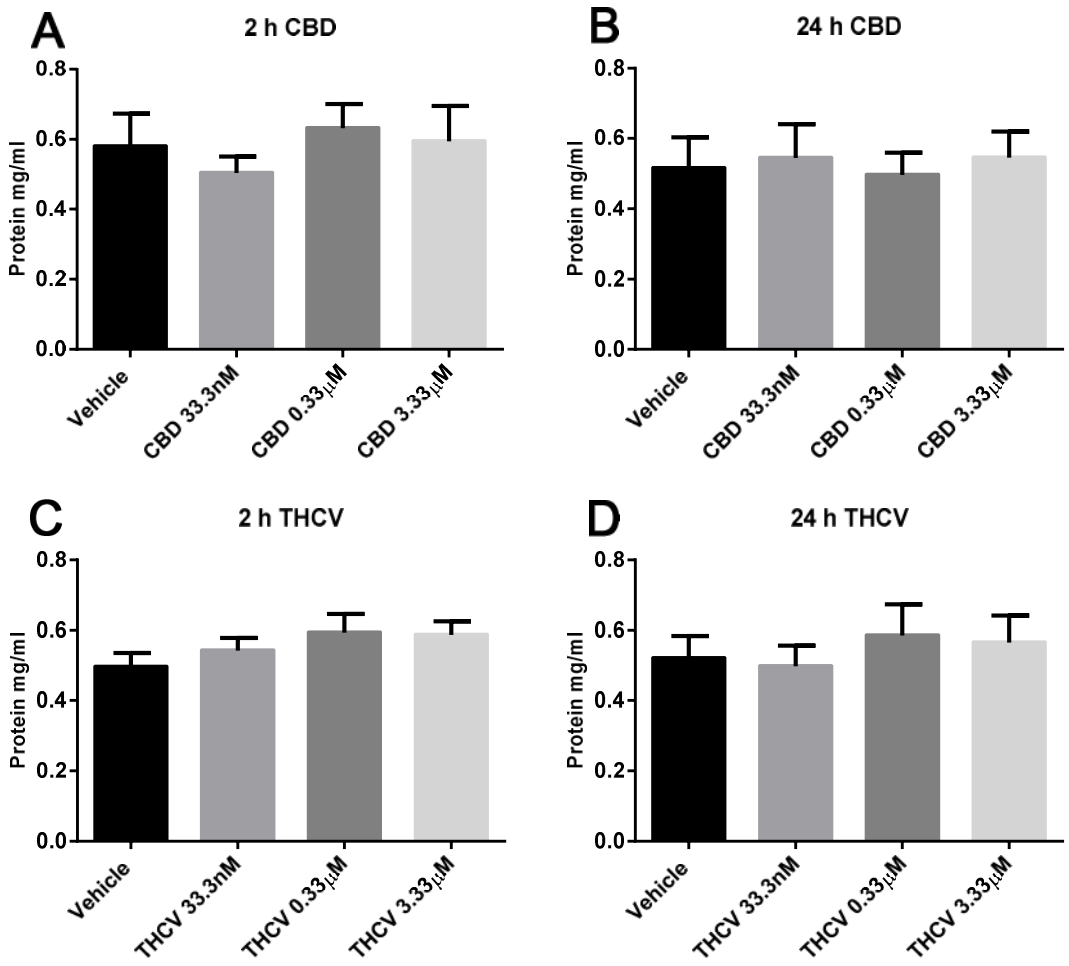


Figure 4.2. Protein content of human adipocytes incubated with increasing concentrations of CBD (A,B) and THCV (C,D) for 2 and 24 hours (n=9-12; based on 4 separate experiments performed in triplicate). Data analysed using one way ANOVA and presented as mean ± SEM.

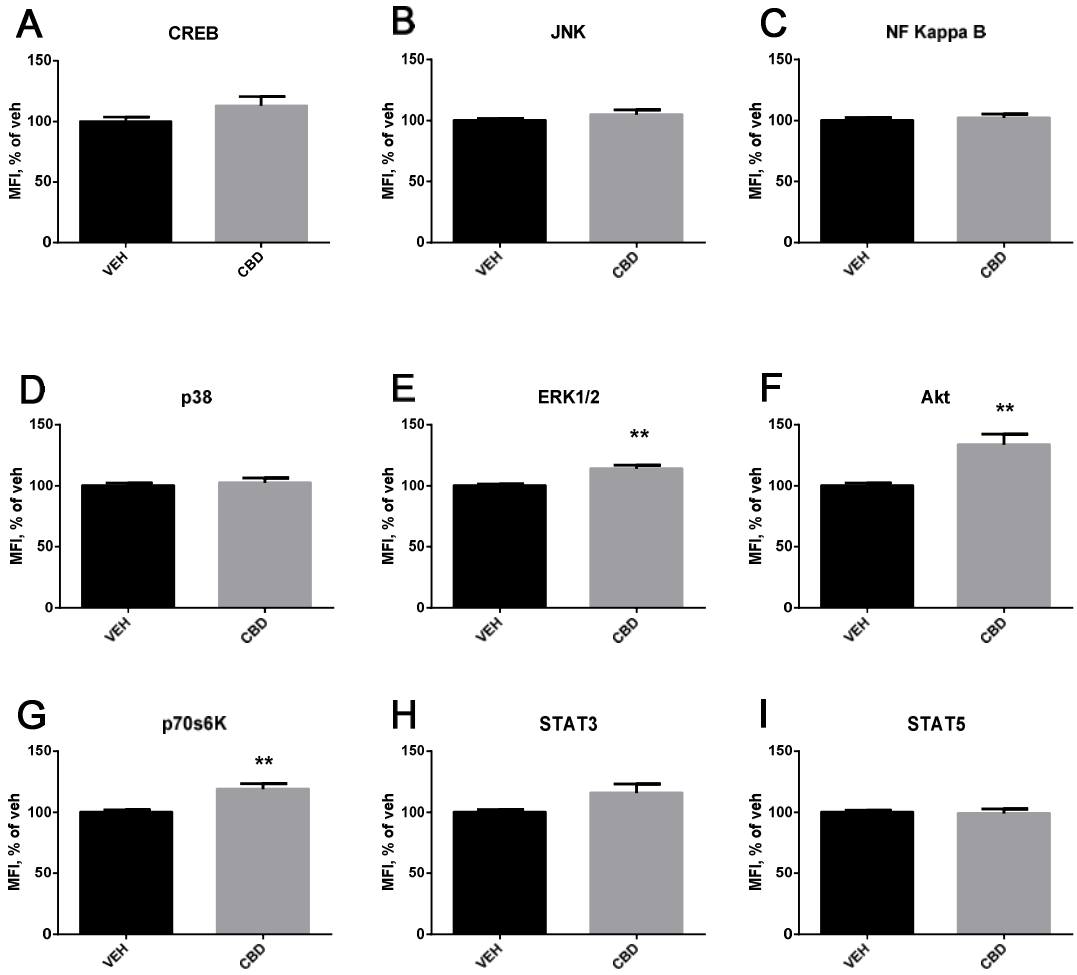


Figure 4.3. Percentage change in the level of phosphorylated signalling proteins after 10 min exposure of fully differentiated human adipocytes to vehicle and 10μM CBD (n=6, based on 2 separate experiments performed in triplicate). Magnetic Bead 9-Plex - cell signalling multiplex assay used to measure these proteins in cell lysates. Data analysed using unpaired t-test and presented as mean ± SEM. (** $p<0.01$).

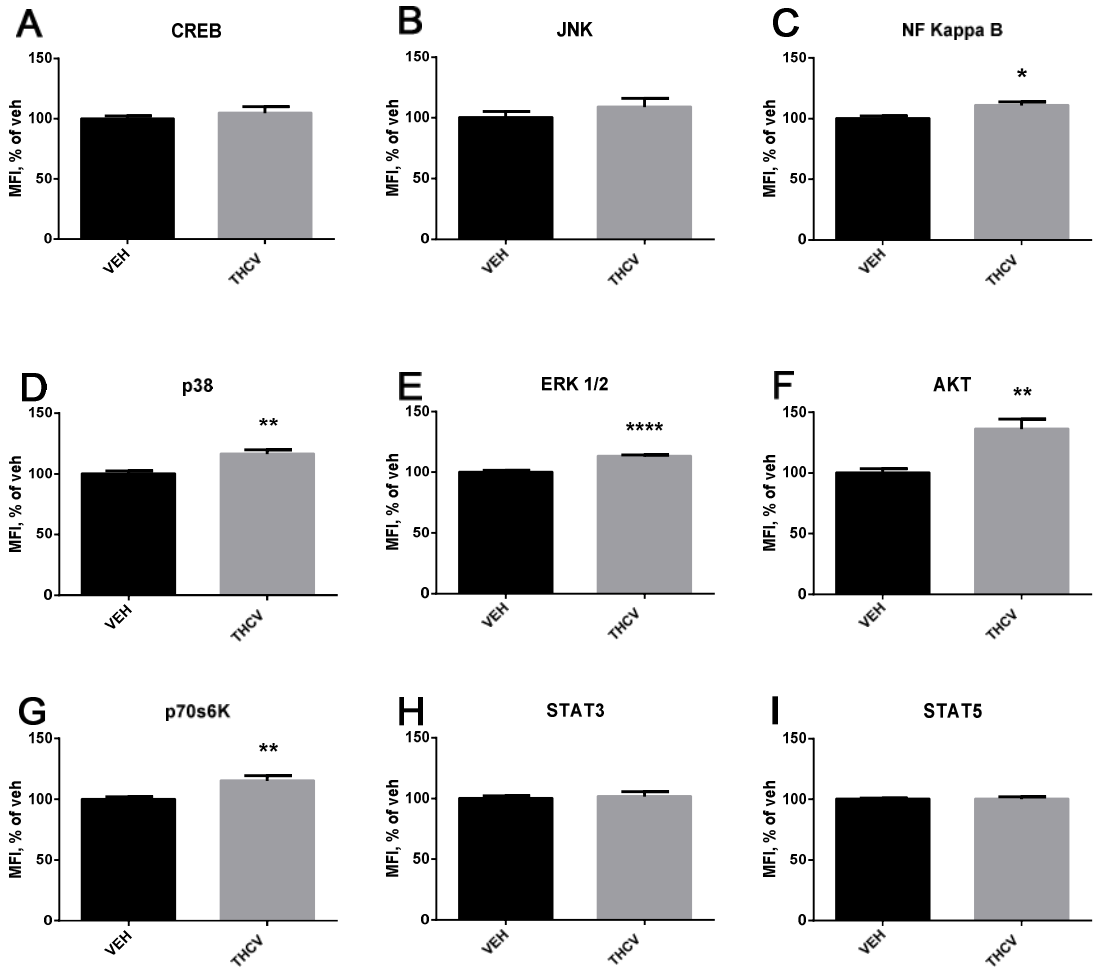


Figure 4.4. Percentage change in the level of phosphorylated signalling proteins after 10 min exposure of fully differentiated human adipocytes to vehicle and 10μM THCV (n=6 based on 2 separate experiments performed in triplicate). Magnetic Bead 9-Plex - cell signalling multiplex assay used to measure these proteins in cell lysates. Data analysed using unpaired t-test and presented as mean ± SEM. (* $p<0.05$; ** $p<0.01$; **** $p<0.0001$)

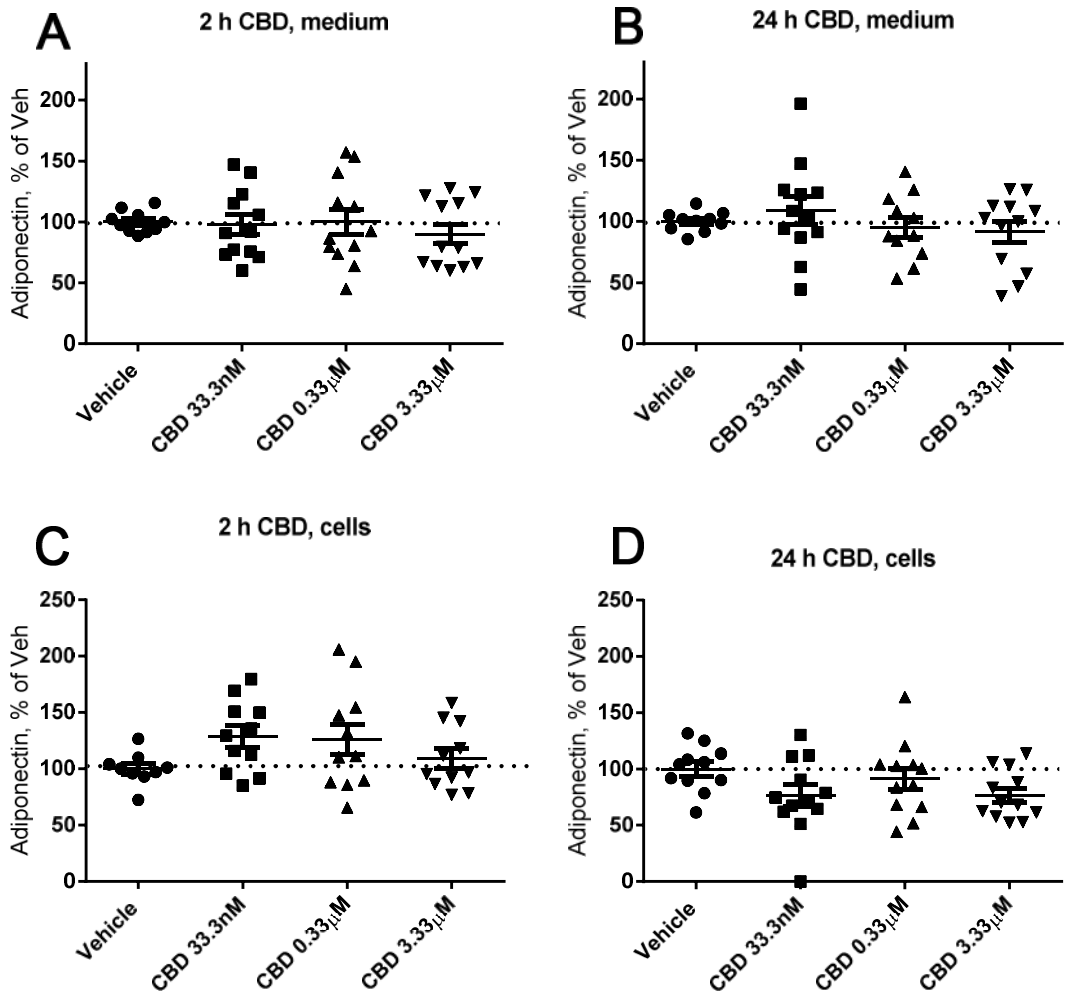


Figure 4.5. Percentage change in the level of adiponectin in medium (A,B) and cells (C,D), after incubating human adipocytes with increasing concentrations of CBD for 2 and 24 hours (n= 9-12 based on 4 separate experiments performed in triplicate). Data analysed using one way ANOVA and presented as mean ± SEM.

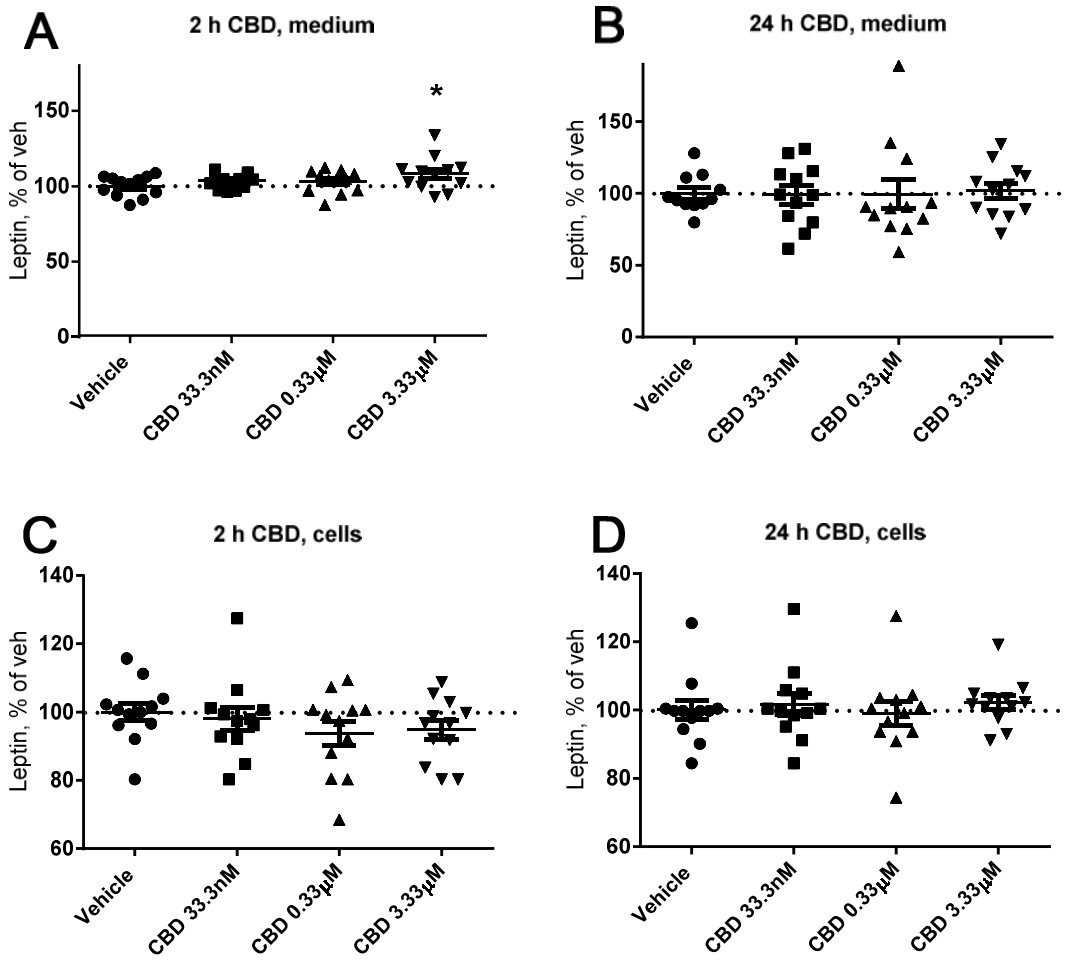


Figure 4.6. Percentage change in the level of leptin in medium (A,B) and cells (C,D) after incubating human adipocytes with increasing concentrations of CBD for 2 and 24 hours (n= 11-12 based on 4 separate experiments performed in triplicate). Data analysed using one way ANOVA and presented as mean ± SEM. (*p<0.05)

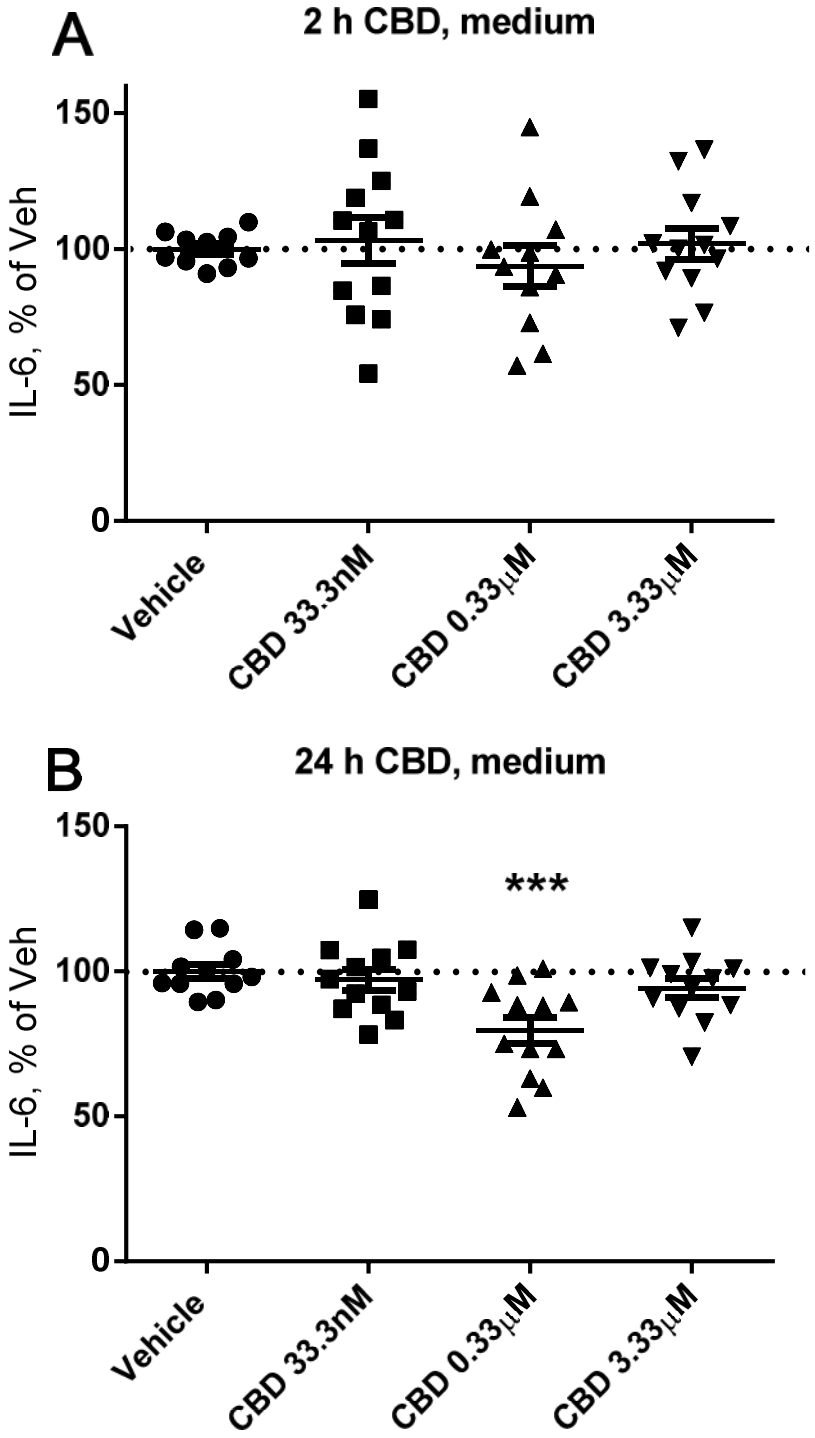


Figure 4.7. Percentage change in the level of IL-6 in medium after incubating human adipocytes with increasing concentrations of CBD for 2 and 24 hours (n=10-12 based on 4 separate experiments performed in triplicate). Data analysed using one way ANOVA and presented as mean ± SEM. (*** $p<0.001$).

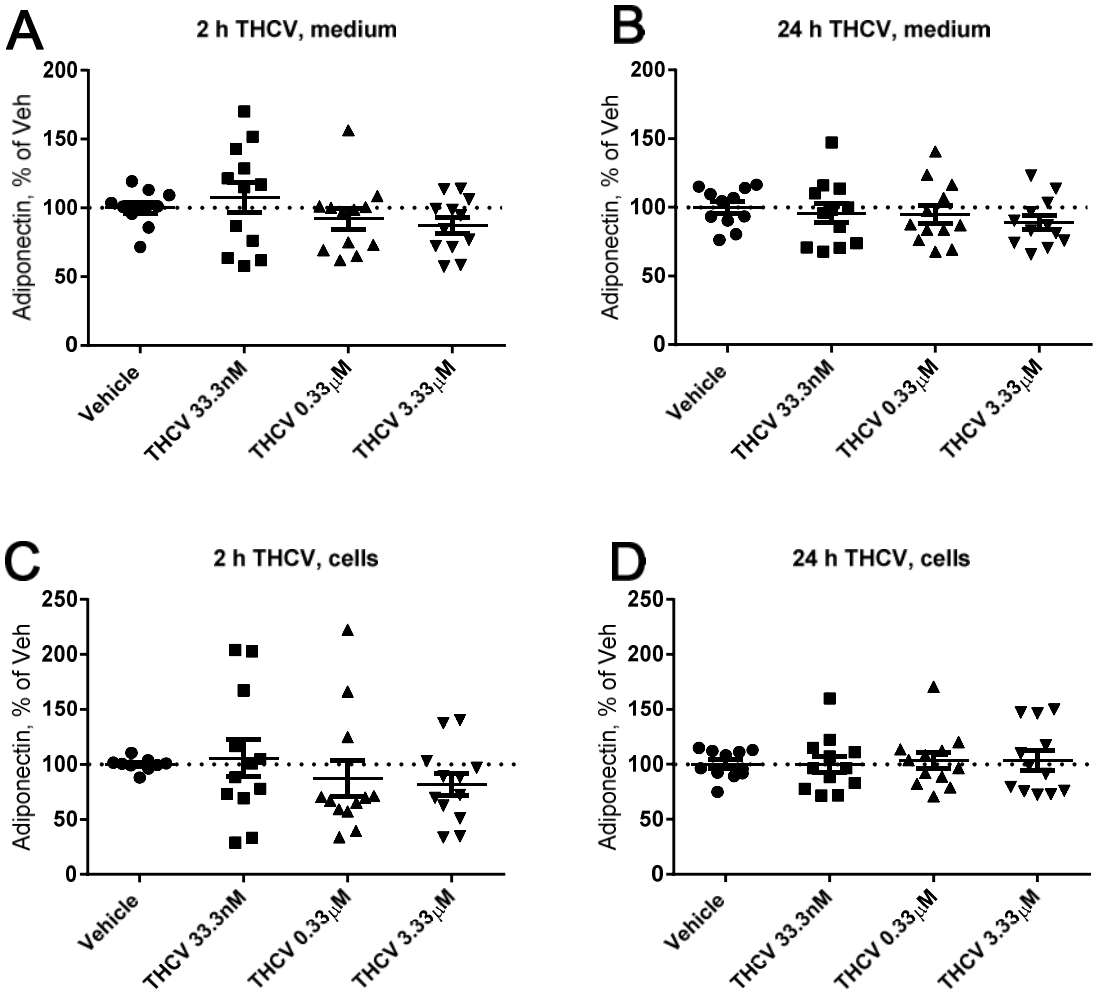


Figure 4.8. Percentage change in the level of adiponectin medium (A,B) and cells (C,D), after incubating human adipocytes with increasing concentrations of THCV for 2 and 24 hours (n= 9 -12 based on 4 separate experiments performed in triplicate). Data analysed using one way ANOVA and presented as mean ± SEM.

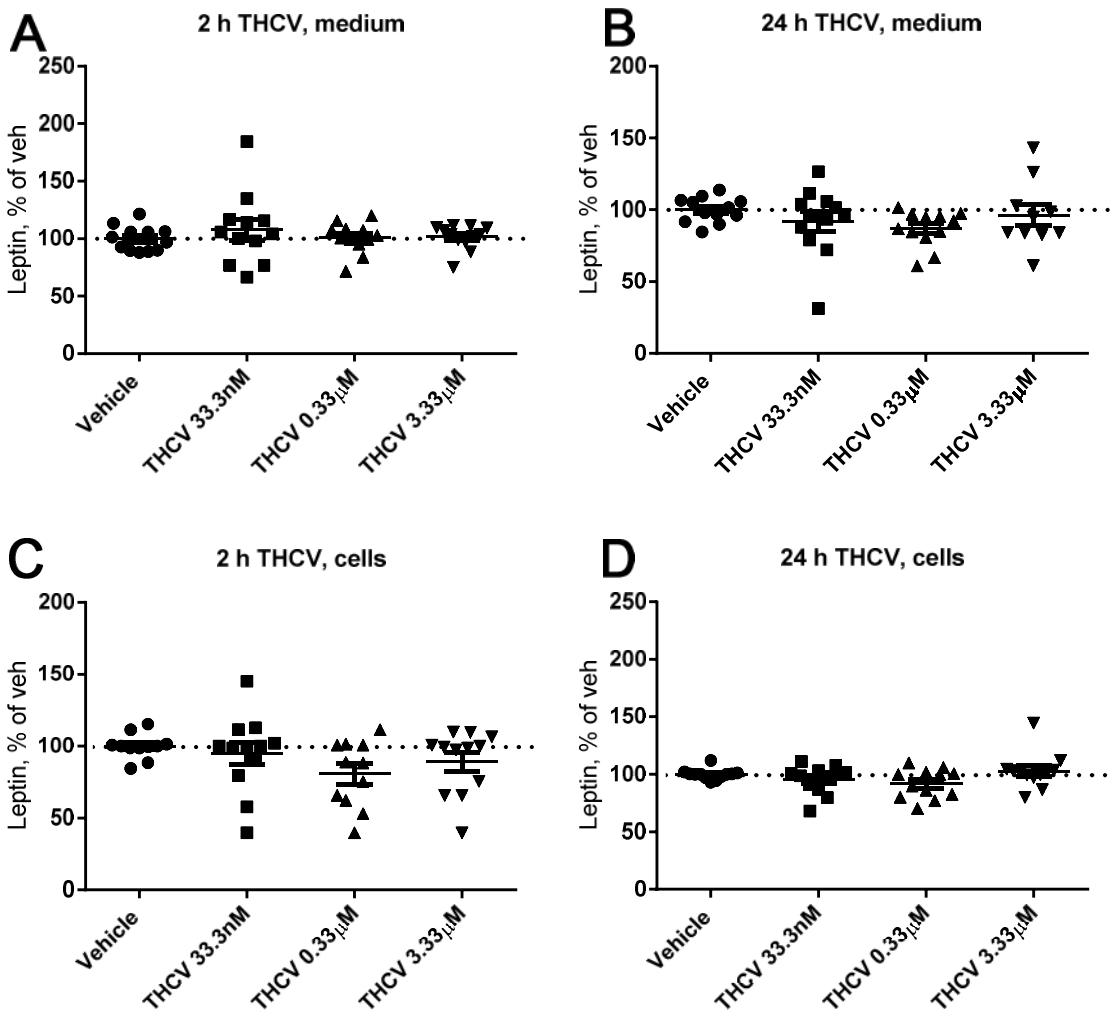


Figure 4.9. Percentage change in the level of leptin in medium (A,B) and cells (C,D) after incubating human adipocytes with increasing concentrations of THCV for 2 and 24 hours (n=11-12 based on 4 separate experiments performed in triplicate). Data analysed using one way ANOVA and presented as mean ± SEM.

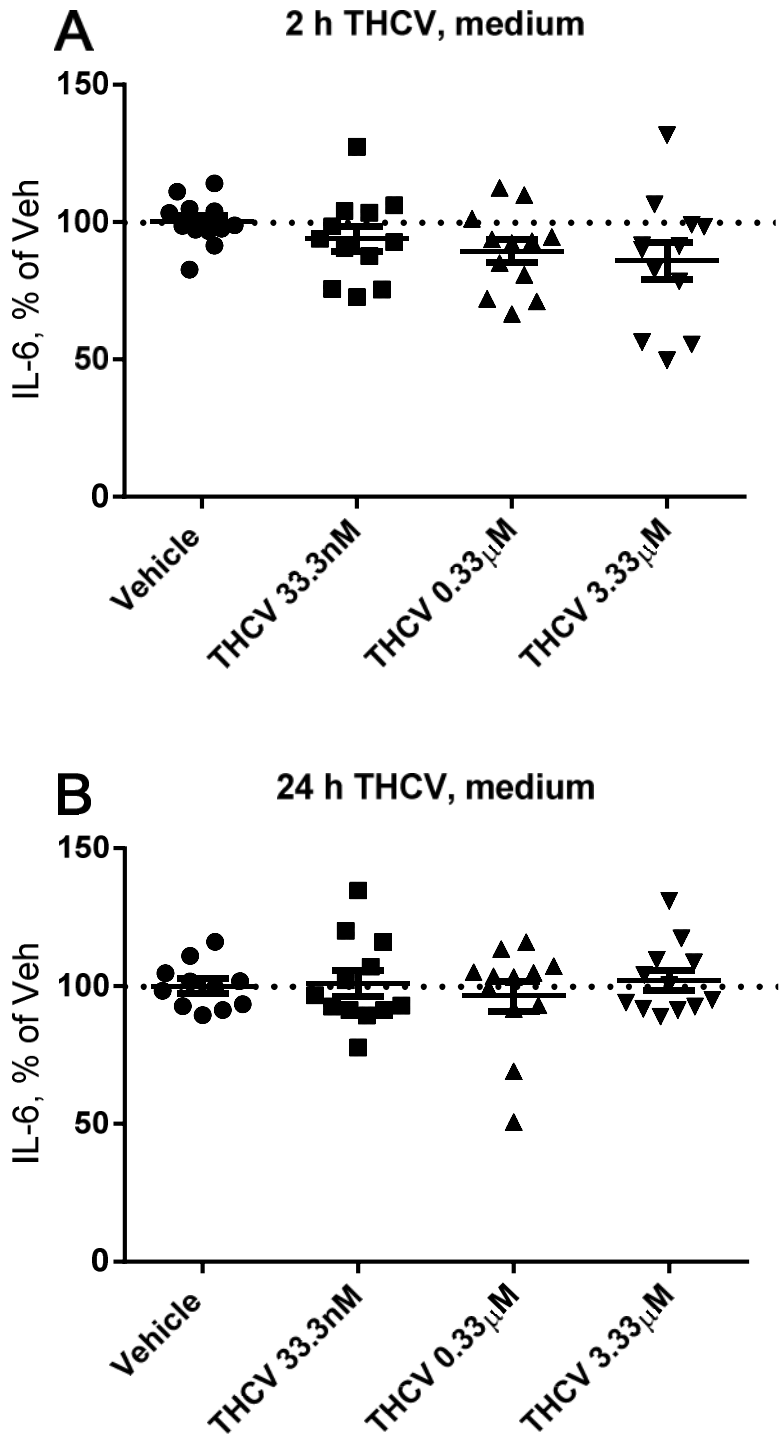


Figure 4.10. Percentage change in the level of IL-6 in medium after incubating human adipocytes with increasing concentrations of THCV for 2 and 24 hours (n=10-12 based on 4 separate experiments performed in triplicate). Data analysed using one way ANOVA and presented as mean ± SEM.

4.5. Discussion

Adipocyte, which is the functional unit of adipose tissue, has an important pathophysiological role in metabolic syndrome and its associated disorders, like type 2 diabetes and dyslipidaemia. It influences various processes by the release of adipokines, such as adiponectin, leptin and IL-6. In our clinical study in subjects with type 2 diabetes and dyslipidaemia, THCV improved glycaemic parameters and increased plasma levels of adiponectin, without having any significant impact on leptin or gut hormones. CBD increased the concentrations of GIP and reduced resistin levels, but had no effect on clinical parameters. The rationale of this study was to see if CBD and THCV interacted with adipocytes, and influenced the release of adipokines. We chose adiponectin, leptin and IL-6 for their roles in insulin sensitivity, weight and appetite homeostasis and obesity related chronic inflammation.

4.5.1. Cell Viability

We did not perform any specific cell viability studies, but measured the protein content of cells exposed to various concentrations of the two phytocannabinoids and placebo. We did not find any significant difference in the protein content of cells, suggesting that none of the compounds was adversely affecting the cells.

4.5.2. Effect of CBD and THCV on downstream signalling

We measured phophorylated signalling proteins after acute exposure of adipocytes to CBD and THCV. The results showed that CBD significantly increased the levels of phosphorylated ERK1/2, Akt and p70S6K. p38 and ERK1/2 play important role in the adipogenesis of human mesenchymal stem cells (Xu *et al.*, 2014). ERK1/2 is also implicated in TNFα mediated lipolysis in human adipocytes (Zhang *et al.*, 2002). Akt has two isoforms, Akt1 and Akt2. Akt2 is responsible for proliferation of preadipocytes to adipocytes, while both Akt1 and Akt2 are essential for insulin-stimulated lipogenesis and

anti-lipolytic effect of insulin (Fisher-Posovszky *et al.*, 2012). Akt is also an important mediator of insulin mediated glucose uptake into adipose tissue (Cong *et al.*, 1997). These data suggest that CBD stimulates downstream signalling proteins that play important roles in proliferation of adipocytes, insulin mediated lipogenesis and glucose uptake into adipose tissue.

THCV caused a significant increase in phosphorylated ERK1/2, NFκB, p38, Akt and p70S6K. p38 mitogen-activated protein kinase (MAPK), plays a role in leptin secretion from human adipose tissue, in response to a combination of TNFα and dexamethasone, implicating that it may have a role in stress related leptin release (Trujillo *et al.*, 2006). THCV increased the levels of p38, but had no effect on leptin secretion, suggesting that its effects may become obvious only under condtions of metabolic stress. p70S6K forms part of the phosphatidylinositol-4,5-biphosphate 3-kinase (PI3)/Akt and mammalian target of rapamycin (mTOR)/p70S6K signalling pathway and is essential for adipogenesis of human mesenchymal cells (Yu *et al.*, 2008). Obesity related hypoxia of adipose tissue and excess release of free fatty acids lead to an inflammatory cascade and activation of various serine kinases via NFκB pathway. This causes altered insulin signalling and insulin resistance (Ye, 2009). The significance of NFκB release from adipocytes in response to THCV is not clear, as THCV has anti-inflammatory properties (Bolognini *et al.*, 2010b) and in our clinical study it improved fasting glycaemia and glycaemic response to oral glucose challenge, without significantly increasing plasma insulin.

4.5.3. CBD affects the release of Leptin and IL-6 from adipocytes

Since both CBD and THCV were able to affect various post receptor signalling proteins, we wanted to see if this had any impact on the release of adiponectin, leptin or IL-6. We found that CBD increased leptin release and inhibited the release of IL-6.

4.5.3.1. Leptin

Leptin is the product of the *ob* gene, secreted by adipocytes (Zhang *et al.*, 1994) and released into systemic circulation (Klein *et al.*, 1996). The exact mechanism by which leptin and other adipokines are secreted by the adipocytes is not known (Rajala and Scherer, 2003). It corsses the blood brain barrier to act on the hypothalamus and produce some of its metabolic effects (Banks *et al.*, 1996). Plasma half life of leptin is about 30 ± 4 min (Klein *et al.*, 1996) and it is cleared from circulation mainly by kidneys (Jensen *et al.*, 1999), in a degraded form, suggesting that it is metabolized prior to clearance. In our study 2 hours exposure of human adipocytes to 3.33μM CBD increased leptin release. The same effect was not seen when adipocytes had been exposed to CBD for 24 hours. This could mean that any secreted leptin was degraded and therefore was not detectable in sufficient quantities after 24 hours exposure. Similarly there was no significant increase in leptin when measured in cell lysates, suggesting that once secreted it does not undergo reuptake into the cells. We did not see any increase in plasma leptin levels in our clinical study, where CBD was given for 13 weeks. This could be related to relatively small dose of CBD (200 mg daily) used in our study. Another explanation would be relatively short half life of CBD, as the duration between the last dose of CBD and plasma samples was approximately 12 hours or more. The first explanation seems more plausible as CBD had no effect on glycaemic control or insulin sensitivity.

4.5.3.2. IL-6

CBD caused a significant reduction in IL-6 secretion from adipocytes, after 24 hour's incubation. IL-6 was not detectable in cell lysates suggesting that once released, it does not undergo reuptake into the cells. CBD is known for its anti-inflammatory properties. It supresses cell mediated and humoral immunity and reduces the development of diabetes in non-obese diabetes prone (NOD) mice (Booz, 2011). It protoects against myocardial

ischaemic reperfusion injury with evidence of reduced inflammation and IL-6 levels in myocardial infarcts (Durst *et al.*, 2007b). More recently CBD has shown its protective effects against hepatic ishcaemia/reperfusion injury, through anti-inflammatory and anti-oxidant properties (Mukhopadhyay *et al.*, 2011). Our finding of suppression of IL-6 production by CBD is in keeping with its known anti-inflammatory properties. Since obesity is associated with a state of chronic inflammation, especially involving the adipose tissue, CBD may have a therapeutic potential in lessening the impact of inflammation and its metabolic consequences and can potentially improve insulin resistance. There was no effet of CBD on plasma levels of IL-6 in our clinical study. Once again this may be related to the relatively small dose of CBD used on our study.

4.5.4. THCV and adipokines

THCV had no effect on adipokine secretion, though in our *in vivo* study, THCV increased plasma levels of adiponectin and improved glycaemic control and 3 hours response to OGTT, without significantly increasing insulin concentrations, suggesting an improvement in insulin sensitivity. This could mean that source of raised plasma adiponectin in our *in vivo* study was not in the adipose tissue. A recent study in rodents showed skeletal muscle to be an important source of adiponectin, which is upregulated under conditions of calorie restriction and exercise (Dai *et al.*, 2013). This is an area that requires further investigation. It would be interesting to see if human skeletal muscle cells secrete adiponectin, under physiological conditions and conditions associated with metabolic stress and whether THCV and CBD has any impact on its release. Another explanation could be that THCV enhances adiponectin secretion from adipose tissue under conditions of metabolic stress, like obesity and diabetes and not under physiological conditions.

There was no effect of THCV on leptin secretion from adipocytes. This could potentially explain why there was no effect on plasma levels of leptin in the clinical study, as leptin is predominantly secreted by the adipocytes. There was trend towards reduction in plasma levels of IL-6 in our *in vivo* study (p=0.07), but THCV had no effect on IL-6 secretion from adipocytes. THCV has anti-inflammatory properties (Bolognini *et al.*, 2010b), but its effects may be more obvious under conditions of metabolic stress and chronic inflammation, as seen in obesity and diabetes. The lack of effect of THCV on adipokine secretion does not mean that THCV has no interaction with human adipocytes. THCV influences various signalling pathways in adipocytes as we saw in this study. It does, however, suggest that this interaction has no influence on the release of various adipokines.

Both CBD and THCV affected downstream signalling in adipocytes, but had no significant effect on adipokine secretion, except for CBD's effect on leptin and IL-6. We therefore did not proceed with exploring the receptor pharmacology.

In our study, CBD increased leptin and reduced IL-6 secretion from adipocytes, while THCV had no effects. Both increased levels of phosphorylated signalling proteins, playing important roles in certain processes relating to adipocyte proliferation. This suggests that both CBD and THCV interact with human adipocytes, but may not directly stimulate adipokine secretion, especially in case of THCV. Further studies are required looking at the effects of CBD and THCV on adipocytes under conditions of metabolic stress.

5. The effects of CBD and THCV on markers of vascular function produced by human aortic endothelial cells

5.1. Introduction

Rather than being a simple physical barrier, vascular endothelium is a dynamic structure, with great structural and functional heterogeneity and is considered an integrated system within itself (Aird, 2007). It plays vital role in various processes, such as, regulation of vascular tone, angiogenesis, transportation of metabolites across the vessel wall, and orchestrates immune and inflammatory responses (Galley and Webster, 2004).

5.1.1. Endothelium derived vasodilators

Endothelium regulates vascular tone by releasing various vasoactive substances. Some of these factors cause vasorelaxation, while others are responsible for vasoconstriction.

5.1.1.1. Nitric Oxide

NO, first identified by Furchgott and Zawadzki, is the main endothelium-derived relaxing factor (Furchgott and Zawadzki, 1980). It is generated, from L-arginine, by the action of endothelial NO synthase (eNOS) (Forstermann and Munzel, 2006). NOS has two other isoforms, neuronal isoform (nNOS), which produces NO to facilitate synaptic neurotransmitter release (Prast and Philippu, 2001), and macrophage or inducible isoform (iNOS), which is only expressed in the presence of inflammatory mediators or stimuli that activate macrophages (Michel and Feron, 1997).

5.1.1.2. Prostacyclin

Prostacyclin is the other endothelium-derived vasodilator substance, generated through the action of cyclooxygenase (COX-2) (Moncada *et al.*, 1977). Its role is limited compared to NO, which in addition to vasodilation inhibits inflammation and thrombosis (Kubes *et al.*,

1991). Expression of COX-2 is dependent on endothelial damage and exposure to inflammatory cytokines (Needleman and Isakson, 1997).

5.1.1.3. Endothelium derived hyperpolarizing factor (EDHF)

Another, yet unidentified, vasodilator is endothelium derived hyperpolarising factor (EDHF). Released from endothelium, this factor modulates the membrane potential and therefore tone of the vascular smooth muscle cells (de Wit and Griffith, 2010).

5.1.2. Endothelium derived vasoconstrictors

Along with the release of vasodilators, endothelial cells mediate vascular tone by releasing various vasoconstrictor substances.

5.1.2.1. Endothelin (ET) - 1

ET-1 is one of the major effectors in this category. It is a potent vasoconstrictor, derived from vascular endothelium that was first identified in 1988 (Yanagisawa *et al.*, 1988). It produces its vasoconstrictor response by acting on ET_A and ET_B receptors in the vascular smooth muscle cells (Davenport *et al.*, 1993). Increased expression of ET-1 is seen in animal models of atherosclerosis (Barton *et al.*, 1998) and in atherosclerotic plaques from human coronary artery disease (Zeiher *et al.*, 1994). Plasma levels of ET-1 are elevated in hypercholesterolaemia and ET-1 is considered a marker of endothelial dysfunction (Lerman *et al.*, 1993). Studies in endothelial cells show that both nascent and oxidised LDL increase the expression of ET-1 (Niemann *et al.*, 2005), while ET-1 stimulates the uptake of oxidised LDL into endothelial cells (Morawietz *et al.*, 2001). ET-1 also enhances oxidative stress, as demonstrated by increasing ROS production in human umbilical vein endothelial cells (Dong *et al.*, 2005). In animal models of hypertension ET_A receptor blockade prevents vascular hypertrophy and attenuates left ventricular hypertrophy, while ET_A receptor antagonists reduce blood pressure in hypertensive patients with chronic

kidney disease (Kawanabe and Nauli, 2011). ET-1 plays important role in vascular inflammation associated with type 2 diabetes and cardiovascular disease, by acting as potent vasoconstrictor, and enhancing oxidative stress and expression of pro-inflammatory cytokines.

5.1.2.2. Thromboxane A (TXA)-2

Thromboxane (TXA_2), derived from prostaglandin H_2 (PGH_2) under the effects of TXA_2 synthase, is another molecule that causes vasoconstriction. PGH_2 itself is derived following the metabolism of arachidonic acid by COX-1 (Feletou *et al.*, 2011). TXA_2 induces platelet aggregation, contraction of vascular smooth muscle cells and has a role in atherogenesis and neovascularisation (Nakahata, 2008). In addition to TXA_2, endothelial cells also control the local angiotensin-II activity (Sitia *et al.*, 2010).

In addition to its effects on vascular tone, vascular endothelium plays important role in homeostasis, through differential activation of pro and anti-coagulant factors in order to limit clot formation where needed and prevent widespread thrombogenesis (Aird, 2007).

5.1.3. Endothelium and adhesion molecules

Endothelial cells also play an important role in the transfer of leukocytes from blood into underlying tissues at sites of inflammation. This coordinated movement is mediated by interactions between leukocytes and endothelial E- and P- selectin. Intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 also play important role (Aird, 2007).

5.1.3.1. E-Selectin

E-selectin also known as endothelial leukocyte adhesion molecule (ELAM) or CD62E, not present constitutively, but expressed on activated endothelial cells (Bevilacqua *et al.*, 1987; Bevilacqua *et al.*, 1989). Its activation is stimulated by TNFα, IL-1 and bacterial

lipopolysaccharide (Wong and Dorovini-Zis, 1996). By interacting with its ligands, it enables leukocytes to roll over endothelial surface, a prerequisite for their adhesion and transendothelial migration (Olofsson *et al.*, 1994).

5.1.3.2. P-Selectin

P-selectin is another related molecule, that is expressed by platelets (Hsu-Lin *et al.*, 1984) and endothelial cells (McEver *et al.*, 1989) on activation, and has been implicated in inflammatory conditions, homeostasis and metastatic spread of tumours (Ludwig *et al.*, 2007).

5.1.3.3. Intercellular adhesion molecule (ICAM) -1

Intercellular adhesion molecule (ICAM)-1, a member of the immunoglobulin superfamily (IgSF) of adhesion molecules, is a cell surface glycoproten, expressed on various cell types including leukocytes and endothelial cells (Mousa, 2008). Its predominant function is recruitment and trafficking of leukocytes, via interaction with integrin molecules, expressed on the surface of various immune cell types (Springer, 1990). Endothelial expression of ICAM-1 is upregulated by pro-inflammatory cytokines including TNFα, IL-1, and IFNγ and ROS (Hubbard and Rothlein, 2000). Animal studies show that ICAM-1 has an important role in the development of atherosclerosis (Bourdillon *et al.*, 2000).

5.1.3.4. Vascular cell adhesion molecule (VCAM) - 1

Like ICAM-1, vascular cell adhesion molecule (VCAM)-1 (CD106) belongs to immunoglobulin superfamily of proteins (Rice and Bevilacqua, 1989; Osborn *et al.*, 1989). Expression of VCAM-1 on activated endothelial cells mediates rolling and adhesion of leukocytes, required for their recruitment and migration into tissues (Ley *et al.*, 2007). VCAM-1 plays an important role in the initiation of atherosclerosis (Cybulsky *et al.*, 2001). VCAM-1 also plays a central role in angiogenesis mediated by oxidative stress

(Dong *et al.*, 2011). Not surprisingly, therefore, increased expression of VCAM-1 and ICAM-1 in plasma has been reported in various conditions associated with vascular inflammation including metabolic syndrome (Kressel *et al.*, 2009), type 2 diabetes (Bruno *et al.*, 2008) and haemodialysis (Papayianni *et al.*, 2002).

5.1.4. Endothelial dysfunction

Endothelial dysfunction is characterised by impaired endothelium-dependent vasorelaxation in response to various biochemical stimuli and manoeuvres that increase shear stress. The mechanisms underlying endothelial dysfunction may vary depending on the cardiovascular disorder, however reduced bioavailability of NO is observed in most of these (Su, 2015). Conditions like obesity, hyperglycaemia and diabetes, hypertension, raised cholesterol and aging all lead to endothelial dysfunction (Park *et al.*, 2000). Vascular endothelial dysfunction plays an important role in the development of atherosclerosis, the hallmark of macrovascular disease (Gimbrone and Garcia-Cardena, 2013).

5.1.5. Endothelial activation

In response to inflammatory stimuli, endothelial cells undergo a phenotypic change called endothelial activation, leading to increased permeability and reduced antithrombotic properties (Pober and Sessa, 2007). This is characterised by expression of cell-surface adhesion molecules like VCAM-1, ICAM-1, and E-selectin, leading to attachment of circulating leukocytes to vessel wall. NO inhibits endothelial activation, through inhibition of NFκB. NFκB is known for its role in the induction of adhesion molecules in response to pro-inflammatory cytokines, turbulent flow and advanced glycation end-products (AGEs) (Liao, 2013). Mediators of endothelial activation include TNFα, IL-1β, bacterial

lipopolysaccharide, oxidized LDL-cholesterol, angiotensin II, ET-1, shear stress and fibronectin and fibrinogen (Pober and Sessa, 2007).

Vascular endothelium is therefore a dynamic structure, that plays a crucial role in maintaining normal homeostasis and its dysfunction and activation play major role in various metabolic and cardiovascular disorders. Two important underlying mechanisms are impaired vascular relaxation and increase vasoconstriction to various stimuli and an interaction of endothelial cells with immune cells. ET-1 and VCAM-1 play significant roles in these two processes respectively.

5.1.6. ECS and cardiovascular system

Components of the endocannabinoid system are present within the cardiovascular system and play important roles in various physiological and pathophysiological processes. Both CB_1 and CB_2 receptors exist in the myocardium and human coronary artery endothelial and smooth muscle cells (Bonz *et al.*, 2003; Mukhopadhyay *et al.*, 2007; Rajesh *et al.*, 2007c; Rajesh *et al.*, 2008). AEA, one of the endocannabinoids, causes vasodilatation through CB_1 (Varga *et al.*, 1995) and TRPV1 (Zygmunt *et al.*, 1999a) receptors, and is implicated in CB_1 mediated hypotension in shock, an affect which is blocked by SR141716A (Malinowska *et al.*, 2008). 2-AG also causes vasodilatation through CB_1 (Jarai *et al.*, 2000) and the vasodilatory effect of cannabinoids is absent in CB_1 knock out mice (Jarai *et al.*, 1999). *In vivo* and *in vitro* CB_1 receptor blockage improves aortic endothelium-dependent vasodilatation, mainly through its antioxidative effects (Tiyerili *et al.*, 2010). Selective CB_1 antagonist, rimonabant, reduces obesity related rises in systolic blood pressure, in Obese Zucker rats (Mingorance *et al.*, 2009) and humans (Ruilope *et al.*, 2008). The role of CBD in modulating the cardiovascular system and its potent anti-inflammatory properties have been discussed in detail in the introduction and chapter 3.

Currently there are no data on the effects of THCV on vascular endothelium. Δ^8-THCV, a synthetic analogue of THCV, prevents hepatic ischemia/reperfusion injury through its anti-inflammatory and anti-oxidative properties. Among the plethora of effects, Δ^8-THCV reduces the expression of chemokines (CCL3 and CXCL2), TNFα, ICAM-1 mRNA levels and tissue neutrophil infiltration (Batkai *et al.*, 2012). In our clinical study in healthy volunteers, CBD reduced resting blood pressure and blood pressure response to stress. In the clinical study in type 2 diabetic subjects, there was no significant change in the cardiovascular parameters, but there was a trend towards lower blood pressure with THCV.

5.2. Aims

Given above findings and the important role of endothelial cell that acts as a functional unit of cardiovascular system, the aim of current study was to see if CBD and THCV interacted with vascular endothelium and influenced the release of ET-1 and VCAM-1. We also probed the receptor pharmacology and investigated the effects of CBD and THCV on downstream signalling proteins.

5.3. Materials and Methods

5.3.1. Cell Culture and Harvesting

Cryopreserved HAoEC (passages 6 and 7) were grown using Endothelial Cell Growth Medium MV-PromoCell® (ready-to-use) with supplimental mix, containing foetal calf serum (0.05 mL/mL), endothelial cell growth suppliment (0.004 mL/mL), recombinant human epidermal growth factor (10 ng/mL), heparin (90 µg/mL) and hydrocortisone (1 µg/mL). The cells were incubated in a humidified environment at 37°C with 5% CO_2. Once 70-80% confluent, the cells were seeded into 12 well cell culture dishes. Once 100% confluent, cells were treated with 100nM, 1µM and 10µM concentrations of either CBD or

THCV for 24 hours. Medium was collected and cells harvested separately, using cell lysis buffer with protease and phosphatase inhibitors (RIPA, Sigma-Aldrich®-UK) and stored at -80°C until analysis for markers of vascular function.

5.3.2. Measurement of protein concentration of cell lysate

Protein concentration of the cell lysates was measured using BCA assay, as described in detail in section 4.3.2.

5.3.3. Measurement of changes in phosphorylated cell signalling proteins after acute exposure of HAoEC to vehicle and 10µM CBD and THCV

The levels of phosphorylated cell signalling proteins were measured in cell lysates as described above in section 4.3.3.

5.3.4. Measurement of markers of vascular function in medium

Duoset ELISA kits from R&D systems® were used to measure VCAM-1 and ET-1, in the medium. All the kits had required components for developing sandwich ELISA, that included capture and detection antibodies, standard and streptavidin-HRP and they all followed the same principle. Since the kit did not come with QC, standard 2 and 6 were used as QC1 and QC2 respectively. The solutions required for the ELISA were developed in the lab or were bought separately. These included; Phosphate buffered saline (PBS, 137mM NaCl, 2.7mM KCL, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2µm filtered), wash buffer (0.05% Tween® 20 in PBS, pH 7.2-7.4), reagent diluent (1% bovine serum albumin in PBS, pH 7.2-7.4, 0.2µm filtered), substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) and stop solution (2 N H_2SO_4). Capture antibody was diluted to the working concentration, using PBS without carrier protein and a 96-well microplate was coated using 100µL per well of diluted capture antibody. The plate was left for overnight incubation at room temperature. Next day the plate was aspirated and washed

3 times using 250µL per well of wash buffer and blocked by adding 250µL of reagent diluent to each well and left for 1 hour. This was followed by another aspiration and wash cycle followed by the addition of 100µL of standards and samples to each well. After 2 hours incubation at room temperature and an aspiration and wash, 100µL of detection antibody was added to each well. The plate was covered with a new adhesive strip and left to incubate for 2 hours at room temperature. The plate was aspirated and washed and 100µL of working dilution of streptavidin was added to each well and the plate left for 20 minutes incubation in a dark room. After another wash, 100µL of substrate solution was added to each well and left for another 20 minutes incubation in a dark room. The reaction was stopped by adding 50µL of stop solution to each well and optical density of each well was determined immediately using a microplate reader set to 450nm with wavelength correction for background absorbance. Data was extrapolated by creating standard curve using nonlinear regression. %CV for QC1 and QC2 for all the experiments was from 4 to 7%. The intra and interexperiment %CV for all experiments was between 2 and 11%.

5.3.5. Measurement of markers of vascular function in medium after incubating HAoEC, for 24 hours, with 3µM CBD and THCV on their own and in the presence of various antagonists

HAoEC were grown as described. Once fully confluent, cells were treated with 3µM CBD or THCV on their own and in the presence of AM251 (CB_1 antagonist), AM630 (CB_2 antagonist/inverse agonist), capsazepine (CAPZ) (TRPV1 antagonist), O-1918 (antagonist at non-CB_1/CB_2 endothelial receptor (CB_e) and inhibitor of phosphatidylinositol 3-kinase/Akt pathway), GW 9662 (PPARγ antagonist), CID 16020046 (GPR55 antagonist) and indomethacin (cyclooxygenase (COX) inhibitor), for 24 hours. Medium and cells were collected as described and stored at -80°C for analysis. Since the response to both CBD and THCV seemed concentration dependent, with significant reduction in VCAM-1 and

ET-1 with 10µM concentration, we decided to use 3µM concentration for these experiments.

5.3.6. Measurement of markers of vascular function in medium after incubating HAoEC, for 24 hours, with 3µM CBD and THCV on their own and in the presence of signalling inhibitors

HAoEC were grown as described. Once fully confluent, cells were treated with 3µM CBD or THCV on their own and in the presence of PD98059 (MAP kinase cascade inhibitor), LY294002 (PI3K/Akt kinase pathway inhibitor), GO6983 (Protein kinase C (PKC) inhibitor) and KT5720 (Protein kinase A inhibitor) from R&D Systems. Medium and cells were collected as described and stored at -80°C for analysis. p38 MAP kinase regulates expression of VCAM-1 in endothelial cells, in response to various factors (Pietersma *et al.*, 1997; Hsu *et al.*, 2011) and plays important role in the development of atherosclerosis and myocardial ischemia induced inflammation and cardiac dysfunction (Seeger *et al.*, 2010). PI3K/Akt signalling is important for cellular proliferation, adhesion, migration and cell survival. It also has important role to play in angiogenesis in normal as well as in cancer tissue (Karar and Maity, 2011). Hyperglycaemia and diabetes are associated with activation of protein kinase C in vascualr endothelial cells (Rask-Madsen and King, 2005). Activation of protein kinase C in endothelial cells inhibits insulin and VEGF mediated Akt activation, Akt-dependent eNOS regulation and causes endothelial dysfunction, seen in obesity related insulin resistance (Naruse *et al.*, 2006). Hyperglycaemia mediated activation of protein kinase C plays important role in the development of various macro and microvascular complications of diabetes, with some clinical trials showing potential for protein kinase C- β inhibitor in diabetic vascular complications (Geraldes and King, 2010). Protein kinase A or cAMP-dependent protein kinase induces expression of various genes, through phosphorylation of CRE-binding factors. In endothelial cells, activation of

PKA inhibits induction of pro-inflammatory E-selectin, VCAM-1 and tissue factor (Ollivier *et al.*, 1996).

5.3.7. Statistical Analyses

All statistical analyses were performed using GraphPad Prism 6 Software. Data for VCAM-1, ET-1 and protein content of cell lysates was extrapolated by creating standard curves, using nonlinear regression model. The data for phosphorylated cell signalling proteins was generated by using Luminex software, and presented as changes in MFI. All data were analysed using one way ANOVA except for cell signalling proteins, where unpaired t-test was used, and presented as mean ± SEM.

5.4. Results

5.4.1. Protein content of HAoEC incubated with increasing concentrations of CBD and THCV

Cells lysates were not available from the first experiment where HAoEC were incubated for 24 hours with increasing concentrations of CBD and THCV. For the remaining two experiments, there was no difference in the protein content of the cells (Figure 5.1 A,B)

5.4.2. Changes in the level of phosphorylated signalling proteins after acute exposure of HAoEC to vehicle and 10µM CBD

Acute exposure of HAoEC to 10µM CBD caused an increase in the level of phosphorylated ERK/MAP kinase 1/2 (Thr185/Tyr187) (p<0.05; Figure 5.2E) and reduction in the level of STAT 5 (p<0.05; Figure 5.2I). The reduction in level of STAT 5 was only significant for the data normalized for protein content of the cells.

5.4.3. Changes in the level of phosphorylated signalling proteins after acute exposure of HAoEC to vehicle and 10µM THCV

Acute exposure of HAoEC to 10µM THCV caused a significant increase in the level of phosphorylated CREB (Ser133) (p<0.0001; Figure 5.3A), JNK (Thr183/Tyr185) (p<0.05;

Figure 5.3B), p38 (Thr180/Tyr182) (p<0.0001; Figure 5.3D), ERK/MAP kinase 1/2 (Thr185/Tyr187) (p<0.0001; Figure 5.3E) and Akt (Ser473) (p<0.0001; Figure 5.3F). The change in signalling proteins was significant irrespective of normalization of data.

5.4.4. Effect of CBD and THCV on VCAM-1 secretion from HAoEC

5.4.4.1. 24 hours' incubation of HAoEC with increasing concentrations of CBD and THCV

24 hours' incubation with 10μM CBD (Figure 5.4A,B; p<0.0001) and 10μM THCV (Figure 5.4C,D; p<0.0001, p<0.05 respectively), caused a significant reduction in the secretion of VCAM-1.

5.4.4.2. 24 hours' incubation of HAoEC with 3μM CBD and 3μM THCV in the presence of various antagonists and COX inhibitor

24 hours' incubation of HAoEC with 3μM CBD alone or in the presence of AM251, AM630, CAPZ, O-1918, GW9662, CID16020046 and indomethacin, had no significant effect on the secretion of VCAM-1 (Figure 5.5A,B). 3μM THCV on its own had no significant effect on the secretion of VCAM-1, however in the presence of CAPZ (p<0.001), O-1918 (p<0.01) and indomethacin (p<0.05), there was a significant reduction in the secretion of VCAM-1 (Figure 5.5C,D). We did not study the effects of antagonists, on their own, on the release of VCAM-1 from HAoEC

5.4.5. Effect of CBD and THCV on ET-1 secretion from HAoEC

5.4.5.1. 24 hours' incubation of HAoEC with increasing concentrations of CBD and THCV

24 hours' incubation with 10μM CBD (Figure 5.6A,B; p<0.05) and 10μM THCV (Figure 5.6C,D; p<0.01), caused a significant reduction in the secretion of ET-1.

5.4.5.2. 24 hours' incubation of HAoEC with 3µM CBD and 3µM THCV in the presence of various antagonists

24 hours' incubation of HAoEC with 3µM CBD (Figure 5.7A,B) and 3µM THCV (Figure 5.7C,D), on their own and in the presence of AM251, AM630, CAPZ, O-1918, GW9662, CID16020046 and indomethacin, had no significant effect on the secretion of ET-1.

5.4.6. Effect of 24 hour's incubation with 3µM CBD and 3µM THCV, on VCAM-1, in the presence of signalling inhibitors

3µM CBD had no effect on the release of VCAM-1 from HAoEC (Figure 5.8A), on its own, and in the presence of signalling inhibitors PD98059, LY294002, and KT5720, except for GO6983. In the presence of GO6983, there was a significant reduction in the release of VCAM-1. 3µM THCV had no effect on the release of VCAM-1, on its own, and in the presence of signalling inhibitors (Figure 5.8 B).

5.4.7. Effect of 24 hour's incubation with 3µM CBD and 3µM THCV, on ET-1, in the presence of signalling inhibitors

At 3µM concentration, both CBD (Figure 5.9 A) and THCV (Figure 5.9B) had no effect on the release on ET-1, from HAoEC, on their own and in the presence of signalling inhibitors PD 98059, LY 294, KT 5720, and GO 6983.

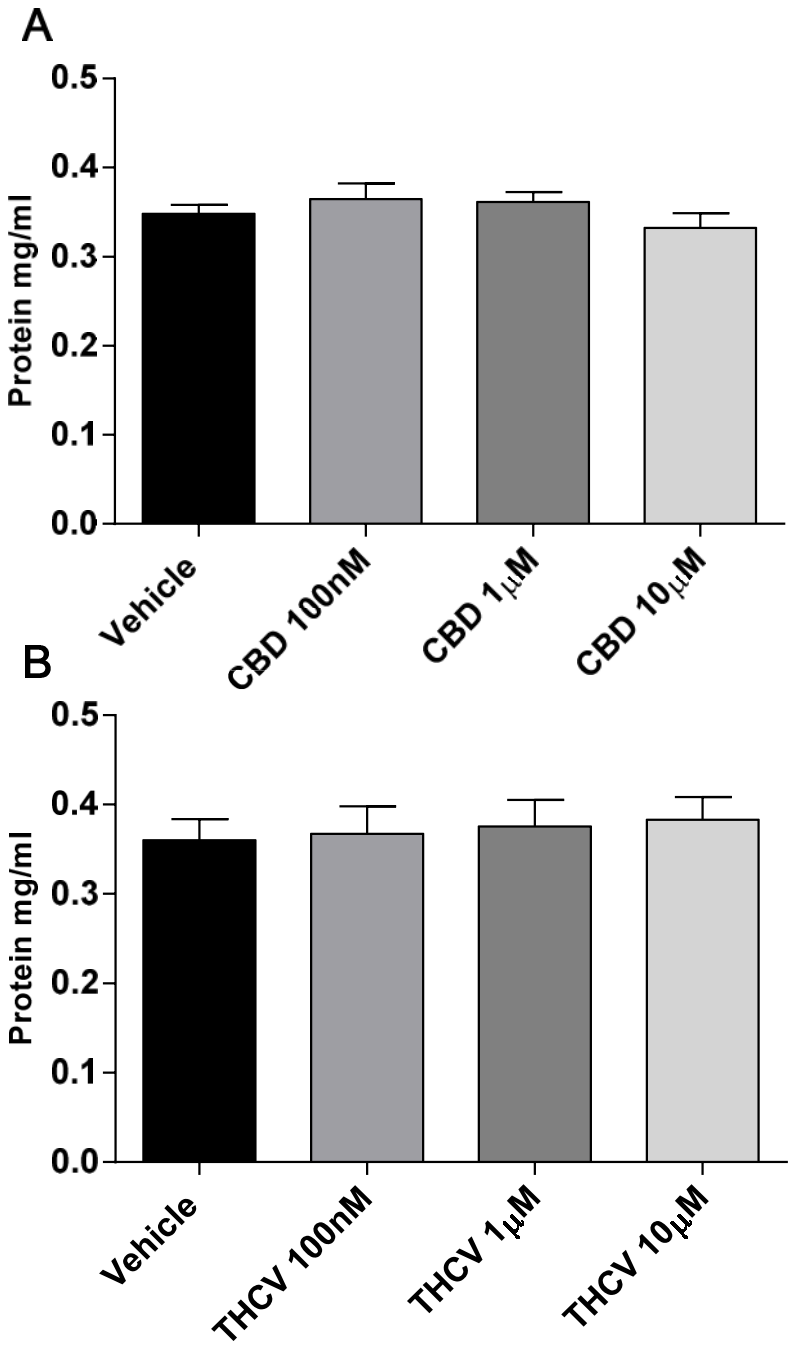


Figure 5.1. Protein content of HAoEC incubated, for 24 hours, with increasing concentrations of CBD (A) and THCV (B). (n=6, based on experiments two and three performed in triplicate- cell lystes from first expreiment were not available). Data analyzed using one way ANOVA and presented as mean ± SEM.

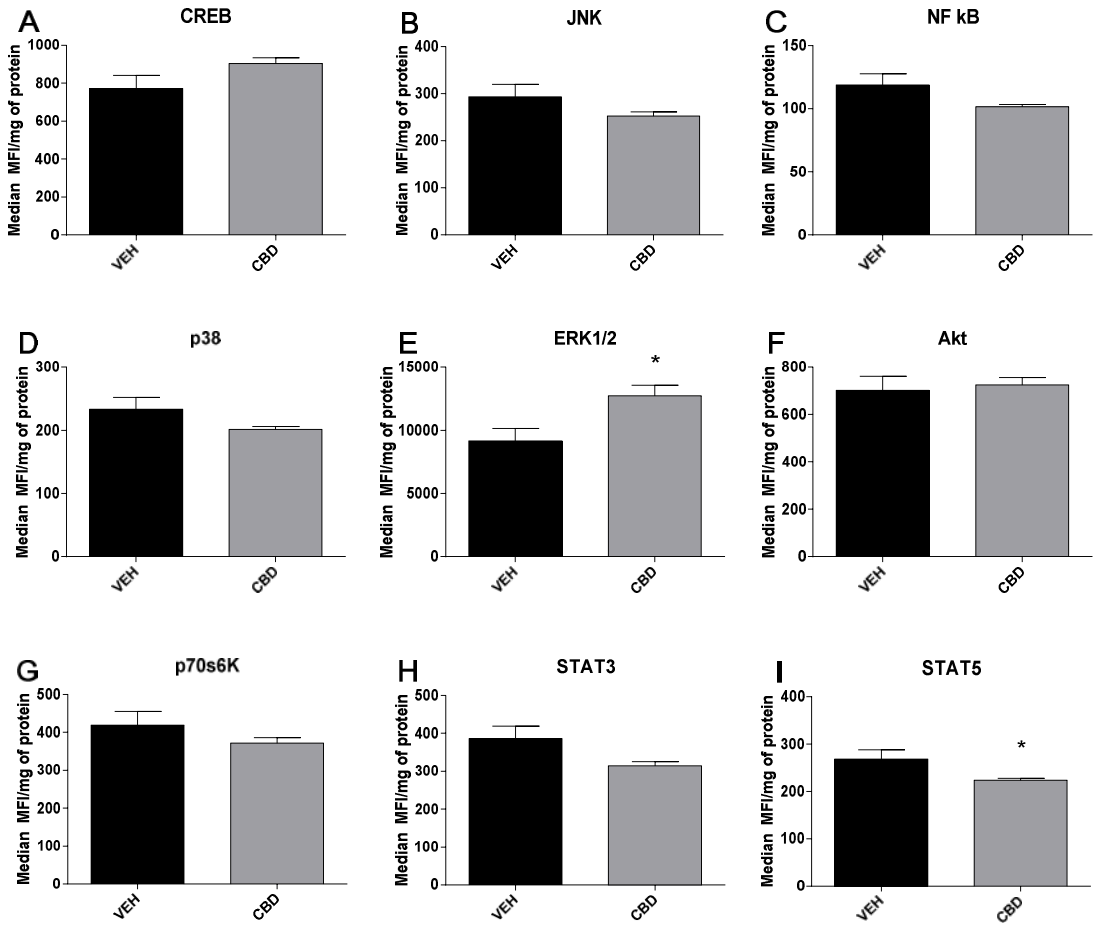


Figure 5.2. Normalized data showing changes in the level of phosphorylated signalling proteins after 10 min exposure of human aortic endothelial cells (HAoEC) to vehicle and 10 μM CBD (n=6 based on a single experiment). Cell Signalling Multiplex Assay used to measure proteins in cell lysates. Data analysed using unpaired t-test and presented as mean ± SEM. (* $p<0.05$).

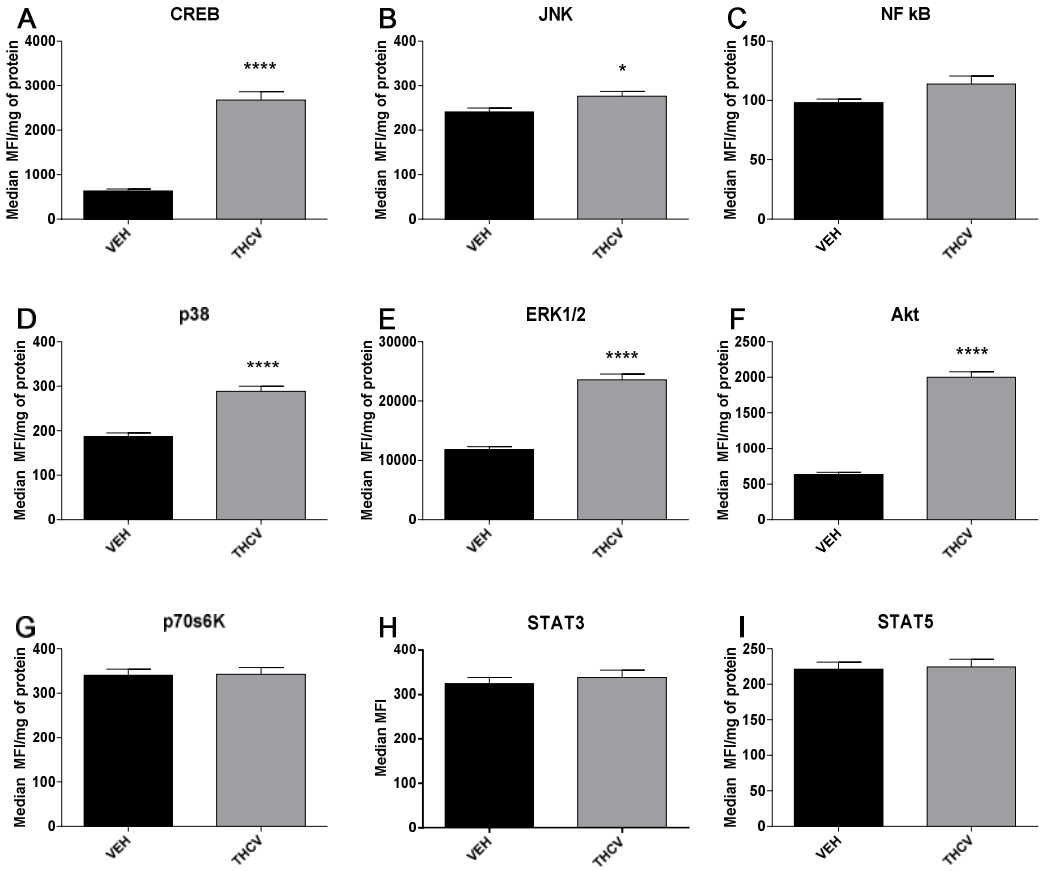


Figure 5.3. Normalized data showing changes in the level of phosphorylated signalling proteins after 10 min exposure of human aortic endothelial cells (HAoEC) to vehicle and 10 μM THCV (n=6 based on a single experiment). Cell Signalling Multiplex Assay used to measure proteins in cell lysates. Data analysed using unpaired t-test and presented as mean ± SEM. (* $p<0.05$, **** $p<0.0001$).

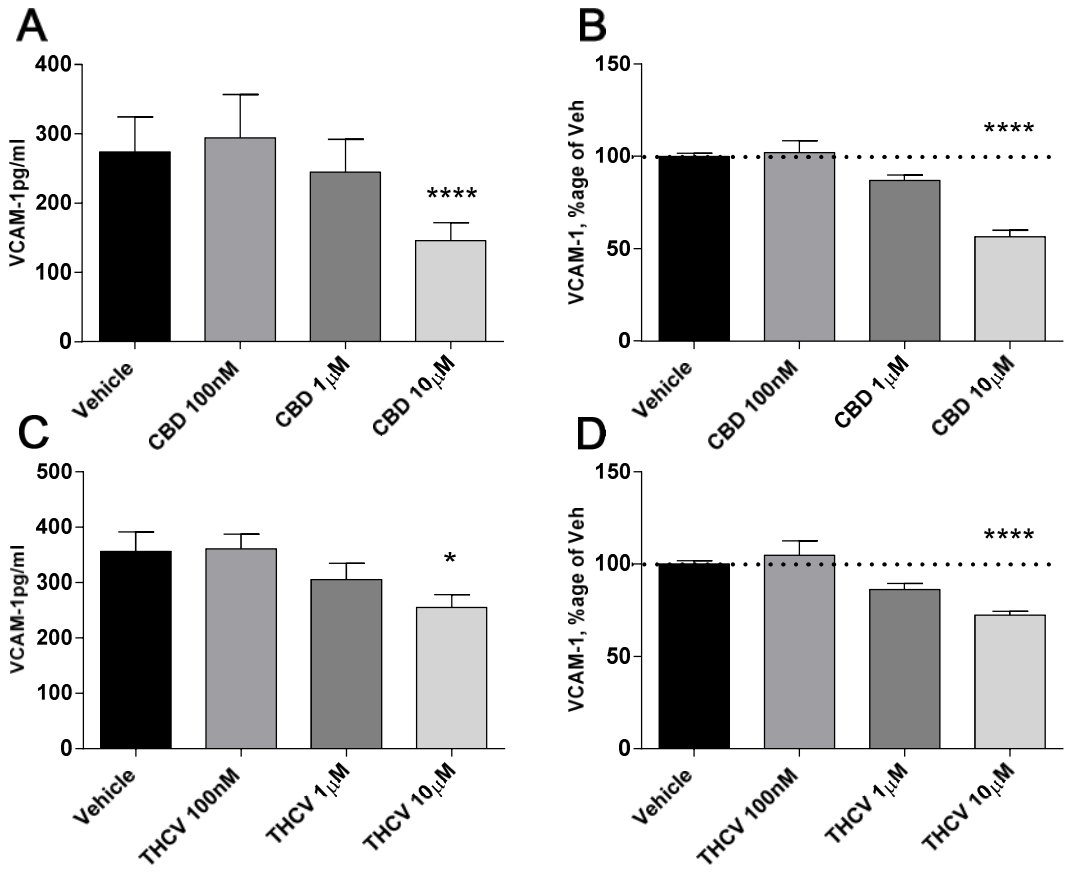


Figure 5.4. Changes in the level of VCAM-1, in medium, after incubating HAoEC with increasing concentrations of CBD (A,B) and THCV (C,D). Panels A and C show actual change, while B and D show percentage change in the level of VCAM-1 (n=9 based on 3 separate experiments performed in triplicate). Data analysed using one way ANOVA and presented as mean ± SEM. (* $p<0.05$, **** $p<0.0001$).

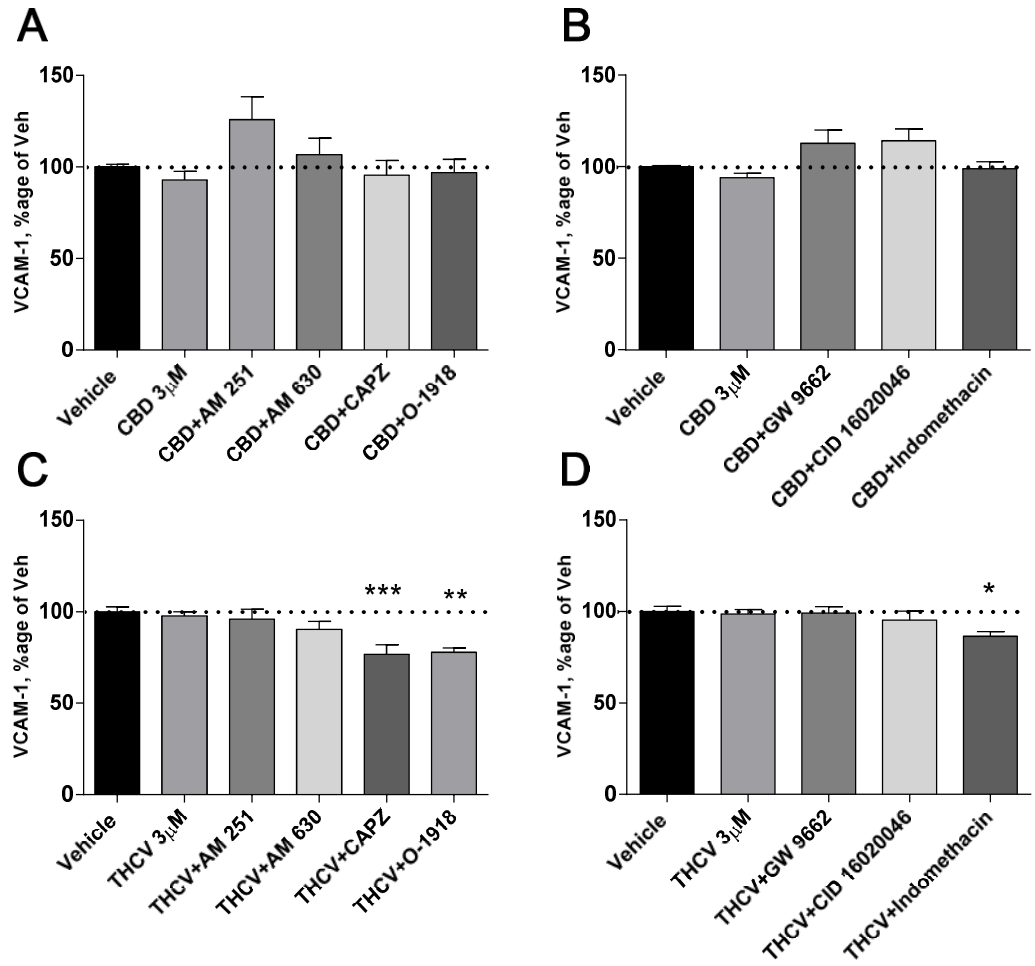


Figure 5.5. Percentage change in the level of VCAM-1, in medium, after incubating HAoEC with 3μM CBD (A,B) and 3μM THCV (C,D), on their own and in the presence of AM251 (CB_1 antagonist) , AM630 (CB_2 antagonist/inverse agonist) , capsazepine (CAPZ) (TRPV1 antagonist), O-1918 (GPR18 antagonist), GW 9662 (PPARγ antagonist), CID 16020046 (GPR55 antagonist) and indomethacin (cyclooxygenase (COX)-1 inhibitor) (n=8; based on 4 separate experiments performed in duplicate). Data analysed using one way ANOVA and presented as mean ± SEM. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

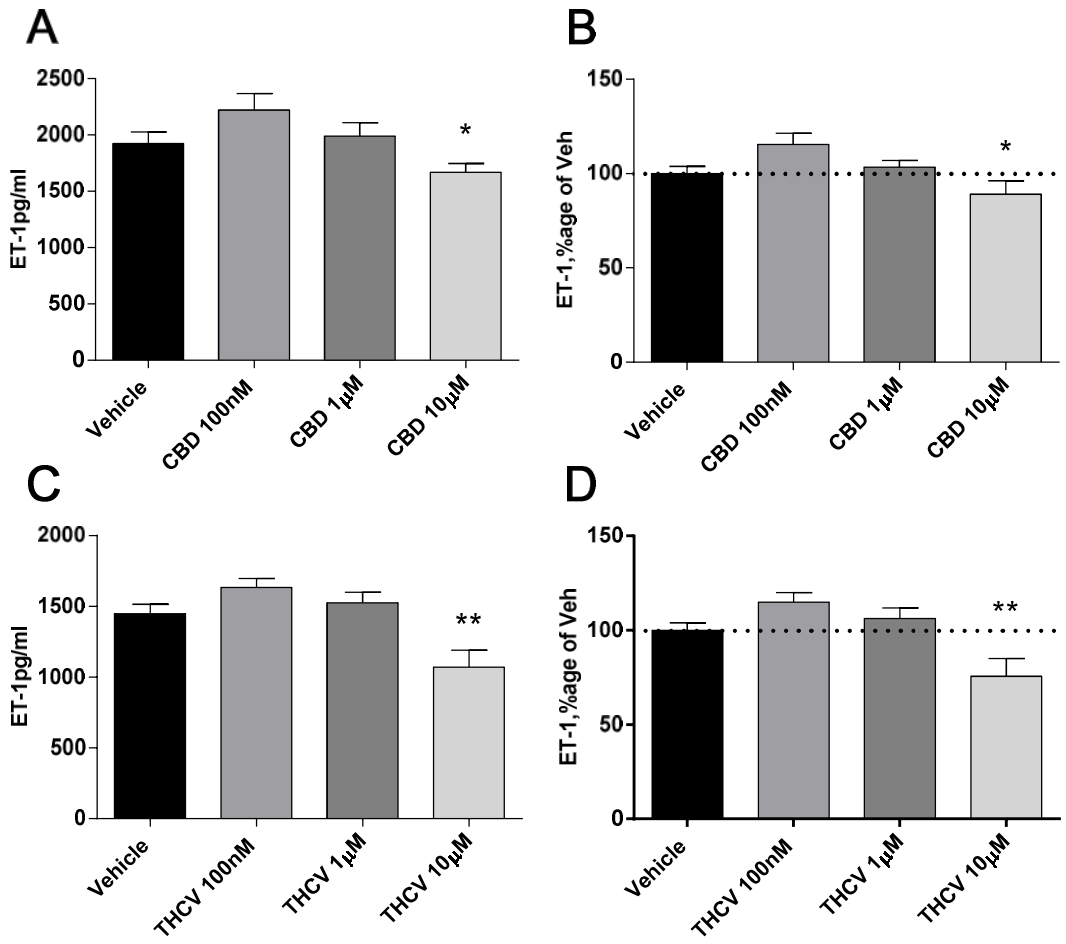


Figure 5.6. Changes in the level of ET-1, in medium, after incubating HAoEC with increasing concentrations of CBD (A,B) and THCV (C,D). Panels A and C show actual change, while B and D show percentage change in the level of ET-1 (n=9 based on 3 separate experiments performed in triplicate). Data analysed using one way ANOVA and presented as mean ± SEM. (* $p<0.05$, ** $p<0.01$).

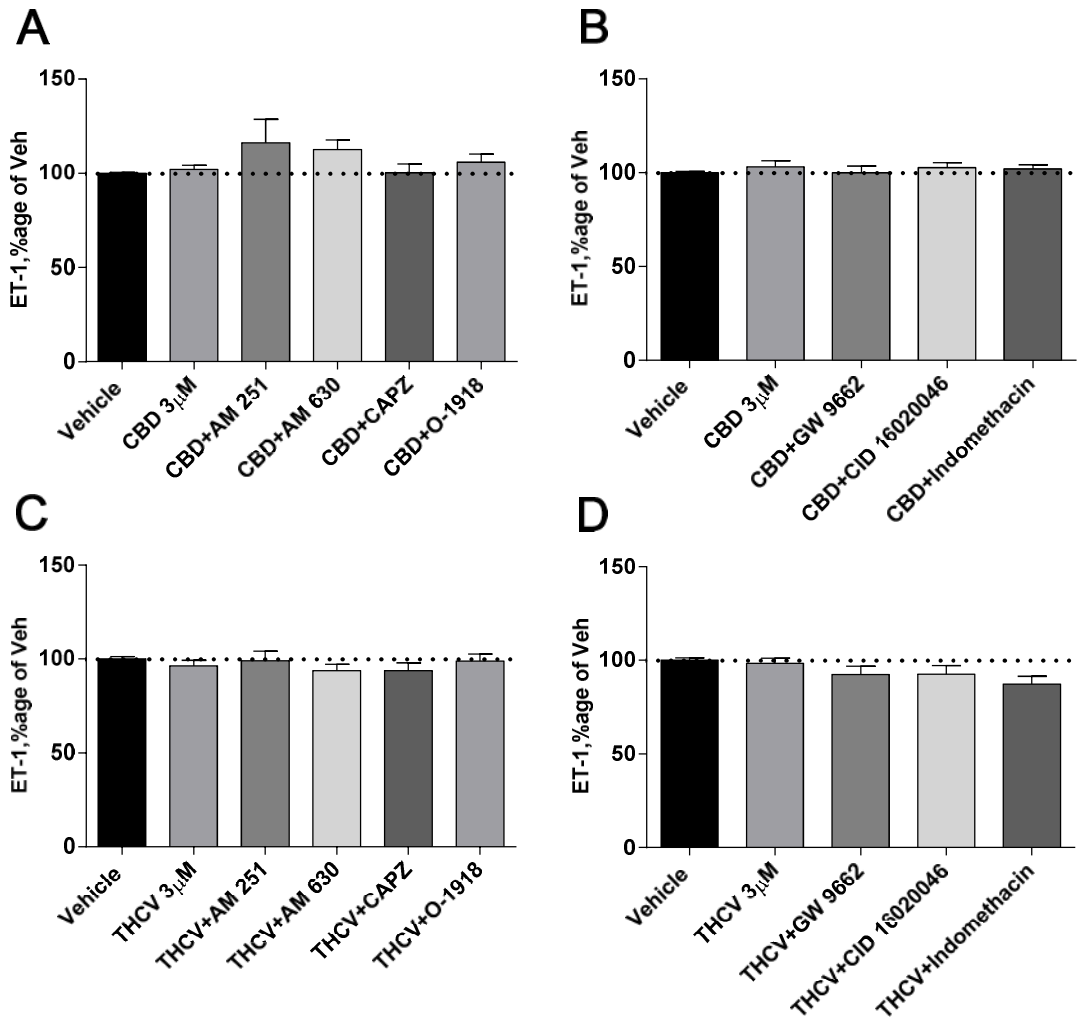


Figure 5.7. Percentage change in the level of ET-1, in medium, after incubating HAoEC with 3μM CBD (A,B) and 3μM THCV (C,D), on their own and in the presence of AM251 (CB_1 antagonist) , AM630 (CB_2 antagonist/inverse agonist) , capsazepine (CAPZ) (TRPV1 antagonist), O-1918 (GPR18 antagonist), GW 9662 (PPARγ antagonist), CID 16020046 (GPR55 antagonist) and indomethacin (cyclooxygenase (COX)-1 inhibitor) (n=8; based on 4 separate experiments performed in duplicate). Data analysed using one way ANOVA and presented as mean ± SEM.

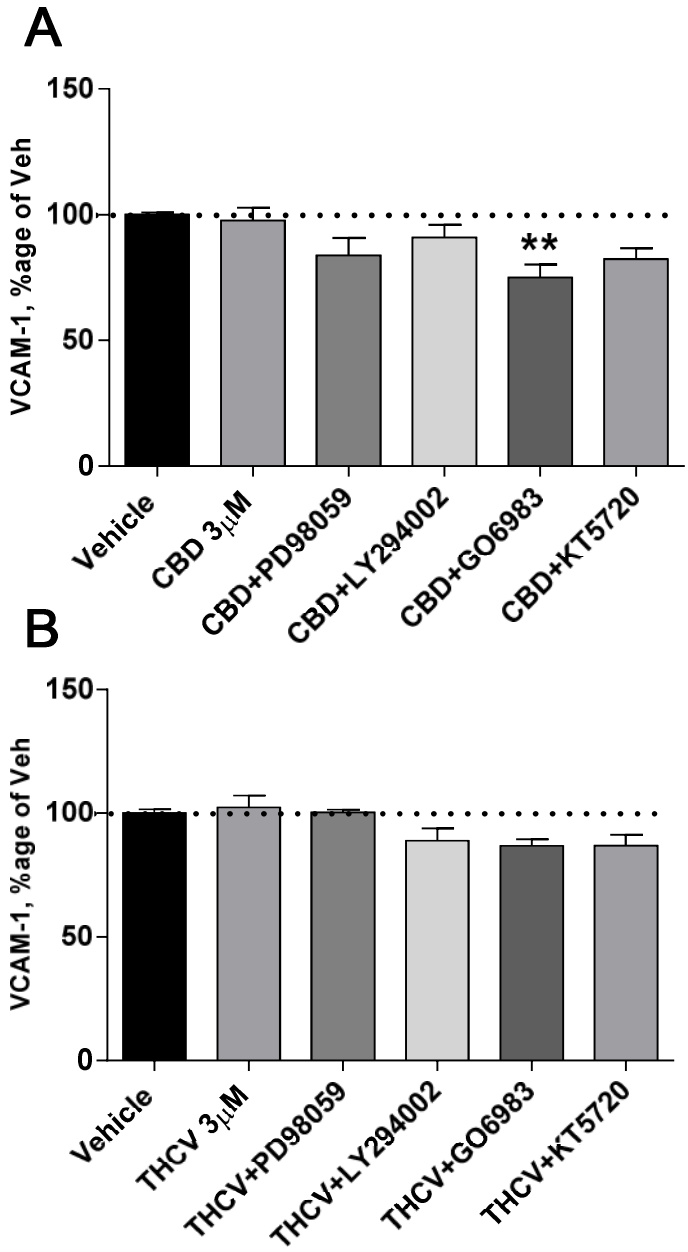


Figure 5.8. Percentage change in the level of VCAM-1, in medium, after incubating HAoEC with 3μM CBD (A) and 3μM THCV (B), on their own and in the presence of PD98059 (MAP kinase cascade inhibitor), LY294002 (PI3K/Akt kinase pathway inhibitor), GO6983 (Protein kinase C (PKC) inhibitor) and KT5720 (Protein kinase A inhibitor). (n=4; based on 2 separate experiments performed in duplicate). Data analysed using one way ANOVA and presented as mean ± SEM. (** $p<0.01$)

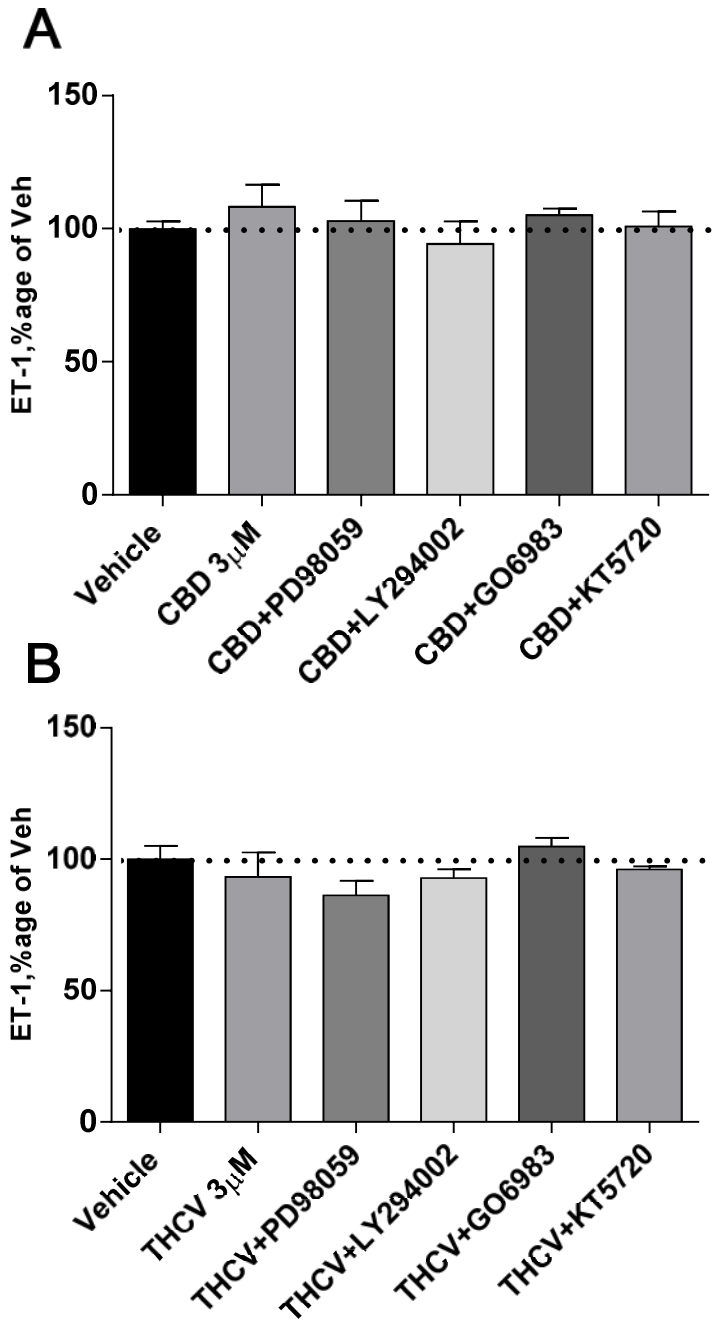


Figure 5.9. Percentage change in the level of ET-1, in medium, after incubating HAoEC with 3μM CBD (A) and 3μM THCV (B), on their own and in the presence of PD98059 (MAP kinase cascade inhibitor), LY294002 (PI3K/Akt kinase pathway inhibitor), GO6983 (Protein kinase C (PKC) inhibitor) and KT5720 (Protein kinase A inhibitor). (n=4; based on 2 separate experiments performed in duplicate). Data analysed using one way ANOVA and presented as mean ± SEM.

5.5. Discussion

Both VCAM-1 and ET-1 play a role in endothelial dysfunction and are important mediators of atherosclerosis, the hallmark of cardiovascular disease. Their levels are elevated in conditions associated with vascular inflammation such as type 2 diabetes and cardiovascular disease. The aim of the current study was to see if CBD and THCV affect the release of VCAM-1 and ET-1 from HAoEC. We also probed the receptor pharmacology and the effects of CBD and THCV on downstream signalling proteins. We found that CBD and THCV stimulated various post receptor signalling pathways, and at 10μM concentration, both CBD and THCV reduced the secretion of VCAM-1 and ET-1 from HAoEC. We were unable to determine the receptors involved.

5.5.1. CBD

CBD, at 10μM concentration, reduced the expression of both VCAM-1 and ET-1, which may explain its known vascular protective and anti-inflammatory properties. In experimental diabetes, CBD reduces lipid peroxidation and expression of ICAM-1 and TNFα levels and inhibits p38 MAP kinase (El-Remessy *et al.*, 2006). p38 MAP kinase, which is downstream target of pro-inflammatory cytokines, is activated on endothelial cells under hyperglycaemic conditions (Nakagami *et al.*, 2001). In diabetic cardiomyopathy, which is associated with activation of inflammatory cascade and increased expresion of adhesion molecules, CBD treatment inhibits the expression of NFκB, ICAM-1, VCAM-1 and TNFα. It reduces the activation of p38 MAP kinase and attenuates inactivation of pro-survival pathway like Akt (Rajesh *et al.*, 2010). In our study acute exposure of HAoEC to CBD increased the levels of phosphorylated ERK1/2. With CBD treatment, there was a trend towards reduction in the levels of JNK, NFκB and p38 MAP kinase, while levels of Akt were not different from vehicle treated cells. There is, however, an important difference between our study and aforementioned studies, that we

did not expose cells to metabolic stress like hyperglycaemia, which stimulates pro-inflammatory signalling pathways. Under these circumstances the effect of CBD may become more prominent. Our findings therefore support the anti-inflmmatory and vasoprotective role of CBD. If CBD can reduce the levels of VCAM-1 and ET-1 under physiological conditions, it is likely to have a greater impact in the context of chronic vascualr inflammation seen in diabetes and vascular disease.

Cardiovascular tissue expresses both CB_1 and CB_2 receptors. Activation of CB_1 receptors in the cardiovascular system has been associated with adverse outcomes, including heart failure and atherosclerosis (Pacher and Steffens, 2009). Deletion of CB_1 receptor or its inhibition by SR141716A, protects against vascular inflammation and cell death and inhibits the expression of adhesion molecules, including VCAM-1, in mouse diabetic retinopathy and human retinal cell line (El-Remessy *et al.*, 2011). On the other hand activation of CB_2 receptors, in the cardiovascular system, protects against inflammatory damage. It inhibits TNFα induced NFκB activation and upregulation of adhesion molecules ICAM-1 and VCAM-1 (Rajesh *et al.*, 2007c) and TNFα mediated vascular smooth muscle proleferation and migration, a phenomenon observed in atherosclerosis (Rajesh *et al.*, 2008). CBD can cause indirect agonism at both CB_1 and CB_2 receptors (McPartland *et al.*, 2015) and has been implicated in vasorelaxation of human mesenteric artery via CB_1 activation (Stanley *et al.*, 2015). It is reasonable to believe that inhibition of VCAM-1 secretion in our study may be via CB_1 or CB_2 receptors.

The effect of CBD on VCAM-1 and ET-1 was not in a classical concentration-dependent manner, with a rise in both VCAM-1 and ET-1 seen with 100nM concnetration, though it did not reach statistical significance. This non-classical concentraion-response, particularly for activating signal trasnduction, has been observed before (Stanley *et al.*, 2015). The same study also showed signal transduction in HAoEC by CBD at 3μM concentration. A

similar pattern was observed with THCV, which also showed a non-classical concentration-response in its effects on the release of VCAM-1 and ET-1. We therefore decided to use 3μM concentration of CBD and THCV for the remaining experiments.

To explore receptor pharmacology, we incubated HAoEC with 3μM CBD, on its own and in the presence of CB_1, CB_2, TRPV1, CB_e , PPARγ and GPR55 antagonists and non-selective COX inhibitor. There was a trend towards reduction in VCAM-1 levels with 3μM CBD that did not reach statistical significance. In the presence of AM251, a CB_1 antagonist, a rising trend in VCAM-1 was observed. The lack of significant effect may have been due to non-classic concentration response. Previous studies have shown CB_1 mediated signal trasduction in endothelial cells by CBD (Stanley *et al.*, 2015) and since we saw an increase in phosporylated ERK1/2 and a reduction in STAT 5, it is reasonable to believe that with 10μM concentration we may have seen a significant reduction in VCAM-1 and any rise in the level of VCAM-1 with simulataneous use of AM251 may have been more significant and informative in terms of receptor pharmacology. There was no change in the level of ET-1 with 3μM CBD, though in the presence of AM251, a trend towards rise in ET-1 levels was observed. Again this result may have been more significant in the presence of 10μM CBD. In the presence of GW9662 (PPARγ antagonist) and CID16020046 (GPR55 antagonist), slight rise in the levels of VCAM-1 was observed. CBD causes time dependent vasorelaxation in rat aortae through PPARγ activation, in a concentration dependent manner, with maximum effect seen at 10μM concentration (O'Sullivan *et al.*, 2009), while GPR55 may have a role in endothelial dependent vasorelaxation in rat mesenteric arteries (AlSuleimani and Hiley, 2015). Although none of these results in our study were significant, this may be due to lower concentration of CBD and the fact that there was a trend towards change in the presence of various antagonists,

may explain the ability of CBD to act on various targets and may also explain non-concentration dependent effects of CBD.

Since we observed a reduction in VCAM-1 and ET-1 with 10μM CBD, we explored its effects on downstream signalling proteins. For these experiments we used 10μM CBD as the duration of exposure was only 10 minutes. Incubation of HAoEC with CBD increased the expression of phosphorylated ERK 1/2 and reduced expression of STAT 5. This is in agreement with a recent study (Stanley *et al.*, 2015), where CBD increased the levels of ERK 1/2 and reduced STAT 5. Activation of ERK 1/2 has been implicated in both CB_1 dependent and independent, cannabinoids induced vasorelaxation. Both 2-arachidonoylglycerol ether and Abn-CBD increase phosphorylation of ERK 1/2 in vascular smooth muscle cells (Su and Vo, 2007). Phosphorylation of ERK 1/2 plays important role in vascular protection and may explain the vasorelaxant and vascular protective properties of CBD. STAT 5 has an important role in angiogenesis (Dudley *et al.*, 2005) and its reduction may have a role in the anti-angiogenic effects of CBD in human umbilical vein endothelial cells (Solinas *et al.*, 2012). There was a trend towards reduction in the levels of phosphorylated p38 and NFκB, though it did not reach statistical significance, which is in keeping with existing data (Stanley *et al.*, 2015). Perhaps the change would have been more obvious if cells were exposed to metabolic stress like hyperglycaemia. The next step was to use signalling inhibitors to see if, by inhbiting the signalling cascade will have any effect on the release of VCAM-1 and ET-1 from HAoEC. Unfortunatley, with 3μM CBD, there was no suppresion of VCAM-1 and ET-1 release, nor was there any increase in the levels of these molecules in the presence of MAP kinase cascade inhibitors. The lack of effect with 3μM CBD may be due to lower concentration of CBD, as both, suppression of VCAM-1 and ET-1 and rise in signalling proteins, were observed with 10μM CBD. The significance of these signalling pathways in CBD

mediated suppression of VCAM-1 and ET-1 and the receptor pharmacology remains unclear. We have, however, shown that CBD is able to suppress VCAM-1 and ET-1 release from vascular endothelial cells, which is in agreement with existing data (Stanley *et al.*, 2015). This suggests that CBD has potent anti-inflammatory and vascular protective properties. Further experiments using 10μM CBD, under physiological and pathological states like hyperglycaemia, might help in clarifying the significance of signalling pathways and help with the identification of receptors involved.

5.5.2. THCV

10μM THCV reduced the secretion of both VCAM-1 and ET-1 from HAoEC. The effect on VCAM-1 and ET-1 did not appear to be in a classical concentration dependent manner. At 10μM concentration, THCV caused some cells to come off the bottom of cell culture plates. Therefore for the remaining experiments, to explore receptor pharmacology, we used 3μM THCV. The protein content of the cells treated with various concentrations of THCV, for 24 hours, was not significantly different from vehicle treated cells, suggesting that THCV did not adversely affect cells viability.

In addition to being a potent vasoconstrictor (Davenport *et al.*, 1993), ET-1 causes endothelial dysfunction by reducing nitric oxide production, increasing its degradation, interacting with angiotensin II and creating a proinflammatory state in endothelial cells. It has been implicated in endothelial dysfunction in atherosclerosis and diabetes (Iglarz and Clozel, 2007). AEA, which causes vasodilatation and hypotension, inhibits the production of ET-1 from human umbilical vein endothelial cells, through a non-CB_1 mechanism (Ronco *et al.*, 2007). In our clinical study THCV had no effect on markers of vascular function and inflammation. The potential explanation for this could be the relatively small dose of THCV (10mg per day) used. Recent animal data from Wargent and colleagues

(Wargent *et al.*, 2013) showed that most of the positive metabolic effects of THCV were seen with 5 and 12.5 mg/kg doses given orally. Suppression of VCAM-1 and ET-1 from HAoEC by THCV shows that this molecule has important anti-inflammatory and vascular protective properties. Since both VCAM-1 and ET-1 play important role in endothelial dysfunction and initiation and progression of atherosclerosis, THCV may have a role, in future, in the treatment of atherosclerotic cardiovascular disease.

We tried to probe the receptor pharmacology by using 3μM THCV on its own and in the presence of CB_1, CB_2, TRPV1, CB_e, PPARγ and GPR55 antagonists and COX inhibitor. Like CBD, THCV also showed a non-classical concentration-response. At 100nM it increased the release of both VCAM-1 and ET-1 from endothelial cells. This may explain why at 3μM concentration, THCV had no effect on their release. In the presence of TRPV1 and CB_e antagonist and COX inhibitor, THCV caused a significant decrease in the release of VCAM-1. TRPV1 has a well documented role in pain and neurogenic inflammation, but there is evidence of its presence on non-neuronal tissue, such as mesenteric arteries and endothelial cells in mice, vascualr smooth muscle cells and panreatic β cells in rats and cerebromicrovascular endothelium in humans (Fernandes *et al.*, 2012). Its activation in high-fat fed apolipoprotein E knockout ($ApoE^{-/-}$) mice ameliorates atherosclerosis (Ma *et al.*, 2011). There is little evidence of THCV's interaction with TRPV1 channels, with one study showing that it acts as human TRPV1 agonist (McPartland *et al.*, 2015). Abn-CBD, an agonist at the putative endothelial cannabinoid receptor (CB_e), inhibits ET-1 mediated vasoconsctriction in retinal arterioles (MacIntyre *et al.*, 2014). While current evidence suggests that activating TRPV1 and CB_e is likley to produce vasoprotective effects, the reduction in the levels of VCAM-1 seen while antagonising their effects, in the presence of THCV, remians unexplained. COX activation, especially COX-1 is known to mediate vascular endothelial dysfunction, with

vascular contraction being one aspect of that (Feletou *et al.*, 2011). Non selective COX inhibition is known to produce anti-inflammatory effects. While CBD causes vasorelaxation in Zucker diabetic fatty rats, via COX activation (Wheal *et al.*, 2014), there is currently no data looking at the effects of THCV on COX pathway. The reduction in VCAM-1 levels seen may be related to additive anti-inflammtory effect of THCV. The overall implication of these results remains unclear, as 3μM THCV on its own was unable to reduce the secretion of both VCAM-1 and ET-1. The likely explanation of lack of effect of THCV is the lower dose used for these experiments.

Acute exposure of HAoEC to 10μM THCV increased the levels of phosphorylated CREB, JNK, p38, ERK 1/2 and Akt. This shows the ability of THCV to activate various pathways in endothelial cells. Activation of ERK 1/2 and Akt may explain the anti-inflammatory and vascular protective properties of THCV, however THCV also increased levels of CREB, JNK, and p38 MAP kinase. CREB or cAMP-response element (CRE)-binding protein is a nuclear transcription factor, which requires phosphorylation of the serine residue at 133 (Ser133) for its transcriptional activation. Phosphorylation of Ser133 is mediated by different protein kinase pathways, including ERK 1/2 (Ono *et al.*, 2006). Activation of CREB via p38/MAPK pathway has been implicated in TNFα mediated expression of VCAM-1 in bovine aortic endothelial cells (Ono *et al.*, 2006). In our study THCV reduced the expression of VCAM-1, therefore increased phosphorylation of CREB may simply be a reflection of stimulation via ERK 1/2. c-Jun N-terminal kinase (JNK) belongs to the family of mitogen-activated protein kinases which is activated by a variety of stress stimuli, including oxidative stress and is involved in apoptosis and cell death (Ho *et al.*, 2000). The significance of increased expression of CREB, p38 MAP kinase and JNK, in response to THCV remains unclear. When we incubated cells with 3μM THCV on its own and in the presence of various signalling protein inhibitors, there was no change in the

levels of VCAM-1 and ET-1. The fact that 10μM THCV reduced the secretion of VCAM-1 and ET-1 from HAoEC and the same results was not observed with 3μM concentratoin, suggests that the concentration of THCV was not enough. Further tests, under similar experimental conditions, comparing the effects of these two concentrations of THCV on HAoEC are required to clarify this further.

In summary we have shown that both CBD and THCV inhibit the release of adhesion molecule VCAM-1, and potent vasoconstrictor ET-1, from HAoEC and activate signalling cascades that will explain their potential beneficial effects. This is in keeping with available data that shows that these phytocannabinoids have anti-inflammatory and in case of CBD vasoprotective properties.

6. Effect of *in vivo* cannabidiol on plasma levels of cytokines, leptin and markers of vascular function in lean and Zucker Diabetic Fatty (ZDF) rats

6.1. Introduction

6.1.1. Obesity and adipose tissue inflammation

Obesity has reached epidemic proportions in the Western world and is rapidly increasing in developing countries, due to a combination of various risk factors such as increased calorie intake and reduced physical activity (Schwarz *et al.*, 2007; Whiting *et al.*, 2011). Obesity increases the risk of insulin resistance, type 2 diabetes, hypertension, dyslipidaemia and cardiovascular disease (Rosen and Spiegelman, 2006). It is characterised by increase in the number and size of adipocytes followed by angiogenesis and increased matrix formation. In addition, there is immune cell infiltration and increased production of pro-inflammatory cytokines (Suganami and Ogawa, 2010). Obese individuals show an increased expression of TNFα, IL-6, monocyte chemotactic protein (MCP)-1, iNOS, TGFβ-1 and certain procoagulant proteins. Macrophage numbers are higher in adipose tissue in obesity accounting for increased pro-inflammatory cytokines (Greenberg and Obin, 2006). The increased expression of pro-inflammatory cytokines (IL-6, TNFα and IL-β) and chemokines such as CCL2, CCL5 and CXCL5 trigger adipose tissue inflammation (Yao *et al.*, 2014), increasing lipolysis and aggravating the problem of high circulating free fatty acids, that impair insulin sensitivity (Guilherme *et al.*, 2008). The positive correlation between central fat distribution and reduced insulin sensitivity is well documented (Kahn *et al.*, 2001).

6.1.2. Adipocytokines

White adipose tissue that acts as a major site for energy storage, also acts as a dynamic endocrine organ (Rajala and Scherer, 2003). It secretes many important hormones, collectively called as adipocytokines or adipokines, leptin and adiponectin being two of

them (Trayhurn and Wood, 2007) . We investigated the effects of both CBD and THCV on leptin and adiponectin in our project. In chapter 2, we showed that THCV increased plasma levels of adiponectin and improved glycaemic control, in type 2 diabetes. In chapter 4, we showed that *in vitro* CBD increased leptin release from human adipocytes, after 2 hours incubation. Leptin a product of *ob/ob* gene that was first discovered in a mouse model of obesity, is secreted mainly by the white adipose tissue (Considine *et al.*, 1996). Binding of leptin to its receptor activates Janus kinase signal transducer and activator of transcription 3 (JAK-STAT3) pathway, regulating energy homeostasis and (Bates *et al.*, 2003), and phosphatidylinositol 3-kinase (PI3K) pathway regulating food intake and glucose homeostasis (Niswender *et al.*, 2001). Sensitivity to leptin is reduced in obese individuals, due to changes at cellular level that attenuate leptin signalling and amplify weight gain (Myers *et al.*, 2010). Adiponectin enhances insulin sensitivity in muscle and liver (Chen *et al.*, 2003a). It increases FFA oxidation in certain tissues and reduces serum FFA, glucose and triglyceride levels (Lihn *et al.*, 2005). It also stimulates NO production (Chen *et al.*, 2003a) and attenuates TNFα induced expression of adhesion molecules in vascular endothelial cells (Ouchi *et al.*, 1999b). Pro-inflammatory cytokines reduce the expression of adiponectin (Brunn *et al.*, 2003). Obesity and insulin resistance are therefore closely linked and two cardinal features of metabolic syndrome and type 2 diabetes (Reaven, 1988; Grundy *et al.*, 2004b).

6.1.3. Adipose tissue inflammation and atherosclerosis

Obesity related chronic inflammation, augmented by the presence of other risk factors such as hypertension, smoking and dyslipidaemia, leads to atherosclerosis (Libby *et al.*, 2011) and related cardiovascular disease, which is responsible for highest mortality in adults in the Western world (Steinberger and Daniels, 2003). During atherosclerosis there is retention of apo-B containing lipoprotein molecules in the subendothelial matrix triggering

inflammatory changes, leading to changes in the morphology and function of vascular smooth muscle cells and infiltration of inflammatory cells (Williams and Tabas, 1995). There is retention of pro-atherogenic lipoproteins, adhesion of monocytes to activated endothelium leading further to the release of chemokines, infiltration of monocytes into vessel wall with formation of macrophages and foam cells [For review see (Tannock and King, 2008)].

6.1.4. Endothelial cell dysfunction and atherosclerosis

Endothelial cell activation and dysfunction play a key role in the development of atherosclerosis, as described in detail in chapter 5. Diabetes plays a key role in the development of endothelial dysfunction, through increased production of ROS, due to overwhelming effect of hyperglycaemia on various cellular antioxidant mechanisms and production of AGE (Funk *et al.*, 2012). Diabetes is well known for its microvascular (neuropathy, retinopathy and nephropathy) and macrovascular (cardiovascular, cerebrovascular and peripheral vascular) complications, arising from accelerated atherogenesis, and is responsible for two to four times greater cardiovascular morbidity than non-diabetic people (Stumvoll *et al.*, 2005).

6.1.5. Role of CBD

CBD has cardioprotective and vascular protective effects mainly through its antioxidant and anti-inflammatory properties (Stanley *et al.*, 2013a), as discussed in detail earlier. In chapter 3, we showed that CBD increases resting heart rate and reduces resting systolic blood pressure and reduces blood pressure response to cold stress in young healthy volunteers. In Zucker Diabetic Fatty (ZDF) rats and their lean counterparts, CBD enhances vasorelxant responses to acetylcholine in aortae, femoral and third order mesenteric arteries (Stanley *et al.*, 2013b). In human coronary artery endothelial cells, CBD attenuates

high glucose associated inflammatory response and inhibits upregulation of VCAM-1, along with other adhesion molecules (Rajesh *et al.*, 2007a). It ameliorates doxorubicin-induced cardiotoxicity in rats through its antioxidant and anti-inflammatory effects. Specifically it attenuates the expression of iNOS, NFκB, Fas ligand and caspase-3 (Fouad *et al.*, 2013).

CBD shows anti-inflammatory, antioxidant and vasoprotective properties, and in our clinical study, in healthy volunteers, it altered cardiovascular parameters, suggesting that it may have a direct interaction with the cardiovascular system. We therefore hypothesized that CBD treatment will ameliorate obesity and diabetes related changes in cytokines and markers of vascular function in ZDF rats (animal model of diabetes and obesity). We also looked at the levels of phosphorylated signalling proteins in homogenised femoral artery tissue, to see if CBD had an effect on downstream signalling.

6.2. Aims

The aim of this study was to see if *in vivo* cannabidiol treatment had an impact on plasma levels of various cytokines and markers of vascular function in ZDF rats and their lean counterparts.

6.3. Materials and Methods

Male ZDF rats, a well known model for studying type 2 diabetes (Peterson *et al.*, 1990), and their lean counter parts were treated, for 7 days, with daily intraperitoneal (IP) injection of 10mg/kg CBD or vehicle, by Dr Amanda Wheal, a researcher working with Dr Saoirse O'Sullivan, as part of her doctorate work. At the end of the treatment period, plasma samples were taken and stored at -80°C. Animals were killed and sections from their aortae, femoral and mesenteric arteries were taken and stored for later analysis. I used

the plasma samples and femoral artery tissue to perform the analyses described in detail below.

6.3.1. Tissue Homogenisation

FastPrep®-24 was used to homogenize the blood vessels (according to manufacturer's instructions), using lysing matrix D. This consists of 1.4mm ceramic spheres in 2mL impact-resistant tubes. Small sections of rat aortae and mesenteric and femoral arteries were weighed and each sample placed in a tube. 300µL of RIPA buffer, with protease and phosphatase inhibitor, was added to each tube. Samples were loaded and secured, and spun for 20 sec at a speed of 6m/s. All the tubes were removed carefully from the machine and kept upright on ice. Lysis buffer, containing the homogenised tissue, was collected and stored at -80°C.

6.3.2. Measurement of protein concentration of tissue homogenate

Protein concentration of the tissue homogenate was measured using BCA assay as described in section 4.3.2.

6.3.3. Measurement of changes in phosphorylated cell signalling proteins, in femoral artery homogenates from lean and ZDF rats, given placebo or 10mg/kg CBD

The levels of phosphorylated cell signalling proteins were measured, in femoral artery homogenates, using the Luminex® xMAP® technology, as described in section 4.3.3.

6.3.4. Measurement of cytokines and chemokines in rat plasma

Plasma levels of granulocyte-colony stimulating factor (G-CSF), exotaxin, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-1α, leptin, macrophage inflammatory protein (MIP)-1α, IL-4, IL-1β, IL-2, IL-6, epidermal growth factor (EGF), IL-13, IL-10, IL-12p70, interferon (IFN) γ, IL-5, IL-17A, IL-18, monocyte chemoattractant protein (MCP)-1, IFNγ-indicible protein (IP)-10, GRO/KC (also known as

chemokine (C-X-C motif) ligand 1 (CXCL1)), VEGF, fractalkine, LIX, MIP-2, TNFα and Regulated on Activation, Normal T Expressed and Secreted (RANTES) were quantified using the Luminex® xMAP® technology, using commercially available panel (Milliplex® MAP Kit- RECYTMAG-65K, Merck Millipore). The assay was performed according to manufacturer's instructions. Based on the results of validation assay, a 1:2 plasma dilution was used for this analysis. 200µL of assay buffer was added to each well of a 96 well plate and left to shake for 10 min at room temperature. Assay buffer was removed and 20µL each of standards, controls, assay buffer and 1:2 diluted samples were added to appropriate wells, followed by addition of 20µL of suspended beads to each well. The plate was left to incubate, on a plate shaker, for 2 hours, at room temperature. It was then washed twice, using hand held magnet to prevent loss of magnetic beads, with 200µL of wash buffer. 25µL of detection antibody was added to each well and the plate was left to incubate for 1 hour, followed by addition of 25µL of streptavadin-phycoerythrin to each well. After 30 min incubation at room temperature, contents were removed and plate washed twice. The beads were resuspended in sheath fluid and plate analysed using the Luminex® system. Where threre was failure of controls, the analysis was performed again.

6.3.5. Measurement of markers of cardiovascular disease (CVD Panel 1) in rat plasma

Plasma levels of brain natriuretic peptide (BNP), von Willebrand Factor (vWF), tissue inhibitor of metalloproteinase (TIMP-1), tissue plasminogen activator inhibitor (tPAI)-1 and VEGF were quantified using the Luminex® xMAP® technology, using commercially available panel (Milliplex® MAP Kit- RCVD1-89K). The assay was performed according to manufacturer's instructions. Based on the results of validation assay, 1:2 plasma dilution was used for this analysis. 200µL of wash buffer was added to each well of a 96 well plate and left to shake for 10 min. This was removed by using vaccum and 25µL each of

standards, controls, assay buffer and plasma samples with 1:2 dilution were added to appropriate wells. 25µL of beads were added to each well, and plate was left to incubate overnight (16-18 hours) at 4°C. Contents were removed and plate washed, using vaccum, to prevent the loss of beads. 25µL of detection antibody was added to each well and plate left to incubate for 2 hours, at room temperature. 25µL of streptavidin-phycoerythrin was added per well and plate left for 30 min at room temperature. The last step was to vaccum and wash and resuspend beads in 100µL of sheath fluid per well. The plate was then analysed using the Luminex® system. There was no failure of controls during this analysis.

6.3.6. Measurement of markers of cardiovascular disease (CVD Panel 2) in rat plasma

Levels of ICAM-1 and E-Selectin were qunatified in rat plasma using Luminex® xMAP® technology, using commercially available panel (Milliplex® MAP Kit- RCVD2-89K). The assay was performed according to manufacturer's instructions. Based on the results of validation assay, plasma samples were diluted 75 times for this analysis. 200µL of wash buffer was added to each well of a 96 well plate and left to shake for 10 min. This was removed by using vaccum and 25µL each of standards, controls, assay buffer and plasma samples with 1:75 dilution were added to appropriate wells. 25µL of beads were added to each well, and plate was left to incubate overnight (16-18 hours) at 4°C. Contents were removed and plate washed, using vaccum, to prevent the loss of beads. 25µL of detection antibody was added to each well and plate left to incubate for 2 hours, at room temperature. 25µL of streptavidin-phycoerythrin was added per well and plate left for 30 min at room temperature. The last step was to vaccum and wash and resuspend beads in 100µL of sheath fluid per well. The plate was then analysed using the Luminex® system.

6.3.7. Measurement of Endothelin (ET)-1 in rat plasma

Plasma levels of ET-1 were measured using Duoset ELISA kits from R&D systems®, as described earlier in chapter 6. Based on the results of validation assay, plasma samples were diluted 4 times for this analysis. Data was extrapolated by creating standard curve using nonlinear regression. %CV for vehicle treated lean and ZDF rats was 21.9 and 18.5% and for CBD treated lean and ZDF rats was 6.5 and 5.7% respectively.

6.3.8. Statistical Analyses

All statistical analyses were performed using the GraphPad Prism 6 Software. Data for ET-1 was extrapolated by creating a standard curve, while data for all other analytes was generated using the Luminex software. All data were analysed using ordinary one way ANOVA with Tukey's post hoc test for multiple comparisons and presented as mean ± SEM.

6.4. Results

6.4.1. Changes in the levels of phosphorylated signalling proteins in homogenised femoral arteries of lean and ZDF rats, after seven days treatment with intraperitoneal CBD (10mg/kg) and vehicle

In lean rats, CBD treatment did not affect any of the signalling proteins. Diabetes reduced the levels of phosphorylated Akt, with significant difference between CBD treated lean rats and vehicle treated diabetic rats (Figure 6.1F, * $p<0.05$; one way ANOVA). CBD treatment in diabetic rats attenuated the fall in Akt and the difference was not statistically significant ($p>0.05$). There was a trend towards reduction in ERK1/2 with diabetes, though it did not reach statistical significance. CBD treatment again seemed to attenuate diabetes related fall in the levels of ERK1/2 (Figure 6.1E).

6.4.2. The effects of intraperitoneal CBD (10 mg/kg) on the plasma levels of cytokines, chemokines and leptin in lean and ZDF rats

Diabetes was associated with a significant rise in plasma levels of leptin (Figure 6.2A, $p<0.01$ and <0.05 respectively vs vehicle treated and CBD treated lean rats), and IP-10 (Figure 6.3F, $p<0.05$) and decline in the levels of TNFα (Figure 6.3E, $p<0.05$) and IL-17α (Figure 6.4G, $p<0.05$). Compared to vehicle, CBD treatment, in lean rats, reduced the levels of IL-2 (Figure 6.4C, $p<0.05$) and IL-17α (Figure 6.4G, $p<0.05$). CBD treatment in diabetic rats attenuated the rise in plasma leptin levels so that the difference between lean and CBD treated diabetic rats was not significant (Figure 6.2A, $p>0.05$). CBD treatment also reduced plasma levels of MCP-1 in diabetic rats (Figure 6.3D, $p<0.05$).

6.4.3. The effects of intraperitoneal CBD (10 mg/kg) on the plasma levels of markers of cardiovascular function (CVD panel 1) in lean and ZDF rats

Diabetes led to a significant rise in plasma levels of vWF (Figure 6.5B, $p<0.05$ to <0.01) and tPAI-1 (Figure 6.5C, $p<0.05$ to <0.001), in both vehicle and CBD treated rats. Compared to vehicle, CBD treatment in lean rats did not significantly affect plasma levels of any of cardiovascular markers. Compared to lean rats, CBD treatment in ZDF rats increased plasma levels of VEGF (Figure 6.5E, $p<0.05$).

6.4.4. The effects of intraperitoneal CBD (10 mg/kg) on the plasma levels of E-Selectin and ICAM-1 (CVD panel 2) and endothelin (ET)-1 in lean and ZDF rats

Plasma levels of ICAM-1 (Figure 6.6B, $p<0.05$) and ET-1 (Figure 6.6C, $p<0.01$) were higher in diabetic rats treated with vehicle. Plasma levels of ET-1 were also higher in diabetic rats treated with CBD (Figure 6.6C, $p<0.01$). CBD treatment in lean rats did not have an impact on any of the parameters, but CBD treatment in ZDF rats attenuated the rise ICAM-1 and therefore the difference was not significant ($p>0.05$, Figure 6.6B).

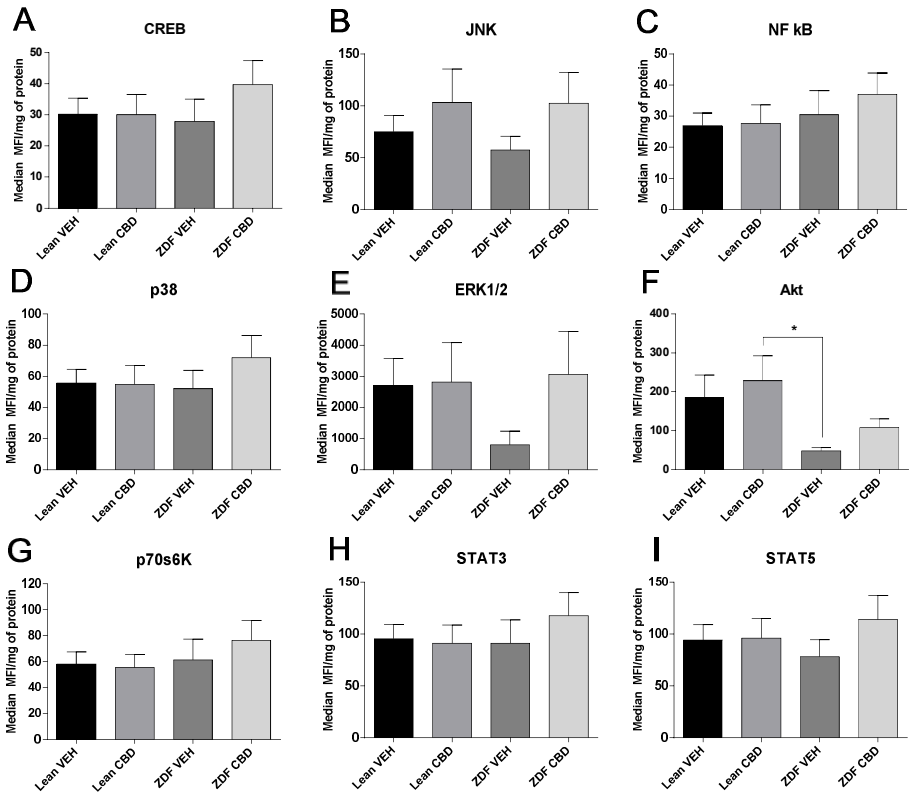


Figure 6.1. Changes in the levels of phosphorylated CREB (Ser133), JNK (Thr183/Tyr185), NF-κB (Ser536), p38 (Thr180/Tyr182), ERK/MAP kinase 1/2 (Thr185/Tyr187), Akt (Ser473), p70 S6 Kinase (Thr412), STAT3 (Ser727) and STAT5A/B (Tyr694/699) in homogenised femoral arteries of lean and Zucker Diabetic fatty (ZDF) rats, treated for seven days with intra-peritoneal CBD (10mg/kg) or vehicle. Values normalised to protein content of homogenised blood vessels. Data analysed using one way ANOVA with Tukey's post hoc test for multiple comparison and presented as mean ± SEM * p<0.05.

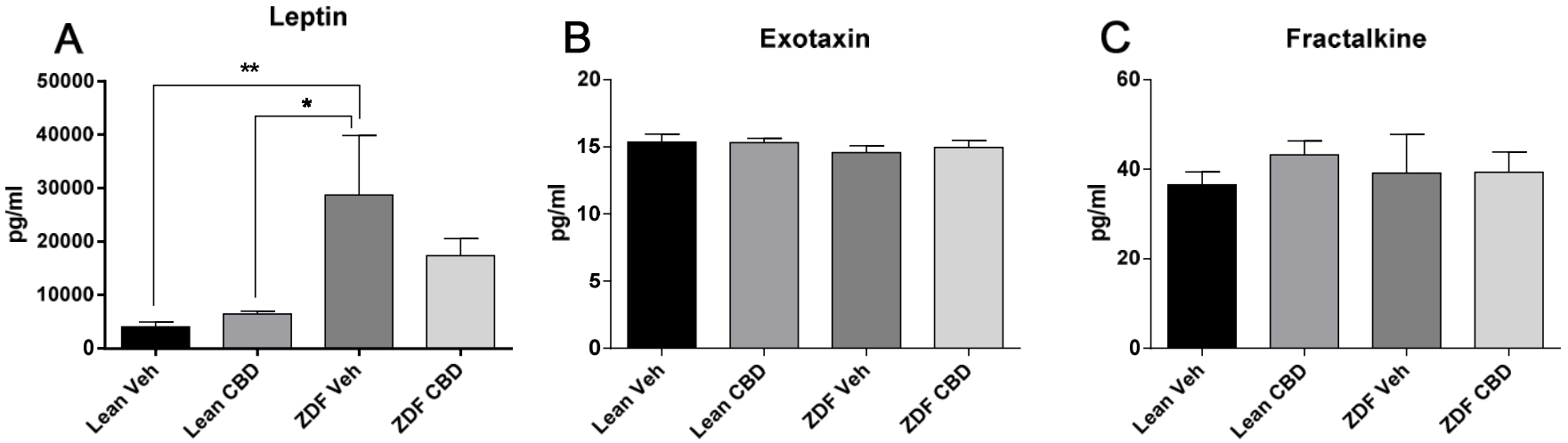


Figure 6.2. Changes in the levels of leptin, exotoxin and fractalkine in the plasma of ZDF lean and ZDF rats, treated for seven days, with intra-peritoneal CBD (10mg/kg) or vehicle. Data analysed using one way ANOVA with Tukey's post hoc test for multiple comparison and presented as mean ± SEM *p<0.05, **p<0.01.

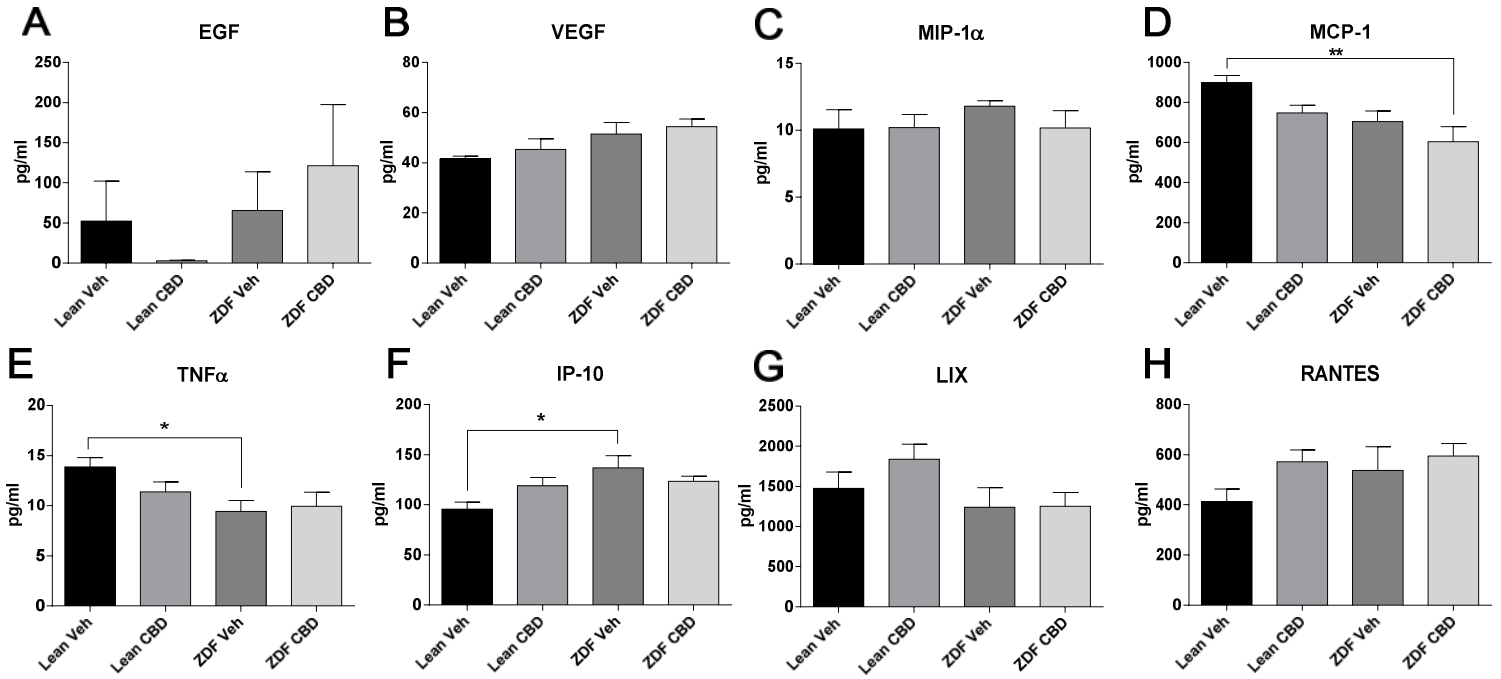


Figure 6.3. Changes in the levels of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), macrophage inflammatory protein (MIP)-1α, monocyte chemotactic protein (MCP)-1, tumour necrosis factor (TNF)-α, interferon gamma-induced protein (IP)-10, lipopolysaccharide-induced CXC chemokine (LIX) and chemokine (C-C motif) ligand 5 also called as RANTES (regulated on activation, normal T cell expressed and secreted) in the plasma of ZDF lean and ZDF rats, treated for seven days, with intra-peritoneal CBD (10mg/kg) or vehicle. Data analysed using one way ANOVA with Tukey's post hoc test for multiple comparison and presented as mean ± SEM *p<0.05, **p<0.01.

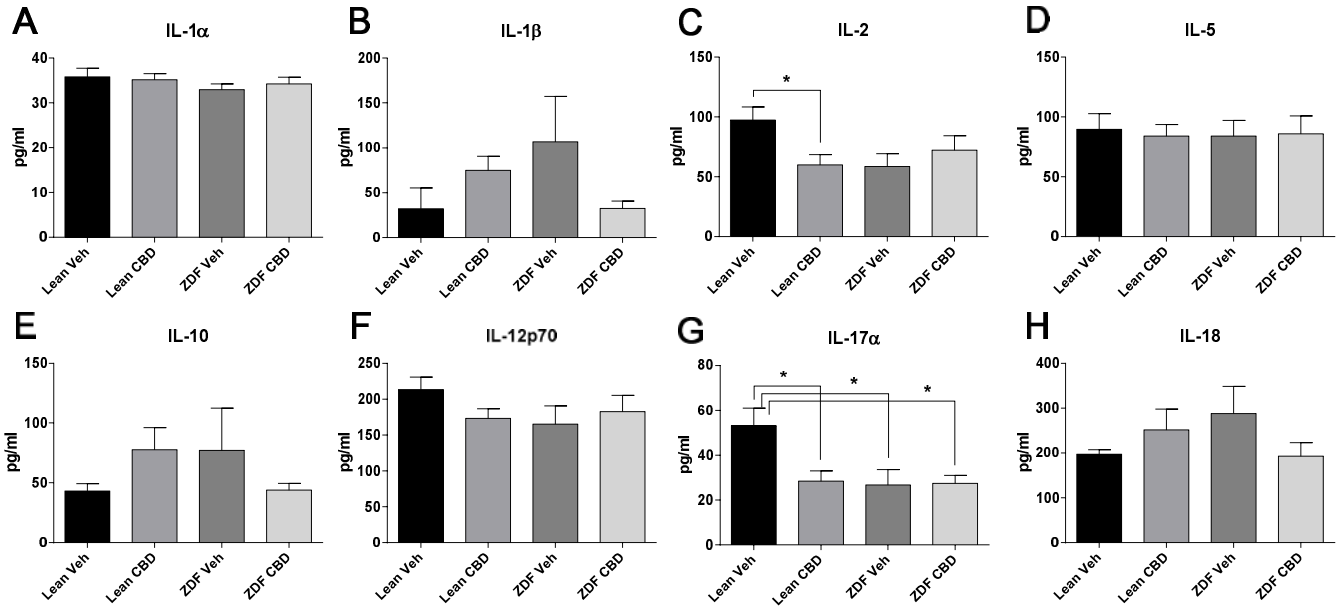


Figure 6.4. Changes in the levels of interleukin (IL)-1α, IL-1β, IL-2 and IL-5, IL-10, IL-12p70, IL-17α and IL-18 in the plasma of ZDF lean and ZDF rats, treated for seven days, with intra-peritoneal CBD (10mg/kg) or vehicle. Data analysed using one way ANOVA with Tukey's post hoc test for multiple comparison and presented as mean ± SEM *p<0.05.

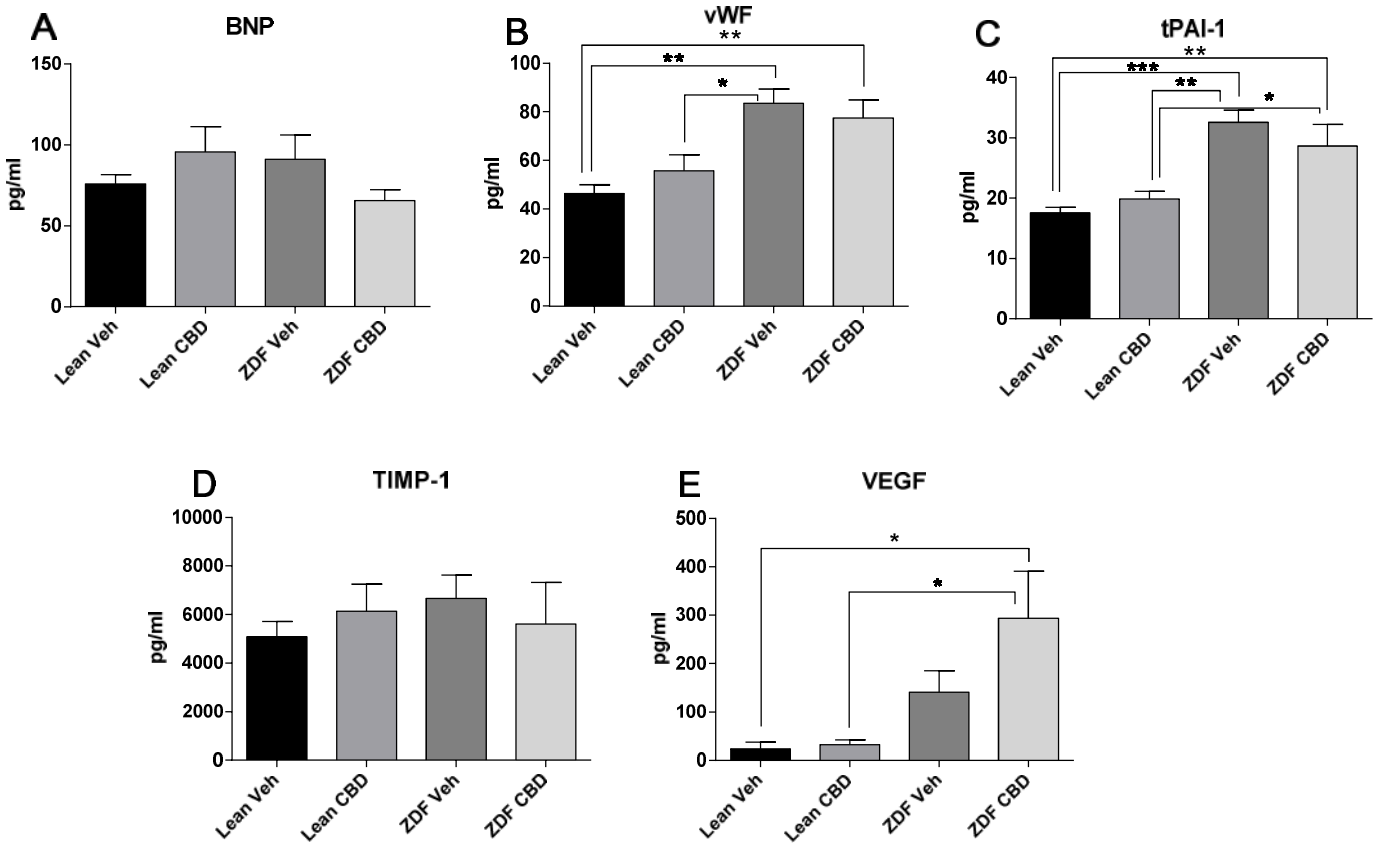


Figure 6.5. Changes in the levels of brain natriuretic peptide (BNP), von Willebrand Factor (vWF), tissue plasminogen activator inhibitor (tPAI)-1, tissue inhibitor of metalloproteinase (TIMP)-1 and vascular endothelial growth factor (VEGF) in the plasma of lean and ZDF rats, treated for seven days, with intra-peritoneal CBD (10mg/kg) or vehicle. Data analysed using one way ANOVA with Tukey's post hoc test for multiple comparison and presented as mean ± SEM *p<0.05,**p<0.01, ***p<0.001.

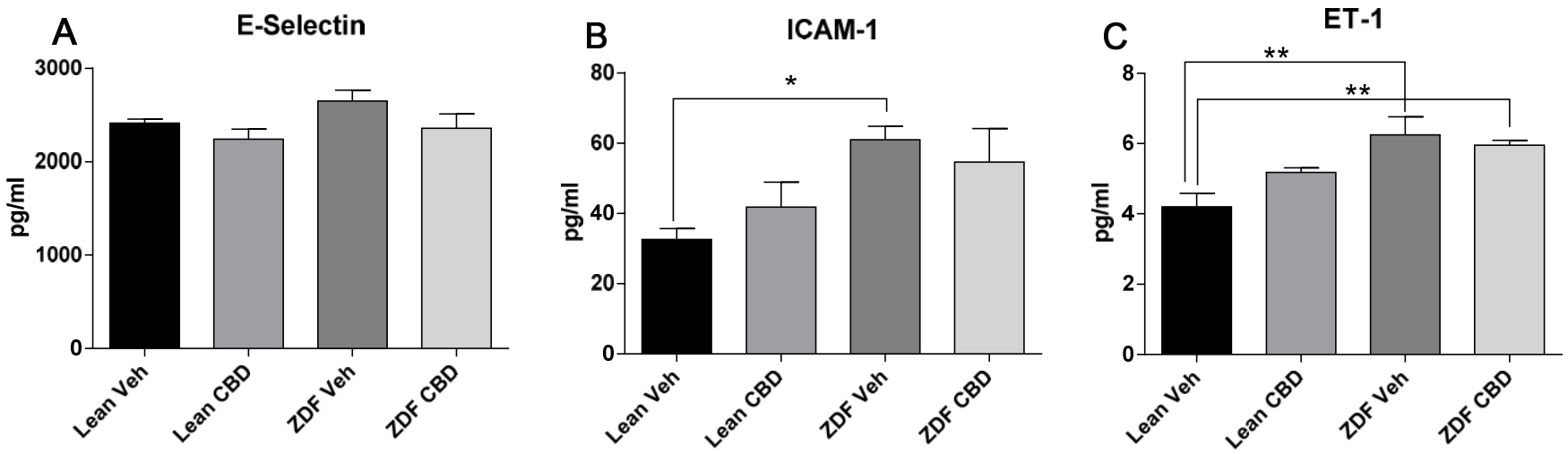


Figure 6.6. Changes in the levels of E-Selectin, intercellular adhesion molecule (ICAM)-1 and endothelin (ET)-1 in the plasma of lean and ZDF rats, treated for seven days, with intra-peritoneal CBD (10mg/kg) or vehicle. Data analysed using one way ANOVA with Tukey's post hoc test for multiple comparison and presented as mean ± SEM *p <0.05, **p<0.01.

6.5. Discussion

The aim of this study was to investigate the effects of CBD in diabetes. We specifically looked at cytokines and markers of cardiovascular function, and investigated how CBD affected post receptor signalling proteins in homogenised femoral arteries.

Our first finding was that CBD increased the levels of phosphorylated Akt in femoral artery tissue. Akt regulates PI3-K mediated cell survival (Yao and Cooper, 1995), and has been implicated in VEGF mediated endothelial cell survival (Gerber *et al.*, 1998). Our finding of increase in Akt is in keeping with existing data. In human aortic endothelial cells, CBD treatment significantly increases phosphorylated Akt (Stanley *et al.*, 2015). In diabetic cardiomyopathy, CBD treatment inhibits the expression of NFκB, ICAM-1, VCAM-1 and TNF-α, reducing the activation of p38 MAP kinase and attenuating inactivation of pro-survival Akt pathway (Rajesh *et al.*, 2010). The rise in Akt with CBD shows its vasoprotective potential and suggests that CBD may have a role in the treatment of cardiovascular complications associated with obesity and diabetes. CBD also attenuated the decline in ERK1/2 levels associated with diabetes. This result did not reach statistical significance, but is in keeping with existing data (Stanley *et al.*, 2015), and reaffirms the vasoprotective properties of CBD. There were no significant effects of CBD on other signalling proteins that were measured.

Leptin is secreted by white adipose tissue and plasma leptin levels reflect body fat mass. Leptin treatment in leptin deficient humans reduces body weight (Farooqi *et al.*, 1999), but the same degree of weight loss is not seen in common obesity, where plasma leptin levels are high, suggesting resistance to the actions of leptin (Heymsfield *et al.*, 1999). In our clinical study in type 2 diabetes (Chapter 3), there was no significant effect of CBD treatment on plasma leptin levels. Study in ZDF rats showed that *in vivo* CBD reduced

plasma leptin levels. In the former study, CBD did not have any impact on other metabolic and cardiovascular parameters either, suggesting that this may have been the result of relatively small dose of CBD used in this study. In human adipocytes, 2 hours incubation with CBD increased leptin release. Taken together, this shows that while CBD increases leptin release from adipose tissue, it improves sensitivity to leptin in leptin resistant state of diabetes and obesity. Both insulin (Aas *et al.*, 2009) and antipsychotics (Kraus *et al.*, 1999), increase plasma leptin, while at the same time causing a rise in body weight. CBD on the other hand is weight neutral. This suggests that rise in leptin may represent increased production from existing adipose tissue rather than an expansion in adipose tissue mass.

Plasma levels of ICAM-1 were significantly higher in diabetic rats. CBD treatment attenuated obesity related rise in ICAM-1. Expressed by endothelial cells, the predominant role of ICAM-1 is recruitment and trafficking of leukocytes to sites of inflammation (Springer, 1990). Endothelial expression of ICAM-1 is upregulated by proinflammatory cytokines including TNF-α, IL-1, and IFNγ and ROS (Hubbard and Rothlein, 2000). Animal studies show that ICAM-1 has an important role in the development of atherosclerosis (Bourdillon *et al.*, 2000). Our finding where CBD attenuated diabetes related rise in ICAM-1 is in keeping with known anti-inflammatory properties of CBD, discussed in great detail in the previous chapters. CBD does not directly interact with CB_1 or CB_2 receptors, but shows indirect agonism (McPartland *et al.*, 2015), and some of its anti-inflammatory effects may be mediated indirectly via these cannabinoid receptors or other mechanisms. This finding shows that CBD may have a potential role in the management of diabetes related vascular problems associated with chronic inflammation.

There was a rise in the plasma levels of VEGF in ZDF rats with CBD treatment. VEGF is a glycoprotein with five members in its family VEGFA, VEGFB, VEGFC, VEGFD and

placental growth factor that bind to three different receptors with tyrosine kinase activity (Zhuang and Ferrara, 2015). VEGF has important physiological and pathophysiological effects in various organ systems. It is a key regulator of angiogenesis promoting growth and survival of vascular endothelial cells and preventing their apoptosis via PI3 kinase-Akt pathway (Gerber *et al.*, 1998). It promotes monocyte chemotaxis, increases vascular permeability and induces vasodilatation via NO release from endothelial cells. Due to its key role in angiogenesis, it plays important role in not only organ development, but also tumour growth (Ferrara *et al.*, 2003). It plays a key role in the development of microvascular complications particularly diabetic retinopathy (Simo *et al.*, 2006) and age related macular degeneration. There are conflicting results for VEGF's role in diabetic nephropathy. In non-diabetic studies, VEGF improved renal function by stimulating angiogenesis (Nakagawa, 2007), while in ZDF rats, reducing VEGF prevents early glomerular hypertrophy, a feature of diabetic nephropathy (Schrijvers *et al.*, 2006). Systemic inhibition of VEGF has raised concerns for adverse vascular outcomes including increased risk of cardiac and arterial thromboembolic events (Ranpura *et al.*, 2010; Choueiri *et al.*, 2010; Cao *et al.*, 2012). In experimental models, systemic inhibition of VEGF accelerates atherosclerosis and disrupts endothelial cell homeostasis by reducing the bioavailability of NO (Winnik *et al.*, 2013). On the other hand, studies show that VEGF-B may have a role in obesity related insulin resistance and type 2 diabetes. In animal models of obesity and type 2 diabetes, antagonising the effects of VEGF-B restores insulin sensitivity and improves glucose tolerance, specifically inhibiting ectopic lipid deposition and improving skeletal muscle glucose uptake (Hagberg *et al.*, 2012). In women with polycystic ovary syndrome, serum VEGF-B levels are higher and positively associated with insulin resistance. Metformin treatment ameliorates insulin resistance and lowers VEGF-B levels (Cheng *et al.*, 2015). In patients with acute coronary syndrome,

serum VEGF levels are elevated and may serve as surrogate marker of myocardial injury (Konopka *et al.*, 2013). The effect of CBD on VEGF is variable. CBD is known to inhibit growth of various tumours, having an anti-angiogenic effect. CBD inhibits HUVEC proliferation via cytostasis and inhibits VEGF-induced angiogenesis (Solinas *et al.*, 2012). In glioma cells, CBD reduces the expression of various proteins including TIMP-1, TIMP-4 and VEGF (Solinas *et al.*, 2013). In human prostate cancer cell line, *in vitro* cannabis extract high in CBD downregulates IL-6, IL-8 and VEGF (Sharma *et al.*, 2015). A recent study, however, shows that CBD increases VEGF production from human brain microvascular endothelial cells, *in vitro*, and protects blood brain barrier from oxygen-glucose deprivation (Hind *et al.*, 2015). Further research is needed to accurately establish the cause and effect relationship between VEGF and cardio-metabolic disorders and the significance of CBD mediated rises in plasma concentrations of VEGF, in the context of obesity and diabetes.

Diabetic rats showed increased plasma levels of IP-10 and this rise was attenuated by CBD treatment. IP-10 or CXCL10 is a chemokine that belongs to CXC family, inducible by pro-inflammatory stimuli such as IFN-γ and TNFα (Wang *et al.*, 2008). It has been implicated in the pathogenesis of various autoinflammatory conditions such as Grave's disease (Antonelli *et al.*, 2009), type 1 diabetes (Tanaka *et al.*, 2009; Roep *et al.*, 2010) and multiple sclerosis (Holman *et al.*, 2011). Elevated plasma levels of IP-10 are seen in diabetes with increased release from monocytes under the influence of hyperglycaemia (Devaraj and Jialal, 2009). It has also been implicated in atherosclerosis (Klinghammer *et al.*, 2013). A recent study looking at the relationship between coronary atherosclerosis and various chemokines showed that trans coronary concentration gradients of circulating IP-10 were significantly higher in coronary artery disease (CAD) positive group than in the CAD negative group, suggesting local expression of IP-10 by coronary atherosclerotic

lesions. Currently there are no studies looking at the effect of CBD on IP-10 in the context of diabetes or atherosclerosis. Our finding that CBD attenuated diabetes related rise in IP-10, is in keeping with the known anti-inflammatory potential of CBD.

CBD treatment in diabetic rats reduced plasma levels of MCP-1. MCP-1 is a member of CC family of chemokines, produced by a variety of normal cells but mainly by endothelial cells, fibroblasts and mononuclear cells, with some tumour cell lines expressing it constitutively (Van Coillie *et al.*, 1999). MCP-1 has important pathological role in obesity and diabetes. Both hyperglycaemia and insulin resistance in type 2 diabetes are associated with a state of subclinical inflammation, characterised by the presence of various pro-inflammatory cytokines including MCP-1 (Daniele *et al.*, 2014). Gene expression of MCP-1 and related chemokines is higher in adipose tissue of obese individuals (Huber *et al.*, 2008) with higher plasma levels compared to lean controls (Catalan *et al.*, 2007). MCP-1 interacts with monocyte chemokine receptor CCR2 to recruit monocytes to endothelial wall, in the early stages of atherosclerotic plaque formation (Gu *et al.*, 1998). MCP-1 therefore plays important role in diabetic atherosclerosis and diabetic microvascular complications with various treatments that inhibit MCP-1 showing a favourable effect (Panee, 2012). Our finding of reduced plasma levels of MCP-1 after CBD treatment is consistent with the anti-inflammatory properties of CBD and the available data. In murine model of acute lung injury, pre-treatment with CBD, reduces neutrophil infiltration and myeloperoxidase activity in lung tissue and decreases the expression of pro-inflammatory cytokines (TNFα and IL-6) and chemokines (MCP-1 and MIP-2) (Ribeiro *et al.*, 2012). In another study, CBD treatment during ongoing acute lung injury had similar effects of improving lung function and reducing inflammation with reduced expression of pro-inflammatory cytokines (TNFα and IL-6) and chemokines (MCP-1 and MIP-2) (Ribeiro *et*

al., 2015). CBD may have a potential future role in conditions associated with chronic inflammation.

CBD treatment in ZDF lean rats reduced the plasma concentrations of IL-2 and IL-17α. IL-2 is a pleiotropic cytokine, produced predominantly by $CD4^+$ T cells (Leonard, 2001). IL-2 signals via three surface receptors, IL-2Rα, IL-2Rβ and IL-2Rγ (Malek and Castro, 2010), and plays an important role in the maintenance of $CD4^+$ regulatory T cells and differentiation of CD4 T cells into various subtypes (Rosenberg, 2011). IL-2 is important for maintaining self-tolerance and controlling autoimmunity (Leonard, 2001). IL-2 immunotherapy has been successfully used to treat metastatic renal cell carcinoma and metastatic melanoma (Rosenberg, 2012). In mouse splenocytes, CBD suppresses or enhances IFNγ and IL-2 production under optimal or suboptimal stimulation respectively (Chen *et al.*, 2012). CBD has immunomodulatory properties and it stops the onset of autoimmune diabetes in animal models (Weiss *et al.*, 2008). In animal model of type 2 diabetes, CBD protects pancreatic islets and β cells from destruction, lowers plasma glucose and increases plasma insulin without increasing body weight (Ziv *et al.*, 2012). In autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, CBD treatment ameliorates the clinical signs of EAE, reduces axonal damage and inflammation and suppresses microglial activity and T cell proliferation (Kozela *et al.*, 2011). Our finding of reduced plasma levels of IL-2 may explain this immunomodulatory role of CBD as it is likely to influence T cell differentiation. CBD may have a role in the treatment of other autoimmune conditions, which requires further studies.

Plasma levels of IL-17α were also lower in diabetic rats, treated with either vehicle or CBD. IL-17α is a member of cytokine family, secreted mainly by T helper type 17 (Th17) cells, playing important role in the pathogenesis of immunoinflammatory diseases including psoriasis. It acts synergistically with other pro-inflammatory cytokines like

TNFα and upregulates the production of various cytokines and chemokines. The role of IL-17α in atherosclerosis remains controversial. In ApoE$^{-/-}$ deficient mice, fed high fat diet, deficiency of IL-17α accelerates early phase of atherosclerosis (Danzaki *et al.*, 2012). Analysis of carotid artery plaques from patients undergoing endarterectomy show the expression of IL-17α is associated with increased inflammation and plaque vulnerability (Erbel *et al.*, 2011). Serum levels of IL-17α are higher in diabetic subjects with hypertension and hypertensive response to angiotensin II is not sustained in IL-17$^{-/-}$ mice compared to wild type mice and blood vessels from IL-17$^{-/-}$ mice show preserved vascular function and reduced oxidative stress (Madhur *et al.*, 2010). IL-17α has also been implicated in the pathogenesis of inflammatory dilated cardiomyopathy (Wu *et al.*, 2014), in myocardial ischemia/reperfusion injury (Liao *et al.*, 2012) and in early and late stages of post myocardial infarction ventricular remodelling (Zhou *et al.*, 2014). In experimental models of multiple sclerosis, CBD suppresses the expression of pro-inflammatory IL-17α and IL-6 and increases the levels of anti-inflammatory IL-10 (Kozela *et al.*, 2013). It attenuates IL-17α mediated mucosal damage in human colonic explant tissue (Harvey *et al.*, 2014). Our finding of reduction in plasma levels of IL-17α is in keeping with the available data and shows the ability of CBD to influence the immune system, especially its ability to regulate T cell function. The finding of low IL-17α levels in diabetic rats is not consistent with current data and its significance remains unclear. Further research is needed to establish the significance of these findings, and potential role of CBD in immune mediated conditions like diabetes type 1.

6.6. Conclusion

In vivo cannabidiol in ZDF rats activates pro-survival Akt. It ameliorates diabetes related rises in leptin, ICAM-1 and IP-10. In diabetic rats, it also reduces the levels of MCP-1, while increasing plasma levels of pro-angiogenic VEGF. In lean ZDF rats, CBD treatment

reduces plasma levels of IL-2 and IL-17α. Except for the finding of low IL-17α in diabetic rats, the rest of findings are in keeping with the known anti-inflammatory, immunomodulatory and vasoprotective potential of CBD. These results highlight the positive metabolic effects of CBD in diabetes and call for further research in this area.

7. Final Discussion

CBD and THCV are two non-psychoactive phytocannabinoids obtained from *Cannabis sativa L* that show positive metabolic effects in preclinical studies. In animal models of obesity, THCV produces fat loss, increases energy expenditure, and reduces fasting plasma insulin and leptin levels. In the same model, CBD improves dyslipidaemia, reduces liver TG and improves liver glycogen and adiponectin. When combined, in a 1:1 ratio, similar effects on dyslipidaemia and fasting insulin are observed. In another study, THCV increased energy expenditure and improved glucose tolerance and insulin sensitivity, without any significant effect on lipids (Wargent *et al.*, 2013). CBD has potent anxiolytic properties and it reduces cardiovascular response to stress in animal models (Resstel *et al.*, 2009). In addition, both CBD and THCV have anti-inflammatory properties. The overall aim of this project, therefore, was to explore the metabolic and cardiovascular effects of these two phytocannabinoids. To achieve this, we did a combination of *in vitro* and *in vivo* studies. *In vitro* work involved human adipocytes and human aortic endothelial cells, while *in vivo* studies were carried out in subjects with type 2 diabetes and dyslipidaemia, healthy young volunteers and in lean and ZDF rats.

We found that chronic use of CBD had no significant impact on metabolic and cardiovascular parameters in diabetic subjects, but acute administration of CBD affected cardiovascular parameters in healthy young volunteers. It interacted with adipocytes and endothelial cells, affecting the release of adipokines and markers of vascular dysfunction,

and affected plasma levels of cytokines and leptin in lean and ZDF rats. THCV improved glycaemic parameters in type 2 diabetes with a positive impact on adiponectin release. It interacted with adipocytes but had no impact on adipokine release, while it reduced the release of markers of vascular dysfunction from endothelial cells.

7.1. CBD

Current evidence shows positive effects of CBD in a range of diverse areas such as epilepsy (Szaflarski and Bebin, 2014), psychosis (Schubart *et al.*, 2014), multiple sclerosis (Flachenecker *et al.*, 2014), vascular system (Stanley *et al.*, 2015), myocardial ischemia and reperfusion injury (Feng *et al.*, 2015) and inflammatory colitis (Jamontt *et al.*, 2010) . In our project, we have highlighted positive effects of CBD in three main areas; glycaemic control, vascular biology and inflammation.

7.1.1. CBD and glycaemic control

In our clinical study, in type 2 diabetes, CBD increased plasma levels of GIP and reduced levels of resistin. GIP has insulinotropic properties (Kreymann *et al.*, 1987), but it stimulates glucagon release (Meier *et al.*, 2003), and causes lipogenesis (Kim *et al.*, 2007; Irwin and Flatt, 2009b). High levels of resistin are associated with obesity and insulin resistance (Steppan *et al.*, 2001). Pioglitazone reduces resistin levels in type 2 diabetes, which is positively correlated with reduction in hepatic fat content and improvement in hepatic insulin sensitivity (Bajaj *et al.*, 2004). Another study showed that reduction in plasma resistin levels may contribute to improving insulin action with rosiglitazone (Jung *et al.*, 2005). It is not surprising that CBD had no positive impact on glycaemic control in our diabetic subjects, because any positive impact of reduction in resistin could have been offset by a rise in plasma GIP levels. The exact mechanism and receptors involved in these effects of CBD are not clear. Cannabinoids are known to interact with GPCRs, some of

which play important role in glucose homeostasis (Moran *et al.*, 2016). One of them, GPR119, is found on GLP-1 producing L cells and GIP-secreting K cells (Parker *et al.*, 2009), that is stimulated by endocannabinoids (Cornall *et al.*, 2013). GPR 119 agonists show insulinotropic properties (Moran *et al.*, 2014), improving glycaemic control and increasing GLP-1 (Kim *et al.*, 2013b). CBD's mediated rise in GIP, seen in our clinical study, may be via its indirect effect on endocannabinoids, which interact with GPCRs, in the gut (Moran *et al.*, 2016). CBD did not have any impact on weight which is in keeping with other studies that show CBD to be weight neutral (Wierucka-Rybak *et al.*, 2014).

In the clinical study, in diabetic subjects, CBD did not have any impact on plasma leptin levels. In adipocyte study, 2 hours incubation with CBD increased the release of leptin from human adipocytes. The same effect was lost when cells were incubated for 24 hours. This indicates an acute effect of CBD on leptin, which is lost with chronic exposure. This may potentially explain why no effect on plasma levels of leptin was observed in our clinical study in type 2 diabetic subjects. Diabetes was associated with significant rise in plasma leptin levels in ZDF rats and CBD treatment attenuated that rise. A similar reduction in leptin levels was not observed in humans. It is, however, important to keep in mind the significant difference in glycaemic parameters between ZDF rats and human subjects with diabetes. While the baseline fasting plasma glucose was 23.2 ± 3.3 mM (mean ±SD), in ZDF rats, it was 8.0 ± 2.3 mM (mean ± SD) in diabetic subjects treated with CBD. ZDF rats, therefore, presented an extreme form of diabetes, not seen in common clinical practice. Another important factor may be the significant difference in dose of CBD used in human and rat study. The dose used in animal study was 10 mg/kg body weight, compared to 200 mg/d in human study. Most recent trials with CBD, in human subjects, have used a dose ranging from 300 to 800 mg per day (Bergamaschi *et al.*, 2011b). In a clinical study, in Huntington's disease, a total daily dose of 10 mg/kg of CBD,

over 6 weeks, led to plasma levels ranging from 5.9 ng/ml to 11.2 ng/ml, with no significant difference between weeks (Consore *et al.*, 1991). CBD undergoes significant first pass effect (Agurell *et al.*, 1986; Harvey and Mechoulam, 1990) with oral bioavailability of around 6% (Zhornitsky and Potvin, 2012). In summary, CBD's lack of effect on glycaemic parameters in type 2 diabetic subjects may be due to its simultaneous effects on GIP and resistin or the relatively low dose of CBD used in this study.

7.1.2. CBD and cardiovascular system

Previous studies in humans have not shown any effect of CBD on cardiovascular parameters such as heart rate and blood pressure. Animal data shows that CBD reduces cardiovascular response to stress. To investigate this in humans, we tested CBD's effect on cardiovascular response to various types of stress. We have found that CBD has an impact on resting cardiovascular parameters and reduces the blood pressure response to stress. It reduces resting blood pressure and stroke volume and blood pressure response to cold stress. In addition, CBD maintains a lower blood pressure, stroke volume, forearm skin blood flow and total peripheral resistance during rest and stress, associated with increased heart rate. This finding may be in keeping with sympathoinhibition with reflex increase in heart rate. Current evidence also shows that CBD has direct vasorelaxant properties (Wheal *et al.*, 2014; Stanley *et al.*, 2015). CBD had no impact on blood pressure and other cardiovascular parameters in the clinical trial, but there are important differences in the two studies. The dose of CBD used in clinical trial was three times less; 200 mg per day as opposed to 600 mg in the acute study. Another important difference was the timing of these tests. In the clinical trial, we did not study acute effects of CBD. We know that tolerance can develop to the cardiovascular effects of THC. Whether tolerance can develop to the cardiovascular effects of CBD over time requires further investigation.

CBD shows vasoprotective properties. *In vitro* incubation of aortae and femoral arteries from ZDF rats, with CBD, restores endothelial function. To see if *in vivo* CBD will have the same results, we gave intraperitoneal CBD to ZDF rats and their lean counterparts. In ZDF rats, CBD treatment attenuated diabetes related fall in the pro-survival Akt, in homogenised femoral arteries, while there was a trend towards rise in ERK1/2 levels. CBD attenuated diabetes related rise in ICAM-1 levels and increased plasma levels of VEGF in ZDF rats. Endothelial expression of ICAM-1 is upregulated by pro-inflammatory cytokines (Hubbard and Rothlein, 2000) and it has important role in the development of atherosclerosis (Bourdillon *et al.*, 2000). VEGF is a key regulator of angiogenesis and promotes survival of vascular endothelial cells. Downregulation of ICAM-1 and upregulation of VEGF will be in keeping with the positive vascular effects of CBD. The role of VEGF, however, is not entirely clear, as it has also been implicated in diabetic microvascular complications. The true significance of CBD mediated rise in plasma VEGF therefore remains unknown and requires further investigation.

Given the vascular effects of CBD, from our clinical study and from previous study in ZDF rats, we investigated if CBD had any effect on HAoEC. CBD increased levels of phosphorylated ERK1/2 and reduced secretion of VCAM-1 and ET-1 from HAoEC. ET-1 is a potent vasoconstrictor and both VCAM-1 and ET-1 mediate endothelial dysfunction seen in vascular disease. CBD had no impact on VCAM-1 in the clinical study in type 2 diabetes, but a direct comparison between these two studies has its limitations. *In vitro* experiments cannot replicate the experimental conditions of a living organism, where there will be other influences affecting the outcome. In case of CBD, it shows the potential to interact with multiple target sites, which may also explain why CBD had no significant impact on VCAM-1 in the clinical study (Bergamaschi *et al.*, 2011b).

Putting all this together, CBD seems to interact with endothelial cells, the functional unit of vascular system. The effects on VCAM-1 and ET-1 production highlight the potential for CBD to minimize endothelial dysfunction, a process that underlies atherosclerosis. At clinical level, CBD lowers blood pressure and reduces blood pressure rises in response to stress. The effect of CBD on vascular system seems multidimensional. It may alleviate anxiety and stress related changes in vasculature, as seen in animal studies (Resstel *et al.*, 2009), while at the same time having a direct interaction with endothelium and vascular smooth muscle, leading to vasorelaxation (Wheal *et al.*, 2014; Stanley *et al.*, 2015). It may attenuate endothelial dysfunction through its potent anti-inflammatory and anti-oxidant properties (Booz, 2011). The effects of CBD on cardiovascular system may be mediated via CB_1 receptors, as seen in some studies (Stanley *et al.*, 2015), but could involve other receptor types as well (Gonca and Darici, 2015). Further research is needed to elucidate underlying mechanisms of CBD's beneficial effects on vascular system, and the future role of CBD as a possible treatment for hypertension or vascular dysfunction associated with chronic inflammation as seen in obesity and diabetes. Any future trial should consider studying the pharmacokinetics of CBD in order to establish the most appropriate dose in a particular clinical setting.

7.1.3. CBD and inflammation

There is a wealth of data showing potent anti-inflammatory potential of CBD, in different organ systems (Mechoulam *et al.*, 2007). In our clinical study, CBD had no impact on plasma levels of inflammatory markers. *In vivo* study in rats showed that CBD treatment reduced plasma levels of IL-2 and IL-17α in lean ZDF and MCP-1 in diabetic rats. The lack of effect in clinical study may have been the result of sub-optimal dose. CBD also reduced the release of IL-6 from human adipocytes. Given the role of IL-6 and visceral adipose tissue in insulin resistance, this may be a significant finding. Further studies in

human adipocytes, in the presence of metabolic stressors like hyperglycaemia, may give information that is more useful. Similarly, clinical study in appropriate patient population with optimal CBD dosing may give different results from our clinical trial. Our findings in ZDF rats and human adipocytes are in keeping with the known anti-inflammatory potential of CBD and may have positive metabolic implications in diabetes and obesity.

Taken together, our data show the potential for CBD to have a positive impact on metabolic parameters in diabetes, via its interaction with adipocytokines and positive impact on chronic inflammation. Through its direct interaction with vasculature and indirect effects via autonomic nervous system and by down regulating pro-inflammatory cytokines, CBD seems to have a vasoprotective effect. It may have a role in hypertension and atherosclerosis, two major mediators of cardiovascular mortality. Further research, using adequate dosing in the appropriate clinical settings, is required to explore the metabolic and cardiovascular potential of CBD.

7.2. THCV

THCV, an analogue of THC, acts as neutral antagonist at CB_1 and partial agonist at CB_2 receptors. In addition, it can cause indirect agonism at CB_1 and CB_2 by increasing endocannabinoid tone. Limited data suggests that it may act as an agonist at human TRPV1 channels (McPartland *et al.*, 2015). There is limited pre-clinical and clinical data on the effects of THCV. We have highlighted its effects on glycaemic control and its interaction with mature human adipocytes and human aortic endothelial cells.

7.2.1. Effect of THCV on adipokines and glycaemic control

In our clinical study, in type 2 diabetes, THCV improved fasting glycaemia and 3 hours glucose response to OGTT. It increased serum adiponectin but had no significant effects on serum insulin levels. This suggests that THCV may improve insulin sensitivity, by

increasing plasma adiponectin levels. Our findings are consistent with the limited published animal data, where THCV had no impact on lipids, but reduced glucose intolerance and enhanced insulin sensitivity and restored insulin signalling in insulin resistant hepatocytes and myotubes (Wargent *et al.*, 2013). Insulin resistance in type 2 diabetes is characterised by impaired insulin signalling cascade at various levels (Frojdo *et al.*, 2009). THCV has the ability to activate various post-receptor signalling cascades, in adipocytes, as we saw in our study. Further studies, therefore, looking at the impact of THCV on insulin mediated glucose transport in adipocytes, skeletal muscles and hepatocytes, and especially their relationship with different signalling cascades, will be able to shed more light on this.

In the adipocyte study, THCV did not have an impact on leptin or adiponectin, but in the clinical study, it increased plasma levels of adiponectin. It is therefore reasonable to believe that THCV may have an indirect effect on the release of adiponectin and on glycaemic control, via a yet unknown pathway, which is especially obvious in the event of metabolic stress. THCV behaves differently to the peripherally restricted CB_1 antagonists, that reduce body weight and fat mass (Mastinu *et al.*, 2012). Although THCV shows a different pharmacology from rimonabant, yet it is possible that some of its effects are centrally mediated, via its interaction with certain neuropeptides, such as neuropeptide Y. CB_1 blockage with AM251 reduces body weight gain and energy storage in obese mice overexpressing NPY, in brain and sympathetic nervous system (Vahatalo *et al.*, 2015). Further studies looking at its effects on some of the neuropeptides, like neuropeptide Y, may give some useful information.

One outcome from the clinical study was that when given in a combination, the beneficial effects of both CBD and THCV were lost. It was interesting to note that 1:1 ratio of CBD and THCV and 20:1 ratio of CBD:THCV were equally ineffective. This is not in keeping

with the animal data (unpublished GW data), where a combination therapy produced positive metabolic effects. This may be related to difference in species. THCV has been reported to behave as both a CB_1/CB_2 agonist and/or a CB_1/CB_2 neutral antagonist while CBD does not bind with great affinity to CB_1 or CB_2, but can antagonise CB_1 receptor agonists (discussed in chapter 2). Could this finding mean that CBD antagonised the effects of THCV? Another possibility is an interference with each other's metabolism leading to reduced plasma half-life. There are currently no studies looking at the interactions of CBD and THCV. Although THCV is an analogue of THC, it has a different pharmacological profile and therefore the nature of its interaction with CBD will be different. Study of the pharmacokinetic profile of CBD and THCV during this trial could have given some useful information. Another aspect of the combination therapy was that there was no increased incidence of adverse events, which again suggests that somehow the two treatments cancelled each other's effect. Further research is needed to explore the nature of interaction between these two phytocannabinoids.

In the adipocyte study, THCV had no impact on the release of adiponectin, leptin or IL-6 from human adipocytes, but it caused a significant increase in the levels of various phosphorylated signalling proteins. This shows the ability of THCV to interact with adipocytes, and activate pathways that may play a role in adipocyte proliferation, but without having an impact on adipokines. THCV increased plasma adiponectin levels in subjects with diabetes. Therefore, future studies should look at the interaction of THCV with human adipocytes under conditions of metabolic stress, like hyperglycaemia and also consider other sources of adiponectin such as skeletal muscle.

7.2.2. Effect of THCV on vascular endothelium

THCV reduced the release of both VCAM-1 and ET-1 from HAoEC, and increased the levels of phosphorylated signalling proteins CREB, JNK, ERK1/2 and Akt. It seems to interact with endothelial cell, the important functional unit of vasculature, by activating post receptor signalling pathways. The reduction in the levels of VCAM-1 and ET-1 will be in keeping with the known anti-inflammatory properties of THCV. In this context, THCV has a similar effect on VCAM-1 and ET-1 as CBD. Given the roles played by VCAM-1 and ET-1 in vascular biology, THCV may have the potential to act as a vasoprotective agent. In fact, in our clinical study, there was a trend towards reduction in blood pressure with THCV, though it did not reach statistical significance. Currently there is no preclinical or clinical data on the vascular effects of THCV and therefore we did not use this for our acute study in healthy volunteers. THCV is less well studied than CBD and although it seems to have a distinct pharmacological profile from rimonabant, further research is needed to make sure that it is not associated with unwanted central effects. In summary, THCV appears to have positive metabolic effects in type 2 diabetes. It interacts with adipocytes and endothelial cells and shows potent anti-inflammatory properties. It may, therefore, have a role in type 2 diabetes and its related cardiovascular disease, both of which share chronic inflammation as a common pathological process.

7.3. Limitations

There are important limitations to this work that require highlighting. Clinical work in this project involves relatively small numbers. Study in diabetic subjects had five treatment arms with only 9-14 patients in each arm. Caution must be exercised in generalizing the results of this trial. Further larger studies are needed to see if the results can be replicated. The dose of CBD used in the clinical study was much less compared to available data. This might have had an impact on the outcome and lack of effect of CBD. Study of the

pharmacokinetics of CBD and THCV would have given some useful information to guide further research. In the clinical study in young volunteers, we only studied the acute effects of CBD on cardiovascular system. Long-term studies are needed before any firm conclusions can be drawn about the effects of CBD on CVS. It has to be established that the effects of CBD on cardiovascular parameters are not subject to the development of tolerance. In addition, studies in real patients where chronic pathophysiological mechanisms are playing their role, will determine if CBD can overcome these and produce favourable effects. In regards to cell work, a lot of questions remain unanswered, such as receptor pharmacology and the effect of these phaytocannbinoids under conditions of metabolic stress.

In conclusion, despite the limitations of this work, it shows for the first time that THCV has positive impact on hyperglycaemia of type 2 diabetes, while CBD is able to interact with the cardiovascular system and lowers resting blood pressure and blood pressure response to stress. Both CBD and THCV interact with adipocytes and endothelial cells, functional units of adipose tissue and vascular system respectively. THCV and CBD may therefore have a role in the future management of type 2 diabetes and hypertension, respectively. Further large-scale studies are needed, in the right clinical setting, to see if the results can be replicated and in case of CBD, to establish that its acute effects on CVS are not subject to tolerance.

8. References

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Appendix 1

Table 2.1. Summary of subject demographics

		CBD (n=13)	THCV (n=12)	1:1 CBD:THCV (n=11)	20:1 CBD:THCV (n=12)	Placebo (n=14)	Total (n=62)
		No. of subjects (%)					
Sex	Male	10 (77)	10 (83)	6 (55)	9 (75)	7 (50)	42 (68)
	Female	3 (23)	2 (17)	5 (45)	3 (25)	7 (50)	20 (32)
Ethnic Origin	White/Caucasian	12 (92)	12 (100)	11 (100)	12 (100)	12 (86)	59 (95)
	Black/African American	0	0	0	0	1 (7)	1 (2)
	Asian	1 (8)	0	0	0	0	1 (2)
	Other	0	0	0	0	1 (7)	1 (2)
Previous cannabis use		1 (8)	0	1 (9)	1(8)	2 (14)	5 (8)

		CBD (n=13)	THCV (n=12)	1:1 CBD:THCV (n=11)	20:1 CBD:THCV (n=12)	Placebo (n=14)	Total (n=62)
		Mean (SD)					
Age (years)		56.80 (9.92)	62.45 (12.58)	59.33 (8.75)	57.96 (8.11)	58.63 (7.72)	58.98 (9.41)
BMI (kg/m^2) (range)		33.24 (27.2 - 46.8)	34.00 (24.2 - 44.0)	36.43 (30.8 - 50.0)	35.37 (26.1 - 41.5)	33.36 (25.2 - 48.2)	34.39 (24.4 - 50.0)
Weight (kg)	Male	97.9 (19.25)	97.9 (19.25)	101.3 (13.89)	105.4 (17.40)	100.4 (9.28)	99.8 (14.60)
	Female	100.0 (2.83)	100.0 (2.83)	99.9 (16.72)	85.7 (11.06)	88.0 (24.80)	94.2 (19.49)
Height (cm)		171.29 (8.00)	170.32 (7.90)	166.47 (8.01)	168.27 (10.34)	168.23 (11.99)	168.97 (9.34)
Duration of Type 2 diabetes (years)		2.8 (3.34)	4.8 (3.55)	4.4 (2.69)	5.1 (3.32)	3.8 (3.45)	4.2 (3.29)
Duration since most recent episode of dyslipidaemia (years)		2.3 (2.60)	4.0 (4.39)	3.9 (4.60)	1.7 (2.18)	2.6 (3.25)	2.9 (3.47)

Table 2.2. Concomitant anti-diabetic and lipid-lowing medications taken by patients while on randomised treatment

Therapeutic class **Generic name**	**1:1 CBD:THCV (n=11)**	**20:1 CBD:THCV (n=12)**	**CBD (n=13)**	**THCV (n=12)**	**Placebo (n=14)**	**Total (n=62)**
	Anti-diabetic treatment taken by n (%) of patients					
Metformin	**10 (91)**	**11 (92)**	**9 (69)**	**9 (75)**	**12 (86)**	**51 (82)**
DPP-4 inhibitors	**1 (9)**	**1 (8)**	**1 (8)**	**1 (8)**	**1 (7)**	**5 (8)**
Other hypoglycaemic treatment	**1 (9)**	**1 (8)**	**0**	**0**	**1 (7)**	**3 (5)**
Exenatide	1 (9)	1 (8)	0	0	0	2 (3)
Liraglutide	0	0	0	0	1 (7)	1 (2)
Sulfonylureas	**4 (36)**	**3 (25)**	**3 (23)**	**5 (42)**	**4 (29)**	**19 (31)**
	Lipid-lowering medications taken by n (%) of patients					
HMG CoA reductase inhibitors	**10 (91)**	**8 (67)**	**9 (69)**	**11 (92)**	**13 (93)**	**51 (82)**
Simvastatin	7 (64)	4 (33)	7 (54)	6 (50)	10 (71)	34 (55)
Atorvastatin	1 (9)	2 (17)	1 (8)	3 (25)	1 (7)	8 (13)
Pravastatin	0	2 (17)	1 (8)	1 (8)	0	4 (6)
Rosuvastatin	2 (18)	0	0	1 (8)	2 (14)	5 (8)

Table 2.3. Clinical data before (baseline) and after (treatment) 13 weeks of randomised treatment. Bonferroni corrections made for multiple comparisons. Data with statistically significant changes shown in bold.

Variable	CBD		THCV		1:1 CBD:THCV		20:1 CBD:THCV		Placebo	
	Baseline	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**
	Primary endpoint									
HDL-C mmol/L	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
HDL-C (PP) mmol/L	1.0 ± 0.3	1.0 ±0.3	1.1 ±0.2	1.1 ±0.2	1.1 ±0.2	1.1 ±0.3	1.0 ±0.1	1.0 ±0.1	1.0 ±0.3	1.1 ±0.2
	Secondary endpoints									
Total-C mmol/L	4.5 ± 0.3	4.3 ± 0.2	3.8 ± 0.3	3.7 ± 0.3	4.2 ± 0.4	3.8 ± 0.2	4.6 ± 0.3	4.2 ± 0.2	4.0 ± 0.2	3.9 ± 0.3
LDL-C mmol/L	2.5 ± 0.2	2.4 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	2.2 ± 0.3	2.0 ± 0.2	2.8 ± 0.2	2.5 ± 0.1	2.2 ± 0.2	2.2 ± 0.2
HDL:LDL ratio	0.5 ± 0.1	0.4 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
UC VLDL-C mmol/L	0.8 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
TG mmol/L	2.3 ± 0.5	2.3 ± 0.4	1.7 ± 0.3	1.8 ± 0.4	2.5 ± 0.5	2.2 ± 0.4	1.9 ± 0.2	1.9 ± 0.2	2.1 ± 0.4	2.0 ± 0.3
Apo A µmol/L	48.6 ± 2.7	43.6 ± 1.8	48.5 ± 2.0	**49.1 ± 1.9**[a]	48.7 ± 3.5	46.9 ± 2.4	48.7 ± 2.9	45.7 ± 1.9	47.3 ± 2.3	43.9 ± 1.9
Apo B µmol/L	3.1 ± 0.2	3.3 ± 0.2	2.6 ± 0.2	2.7 ± 0.3	3.0 ± 0.3	2.9 ± 0.2	3.4 ± 0.2	3.4 ± 0.2	2.9 ± 0.2	3.0 ± 0.2
Apo B:Apo A ratio	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.0
NEFA mmol/L	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0

Variable	CBD		THCV		1:1 CBD:THCV		20:1 CBD:THCV		Placebo	
	Baseline	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**
Liver TG (%)	26.7 ± 17.1	22.2 ± 17.1	11.7 ± 8.2	14.0 ± 15.5	33.3 ± 18.3	32.2 ± 26.3	25.0 ± 14.6	25.4 ± 17.4	20.5 ± 15.1	18.5 ± 15.4
Fasting glucose mmol/L	8.0 ± 0.6	8.4 ± 0.8	7.4 ± 0.7	**6.7 ± 0.6[a]**	8.5 ± 0.8	8.9 ± 0.7	8.4 ± 0.8	8.8 ± 0.9	7.6 ± 0.4	8.0 ± 0.5
Fructosamine µmol/L	259.5 ± 9.5	256.8 ± 12.4	238.2 ± 7.2	239.3 ± 8.3	254.4 ± 11.3	256.0 ± 17.5	253.3 ± 10.5	268.8 ± 17.5	241.4 ± 5.4	253.7 ± 8.6
HbA1c (%)	6.9 ± 0.3	7.0 ± 0.3	6.6 ± 0.2	6.6 ± 0.2	7.1 ± 0.4	7.4 ± 0.5	7.2 ± 0.3	7.3 ± 0.4	7.0 ± 0.2	7.3 ± 0.3
Glucose - 2 h OGTT mmol/L	15.4 ± 1.0	14.9 ± 1.2	12.6 ± 1.0	12.8 ± 1.0	16.3 ± 1.4	17.2 ± 1.5	14.0 ± 1.0	15.4 ± 1.3	15.4 ± 0.8	16.2 ± 0.8
Insulin - 2 h OGTT pmol/L	708.6 ± 166.1	571.7 ± 114.7	806.2 ± 119.8	885.0 ± 205.9	977.3 ± 220.3	1074.0 ± 343.5	814.4 ± 177.3	874.8 ± 219.0	821.9 ± 106.2	789.7 ± 129.5
Fasting insulin pmol/L	111.5 ± 12.8	123.7 ± 16.9	152.9 ± 27.2	203.4 ± 59.6	175.3 ± 26.0	185.7 ± 22.6	197.6 ± 31.1	192.2 ± 20.9	171.7 ± 28.1	179.6 ± 21.0
C-peptide nmol/L	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
HOMA2-IR	2.3 ± 0.3	2.6 ± 0.4	3.0 ± 0.5	3.8 ± 1.0	3.5 ± 0.5	3.7 ± 0.5	4.2 ± 0.8	4.0 ± 0.5	3.4 ± 0.6	3.6 ± 0.4
HOMA2 insulin sensitivity	51.1 ± 6.1	53.0 ± 10.1	47.3 ± 9.4	53.5 ± 13.4	34.9 ± 5.2	31.2 ± 4.8	30.2 ± 3.3	28.9 ± 3.5	42.4 ± 7.8	37.8 ± 8.9
HOMA2 B cell function	67.9 ± 7.6	69.6 ± 8.7	105.1 ± 18.7	**144.4 ± 33.3[b]**	95.7 ± 15.3	87.5 ± 16.4	103.7 ± 17.5	97.9 ± 15.2	96.4 ± 11.1	94.7 ± 10.9

Variable	CBD		THCV		1:1 CBD:THCV		20:1 CBD:THCV		Placebo	
	Baseline	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**
BMI kg/m^2	33.3 ± 1.5	33.1 ± 1.4	34.0 ± 1.9	33.8 ± 1.9	36.4 ± 1.7	36.1 ± 1.7	35.4 ± 1.3	35.2 ± 1.4	33.4 ± 1.9	32.9 ± 2.1
Waist circumference (cm)	107.7 ± 3.0	108.0 ± 2.9	115.3 ± 3.8	114.9 ± 4.0	115.4 ± 2.9	116.2 ± 3.6	113.7 ± 3.8	113.5 ± 3.8	109.2 ± 3.5	106.3 ± 3.1
Waist-to-hip ratio	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Neck circumference (cm)	42.4 ± 0.9	42.1 ± 1.0	42.8 ± 1.1	42.8 ± 1.0	42.7 ± 1.0	42.2 ± 1.1	42.8 ± 1.1	43.1 ± 1.1	41.7 ± 1.3	40.5 ± 1.2
Visceral abdominal fat (l)	7.8 ± 0.8	8.2 ± 0.8	8.3 ± 1.2	8.1 ± 1.2	7.7 ± 0.8	7.8 ± 0.7	8.6 ± 0.9	9.6 ± 1.1	7.0 ± 0.7	7.2 ± 1.2
Appetite 0-10 NRS score	5.6 ± 0.3	4.9 ± 0.3	5.4 ± 0.5	5.0 ± 0.5	4.5 ± 0.4	3.6 ± 0.5	5.0 ± 0.6	4.2 ± 0.5	5.1 ± 0.3	4.5 ± 0.4
Systolic BP (mmHg)	133.4 ± 4.9	132.2 ± 3.9	135.9 ± 4.2	132.8 ± 5.4	126.4 ± 3.7	134.3 ± 4.1	132.7 ± 3.3	134.2 ± 4.7	137.2 ± 3.4	140.4 ± 3.4
Diastolic BP (mmHg)	70.0 ± 2.7	70.6 ± 2.7	70.6 ± 3.9	71.0 ± 3.0	73.2 ± 2.1	77.5 ± 2.5	73.5 ± 3.1	72.1 ± 3.3	73.0 ± 2.8	72.3 ± 3.2
Pulse rate (bpm)	72 ± 5	71 ± 5	75 ± 4	74 ± 4	80 ± 4	77 ± 3	77 ± 4	82 ± 5	72 ± 3	76 ± 2
BDI-II score	3.8 ± 1.0	4.6 ± 1.0	2.8 ± 1.1	**3.3** ± 1.0	4.5 ± 1.6	4.7 ± 1.5	2.8 ± 0.8	**8.0 ± 2.3**[b]	3.5 ± 1.0	3.5 ± 0.9
CRP (ng/mL)	7073 ± 8345	7301 ± 7578	13915 ± 22142	6026 ± 5488	12369 ± 10425	18958 ± 19162	21166 ± 43390	8224 ± 6330	6369 ± 6217	10512 ± 10718
TNFα (pg/mL)	7.0 ± 1.6	7.0 ± 1.9	7.3 ± 1.4	7.1 ± 2.2	6.8 ± 2.5	7.2 ± 2.3	6.6 ± 2.4	6.9 ± 2.5	7.2 ± 2.6	7.0 ± 2.5
IL-6 (pg/mL)	3.9 ± 6.8	6.2 ± 8.0	12.5 ± 13.1	9.2 ± 9.5	4.3 ± 4.1	4.4 ± 4.8	6.9 ± 13.5	12.0 ± 20.5	4.0 ± 4.4	7.2 ± 11.8

Variable	CBD		THCV		1:1 CBD:THCV		20:1 CBD:THCV		Placebo	
	Baseline	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**
GLP-1 (pg/mL)	16.7 ± 34.5	20.2 ± 25.3	22.0 ± 30.6	35.7 ± 51.9	18.5 ± 26.1	20.4 ± 25.9	20.2 ± 30.4	32.5 ± 33.7	24.0 ± 29.0	27.6 ± 33.0
GIP (pg/mL)	32.4 ± 15.0	54.0 ± 35.0	40.5 ± 18.6	36.1 ± 21.8	53.6 ± 27.6	49.5 ± 21.2	45.8 ± 27.2	53.3 ± 29.9	48.8 ± 21.4	45.7 ± 27.2
Ghrelin (pg/mL)	15.0 ± 22.5	15.4 ± 17.1	18.1 ± 17.0	14.7 ± 18.3	9.8 ± 15.8	8.0 ± 11.3	4.7 ± 10.6	7.0 ± 14.0	15.7 ± 21.3	12.9 ± 7.9
RBP-4 (ng/mL)	8874 ± 1186	9158 ± 1317	8692 ± 2186	**9355** ± 1949	8653 ± 1311	8939 ± 1676	9177 ± 1732	8742 ± 1282	9436 ± 2108	8738 ± 1328
Orexin (ng/mL)	0.6 ± 0.3	0.7 ± 0.6	1.0 ± 0.6	1.0 ± 0.6	1.0 ± 0.5	0.8 ± 0.4	0.6 ± 0.5	0.8 ± 1.2	0.8 ± 0.4	0.8 ± 0.5
Ketones (mM)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
AEA nmol/L	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
2-AG nmol/L	5.0 ± 2.9	4.7 ± 2.9	4.3 ± 1.7	4.1 ± 1.4	6.2 ± 3.1	5.0 ± 1.5	3.8 ± 1.5	3.8 ± 1.7	5.0 ± 3.3	5.4 ± 3.4
OEA nmol/L	2.4 ± 1.1	1.8 ± 0.7	2.4 ± 1.0	2.3 ± 0.6	2.5 ± 0.8	2.2 ± 0.7	2.2 ± 0.5	2.2 ± 0.8	2.4 ± 0.5	2.1 ± 0.5
PEA nmol/L	2.7 ± 2.0	1.9 ± 0.7	2.7 ± 1.1	2.5 ± 0.7	2.6 ± 0.7	2.3 ± 0.6	2.5 ± 1.2	2.6 ± 1.7	2.9 ± 1.3	2.0 ± 0.5

Data are mean ± SD; [a]$p<0.05$, [b]$p<0.01$ compared with placebo; Abbreviations: AEA, N-arachidonoylethanolamine; Apo, Apolipoprotein; BDI-II, Beck Depression Inventory-II; BMI, Body mass index; BP, blood pressure; CRP, C reactive protein; GIP, glucose dependent insulinotropic peptide, GLP-1, glucagon like peptide 1, HbA1c, glycosylated haemoglobin A1c; HDL, high density lipoprotein; HOMA2-IR, homeostatic assessment model 2 - insulin resistance; IL-6, interleukin 6; LDL-C, low density lipoprotein cholesterol; NEFA, non-esterified fatty acid; OEA, oleoyl-ethanolamine; OGTT, oral glucose tolerance test; PEA, palmitoyl-thanolamine; RBP-4, retinol binding protein 4, TNFα, tumour necrosis factor α; UC, ultracentrifugation; VLDL, very low density lipoprotein; 2-AG, 2-arachidonoylglycerol

Appendix 2

Table 3.4. Treatment-emergent (all-causality) and treatment related adverse events by primary system organ class (SOC) and MedDRA Preferred Terms (PTs) occurring in one or more patients in the active or placebo treatment groups

	1:1 CBD:THCV (5 mg:5 mg) (n=11)		**20:1 CBD:THCV (100 mg:5 mg) (n=12)**		**CBD alone (100 mg) (n=13)**		**THCV alone (5 mg) (n=12)**		**Placebo (n=14)**	
SOC	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**
	Number of patients									
Total patients with at least one AE	**7**	**7**	**8**	**5**	**11**	**7**	**11**	**8**	**13**	**7**
Cardiac disorders	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**
Myocardial infarction	0	0	1	0	0	0	0	0	0	0
Myocardial ischaemia	0	0	0	0	0	0	0	0	1	0
Familial and genetic disorders	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Dermoid cyst	0	0	1	0	0	0	0	0	0	0
Endocrine disorders	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**
Hyperparathyroidism	0	0	0	0	1	0	0	0	0	0
Eye disorders	**0**	**0**	**1**	**0**	**3**	**2**	**0**	**0**	**0**	**0**
Retinal haemorrhage	0	0	1	0	0	0	0	0	0	0

	1:1 CBD:THCV (5 mg:5 mg) (n=11)		20:1 CBD:THCV (100 mg:5 mg) (n=12)		CBD alone (100 mg) (n=13)		THCV alone (5 mg) (n=12)		Placebo (n=14)	
SOC	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**
	Number of patients									
Conjunctival haemorrhage	0	0	0	0	1	1	0	0	0	0
Vision blurred	0	0	0	0	1	0	0	0	0	0
Visual impairment	0	0	0	0	1	1	0	0	0	0
Gastrointestinal disorders	**1**	**1**	**3**	**2**	**3**	**2**	**3**	**3**	**2**	**2**
Abdominal distension	0	0	0	0	0	0	1	1	0	0
Abdominal pain	0	0	1	1	0	0	0	0	0	0
Abdominal pain upper	0	0	0	0	0	0	1	1	0	0
Change of bowel habit	0	0	1	0	0	0	0	0	0	0
Diarrhoea	0	0	0	0	1	1	2	2	0	0
Flatulence	1	1	0	0	1	1	0	0	0	0
GORD	0	0	0	0	1	1	0	0	0	0
Nausea	0	0	1	1	0	0	0	0	1	1

	1:1 CBD:THCV (5 mg:5 mg) (n=11)		20:1 CBD:THCV (100 mg:5 mg) (n=12)		CBD alone (100 mg) (n=13)		THCV alone (5 mg) (n=12)		Placebo (n=14)	
SOC	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**
	Number of patients									
Vomiting	0	0	0	0	1		0	0	1	1
Rectal haemorrhage	0	0	0	0	0	0	1	0	0	0
General disorders	**3**	**3**	**2**	**1**	**1**	**1**	**1**	**1**	**4**	**2**
Fatigue	1	1	1	1	0	0	1	1	1	0
Feeling abnormal	0	0	0	0	0	0	0	0	1	1
Irritability	1	1	0	0	0	0	0	0	0	0
Malaise	1	1	0	0	1	1	0	0	1	1
Oedema peripheral	0	0	0	0	0	0	1	0	1	0
Vessel puncture site reaction	0	0	1	0	0	0	0	0	0	0
Hepatobiliary disorders	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**
Hepatic steatosis	0	0	0	0	0	0	0	0	1	0

	1:1 CBD:THCV (5 mg:5 mg) (n=11)		20:1 CBD:THCV (100 mg:5 mg) (n=12)		CBD alone (100 mg) (n=13)		THCV alone (5 mg) (n=12)		Placebo (n=14)	
SOC	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**
	Number of patients									
Infections	**1**	**0**	**2**	**0**	**5**	**0**	**4**	**1**	**2**	**0**
Ear infection fungal	0	0	0	0	0	0	0	0	1	0
Genital candidiasis	0	0	1	0	0	0	0	0	0	0
Influenza	0	0	1	0	1	0	1	0	2	
Laryngitis	0	0	0	0	0	0	1	1	0	0
Lower respiratory tract infection	0	0	0	0	1	0	1	0	0	0
Nasopharyngitis	1	0	1	0	1	0	2	0	0	0
Tinea pedis	0	0	0	0	1	0	0	0	0	0
Urinary tract infection	0	0	0	0	1	0	0	0	0	0
Injury and procedural complications	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**2**	**0**
Foreign body	0	0	0	0	0	0	0	0	1	0
Joint sprain	0	0	0	0	1	0	0	0	1	0

	1:1 CBD:THCV (5 mg:5 mg) (n=11)		20:1 CBD:THCV (100 mg:5 mg) (n=12)		CBD alone (100 mg) (n=13)		THCV alone (5 mg) (n=12)		Placebo (n=14)	
SOC	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**
	Number of patients									
Investigations	**1**	**1**	**1**	**1**	**0**	**0**	**1**	**0**	**1**	**1**
ALT increased	1	1	0	0	0	0	0	0	0	0
Blood TSH decreased	0	0	0	0	0	0	0	0	1	1
Cardiac murmur	0	0	1	1	0	0	0	0	0	0
Weight increased	0	0	0	0	0	0	1	0	0	0
Metabolism and nutrition disorders	**1**	**1**	**2**	**1**	**2**	**2**	**5**	**4**	**4**	**3**
Decreased appetite	1	1	0	0	2	2	4	4	2	2
Diabetes mellitus inadequate control	0	0	1	0	0	0	0	0	0	0
Gout	0	0	0	0	0	0	0	0	1	0
Hypercholesterolaemia	0	0	1	0	0	0	0	0	0	0
Increased appetite	0	0	1	1	0	0	1	0	1	1

	1:1 CBD:THCV (5 mg:5 mg) (n=11)		20:1 CBD:THCV (100 mg:5 mg) (n=12)		CBD alone (100 mg) (n=13)		THCV alone (5 mg) (n=12)		Placebo (n=14)	
SOC	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**
	Number of patients									
Musculoskeletal disorders	**2**	**1**	**2**	**2**	**2**	**1**	**1**	**0**	**2**	**1**
Arthralgia	0	0	2	2	1	1	0	0	0	0
Arthritis	0	0	0	0	0	0	0	0	1	1
Joint instability	0	0	0	0	1	0	0	0	0	0
Joint swelling	0	0	1	1	0	0	0	0	0	0
Muscle spasms	1	0	0	0	0	0	1	0	0	0
Muscle twitching	1	1	0	0	0	0	0	0	0	0
Musculoskeletal pain	0	0	0	0	0	0	0	0	2	0
Myalgia	0	0	0	0	1	1	0	0	0	0
Nervous system disorders	**2**	**2**	**1**	**1**	**2**	**2**	**3**	**3**	**3**	**3**
Carpal tunnel syndrome	0	0	0	0	0	0	0	0	1	1
Disturbance in attention	0	0	0	0	1	1	0	0	0	0

	1:1 CBD:THCV (5 mg:5 mg) (n=11)		20:1 CBD:THCV (100 mg:5 mg) (n=12)		CBD alone (100 mg) (n=13)		THCV alone (5 mg) (n=12)		Placebo (n=14)	
SOC	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**
	Number of patients									
Dizziness	1	1	0	0	0	0	1	1	2	2
Headache	1	1	1	1	1	1	1	1	1	1
Lethargy	0	0	0	0	0	0	1	1	0	0
Psychiatric disorders	**1**	**1**	**2**	**0**	**2**	**2**	**0**	**0**	**2**	**0**
Abnormal dreams	1	1	0	0	0	0	0	0	1	0
Affect lability	0	0	0	0	1	1	0	0	0	0
Depression	0	0	1	0	0	0	0	0	0	0
Insomnia	0	0	0	0	0	0	0	0	1	0
Sleep disorder	0	0	1	0	1	1	0	0	0	0
Renal disorders	**1**	**1**	**0**	**0**	**0**	**0**	**2**	**0**	**3**	**1**
Haematuria	0	0	0	0	0	0	2	0	2	0
Hypertonic bladder	0	0	0	0	0	0	0	0	1	1
Pollakiuria	1	1	0	0	0	0	0	0	0	0

	1:1 CBD:THCV (5 mg:5 mg) (n=11)		20:1 CBD:THCV (100 mg:5 mg) (n=12)		CBD alone (100 mg) (n=13)		THCV alone (5 mg) (n=12)		Placebo (n=14)	
SOC	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**
	Number of patients									
Respiratory, thoracic and mediastinal disorders	**1**	**1**	**2**	**2**	**1**	**0**	**0**	**0**	**2**	**0**
Cough	0	0	0	0	0	0	0	0	1	0
Dry throat	1	1	0	0	0	0	0	0	0	0
Dyspnoea	0	0	0	0	0	0	0	0	1	0
Dyspnoea exertional	0	0	1	1	0	0	0	0	0	0
Oropharyngeal pain	0	0	1	1	0	0	0	0	0	0
Wheezing	0	0	0	0	1	0	0	0	0	0
Skin and subcutaneous tissue disorders	**0**	**0**	**3**	**1**	**0**	**0**	**0**	**0**	**0**	**0**
Eczema	0	0	1	0	0	0	0	0	0	0
Rosacea	0	0	1	0	0	0	0	0	0	0
Skin odour abnormal	0	0	1	1	0	0	0	0	0	0

	1:1 CBD:THCV (5 mg:5 mg) (n=11)		20:1 CBD:THCV (100 mg:5 mg) (n=12)		CBD alone (100 mg) (n=13)		THCV alone (5 mg) (n=12)		Placebo (n=14)	
SOC	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**	**All-causality**	**Treatment related**
	Number of patients									
Vascular Disorders	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**
Hypertension	0	0	0	0	0	0	1	0	0	0