EFFECT OF POLYPHENOLS ON GLUCOREGULATORY BIOMARKERS BLOOD PRESSURE AND LIPID PROFILE IN OVERWEIGHT AND OBESE SUBJECTS

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ABSTRACT

This thesis describes a series of *in vitro*, animal and humans studies conducted with the aim of investigating the effect of polyphenol-rich green coffee bean extract (GCBE) and dark chocolate (DC) on biomarkers of glucose metabolism, lipid profile and blood pressure (BP) in overweight and obese individuals.

Green coffee and *Theobroma cacao* bean extracts were found to be rich in polyphenols and to act as effective free radical scavenging compounds *in vitro*. A potential role for GCBE in inhibiting pancreatic lipase was identified *in vitro*. Preliminary human studies revealed a differential effect of GCBE and DC on fasting glucose, total cholesterol, BP and urinary glucocorticoids. Accordingly, consumption of 200mg GCBE containing 90mg chlorogenic acid (CGA) twice daily for 14 days by healthy overweight and obese volunteers reduced systolic BP (P=0.043), urinary free cortisone (P=0.0015) and waist circumference (-0.78cm; P=0.013) but raised salivary cortisone (P=0.042) without significantly affecting capillary fasting glucose, total cholesterol or urinary antioxidant excretion (P>0.05). The ability of CGA to differentially regulate cortisol metabolism was further highlighted in male C57BL6 mice wherein daily administration of a diet containing 0.15% CGA for 17 days marginally increased cortisol in kidney (P=0.108; eta^2=0.26) and reduced hepatic cortisol (P=0.219; eta^2=0.14). In the preliminary single-blind randomised cross-over DC study, 2-week consumption of 20g DC containing 500mg or 1000mg polyphenols by overweight and obese individuals produced equal reductions in capillary fasting glucose, systolic and diastolic BP. This was further confirmed by the long-term placebo-controlled trial wherein ingestion of 20g DC (500mg polyphenols) for 4 weeks reduced fasting glucose (P=0.028), insulin resistance (P=0.005), systolic (P=0.020), diastolic BP (P=0.008) and improved insulin sensitivity (QUICKI, P=0.04; revised-QUICKI, P=0.026) and urinary antioxidant capacity (total phenolics, P=0.046; ferric-reducing capacity, P=0.048) without significantly affecting lipid profile (P>0.05). A particular contribution of the main study is the finding that overweight and obese individuals respond more effectively to polyphenol-rich DC, compared to lean individuals, but more adversely to polyphenol-deficient placebo. The latter was marked by the rise in fasting insulin, insulin resistance and salivary cortisol.

In conclusion, this thesis supports a role for polyphenol-rich GCBE and DC in counteracting overweight and obesity-related complications. The role of GCBE and CGA in modulating glucocorticoid metabolism emerges as a novel and potentially relevant field of research to the prevention of overweight and obesity-related complications.

Keywords: dark chocolate, green coffee bean extract, polyphenols, glucose, insulin, lipid, blood pressure, glucocorticoids, obesity

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11βHSD	11-beta hydroxysteroid dehydrogenase
1O2	Singlet oxygen
5α-DHF	5-alpha dihydrocortisol
5α-THF	5-alpha tertahydrocortisol
AAPH	2, 2'-azobis-(2-amidinopropane) dihydrochloride
Accutrend GC	Accutrend glucose-cholesterol
Acetyl-CoA	Acetyl co-enzyme
ADP	Adenosine Diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AOC	Total antioxidant capacity
aPKC	Atypical protein kinase C
ATP	Adenosine triphosphate
AUC	Area under curve
BH2	Dihydrobiopterin
BH4	Tetrahydrobiopterin
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin
C	Carbon
cAMP	Cyclic adenosine monophosphate
CETP	Cholesterol ester transfer protein
CGA	Chlorogenic acid
cGMP	Cyclic guanidine monophosphate
CIR120	Corrected insulin response
CQA	Caffeoylquinic
CV	Coefficient of variation
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DC	Dark chocolate
DEFRA	Department for Environment, Food and Rural Affairs
DHE	Dihydrocortisone
DHF	Dihydrocortisol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1, 1 –diphenyl-2-picilhydrazyl
E	Cortisone
ELISA	Enzyme-linked immuno-sorbent assay
eNOS	Endothelium nitric oxide synthase
ET	Electron transport
EtOH	Ethanol
F	Cortisol
FC	Folin-Ciocalteu
Fe2+	Ferrous ion
Fe3+	Ferric ion
FFA	Free fatty acids
FG	Fasting glucose
FOXO1	Forkhead box O transcription factor 1
FRAP	Ferric-reducing capacity of plasma
G-6-P	Glucose-6-phosphate
GA	Gallic acid

GAE	Gallic acid equivalents
GCBE	Green coffee bean extract
GC-MS	Gas chromatography-mass spectrometry
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
H+	Hydrogen ion
H2O2	Hydrogen peroxide
H3O	Hydronium
HAT	Hydrogen atom transfer
HC	Hip circumference
HCL	Hydrochloric acid
HDL	High-density lipoprotein
HK	Hexokinase
HO.	Hydroxyl radical
HOMA-IR	Homeostasis-model assessment of insulin resistance
HPA	Hypothalamic-adrenal axis
HPLC	High performance liquid chromatography
HSL	Hormone sensitive lipase
IC50	Inhibitory concentration 50
ICCO	International cacao organisation
ICO	International coffee organisation
IGF	Insulin growth factor
IL-6	Interleukin-6
INOS	Inducible nitric oxide synthase
INTERSALT	International Study of Salt and Blood Pressure
IR	Insulin receptor
IRS	Insulin receptor substrate
ISI	Insulin sensitivity index
JNK	Jun N-terminal Kinase
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
LDL	Low-density lipoprotein
LoD	Limits of detection
LoQ	Limits of quantification
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
mRNA	Messenger ribonucleic acid
Na	Sodium
NaCl	Sodium chloride
NAD(H)	Nicotinamide Adenine
NAD(P)H	Nicotinamide adenine dinucleotide phosphate hydrogenase
NaOH	Sodium hydroxide
NEFA	Non-esterified fatty acids
NG	Negligible
NHANES	The National Health and Nutrition Examination Survey
NO	Nitric oxide
NOS	Nitric oxide synthase
O2-	Superoxide anion
ONOO-	Peroxynitrate
ORAC	Oxygen radical absorbance capacity
P13K	Phosphatidylinositol 3-kinase
PBS	Phosphate buffer saline

PDH	Pyruvate dehydrogenase
PEP-CK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKCα	Protein kinase C alpha
PKCβ	Protein kinase C beta
PKCδ	Protein kinase C gamma
PKCζ	Protein kinase C zeta
PKCλ	Protein kinase C lambda
PKCϴ	Protein kinase C theta
QUICKI	Quantitative insulin sensitivity check index
Revised-QUICKI	Revised quantitative insulin sensitivity check index
ROO.	Peroxyl radical
ROS	Reactive oxygen species
R.S.D	Relative standard deviation
SBP	Systolic blood pressure
SCLT1	Sodium-coupled glucose transporter
SD	Standard deviation
SEM	Standard error of mean
SPANOVA	Mixed between-within subjects analysis of variance
SREBP-1c	Sterol regulatory element binding protein-1c
TBARS	Thiobarbituric acid-reactive substances
TC	Total cholesterol
TCBE	Theobroma cacao bean extract
TC: HDL	Total cholesterol-to- high density lipoprotein ratio
TG	Triglycerides
THE	Tetrahydrocortisone
THF	Tertahydrocortisol
TMB	3,3,5,5 Tetramethyl-benzidine
TNF-α	Tumour necrosis factor alpha
TOC	Trolox equivalent antioxidant capacity
TPTZ-Fe2+	Ferrous-2, 4, 6-tri-2-pyridyl-s-triazine
TPTZ-Fe3+	Ferric-2, 4, 6-tri-2pyridyl-s-triazine
UDP	Uridine diphosphate
USDA	US Department of Agriculture
VLDL	Very low density lipoprotein
WC	Waist circumference
WHO	World health organisation
WHR	Waist-to-hip ratio

1. General Introduction

1.1. Background and structure of the thesis

Over the last two decades, the emergence of the concept that plant-derived polyphenols act via various biochemical, physiological and endocrinological pathways to promote health and prevent chronic diseases has stimulated much interest in this field. However, the diversity and complexity of these compounds implies that much remains to be elucidated concerning the mechanisms by which these compounds influence health. With the rising obesity epidemic, improving our knowledge of the relation between diet and health is becoming increasingly ever more important. This is especially evident when considering that obesity is associated with various metabolic abnormalities including insulin resistance, endothelium dysfunction and dyslipidaemia, which are in turn implicated in the development of diabetes, hypertension and cardiovascular disease.

This thesis consists of five chapters. Chapter 1 begins by providing an overview of the definition, classification, prevalence and consequences of obesity. This step is crucial to highlighting the global burden of the overweight and obesity epidemic whilst emphasising the need to prevent overweight and obesity-related chronic conditions through appropriate dietary strategies and public health strategies aimed at promoting health in this high-risk population. In section 1.3, the pathophysiology of obesity is examined. The role of insulin resistance as the centre of obesity-related complications is described (section 1.4-1.6). The molecular and biochemical pathways underlying the association between insulin resistance, hypertension and dyslipidaemia are critically evaluated. This provides the rationale for investigating the three main variables stated in the title of the thesis: glucoregulatory biomarkers, blood pressure and lipid profile. Next in sections 1.7-1.9, the etiology of insulin resistance is discussed with specific reference to the role of free fatty acids (FFA)s, cortisol and oxidative stress in promoting obesity-related insulin resistance, three variables that are subsequently examined in the main study and which reflect potential mechanistic pathways that underlie insulin resistance and overweight and obesity-related hypertension and dyslipidaemia. This discussion sets the scene for Section 1.10 which begins by defining and classifying polyphenols and moves towards examining the potential role of these powerful antioxidants in preventing type-II diabetes, hypertension and cardiovascular disease in overweight and obesity. Particular attention is drawn to green coffee bean extract (GCBE) and dark chocolate (DC) as important dietary sources of polyphenols. Conflicts and gaps in

the literature are indentified. The main concepts discussed in Chapter 1 are then summarised and brought forward in section 1.120. The hypothesis and overall aim of the PhD research are stated and specific rationale for each of the *in vitro*, animal and human studies are highlighted (Section 1.13). In, Chapter 2 the general materials and methods used in this thesis are described. The principles of the antioxidant and pancreatic lipase assays are highlighted and details are provided concerning the collaborative mice study that was conducted at the Queen's Medical Research Institute, Edinburgh, UK. Section 2.3 then describes the preliminary GCBE and DC human studies and the main DC study. The rationale behind the selected study designs are discussed and the strengths and limitations of such designs are pointed out. A description of subjects sources and selection, inclusion and exclusion criteria is provided and the basis for selecting the GCBE and DC polyphenol doses is underlined. The main findings of the thesis are then outlined in Chapter 3. Chapter 4 builds on these results by discussing the implications and the limitations of the studies and by providing directions for future research. The overall findings of the thesis are then summarised in Chapter 5 and the conclusion stated.

1.2. Overweight and Obesity

1.2.1 Definition

Obesity can be defined as excess accumulation of body fat arising from a sustained or a periodic positive energy balance that is when energy intake exceeds energy expenditure (WHO, 2000). The most common method of classifying overweight and obesity is based on Body Mass Index (BMI). Accordingly, the World Health Organisation (WHO) classifies individuals with BMI 25-29.99 kg/m^2 as overweight while individuals with BMI$\geq$ 30 kg/m^2 are termed obese. Obesity could also be categorized further into abdominal or peripheral obesity using waist circumference (WC) as a surrogate measure of body fat distribution (see section 2.11.4). The importance of distinguishing between abdominal and peripheral obesity is discussed in section 2.11.4. These standard WHO definitions will be adopted throughout this thesis, unless otherwise stated.

1.2.2 Prevalence

In recent years the prevalence of obesity has increased reaching epidemic levels. Worldwide, an estimated 1.6 billion adults are overweight and at least 400 million are obese (WHO, 2006). These rates are predicted to increase reaching 2.3 billion overweight adults and 700 million obese by 2015 (WHO, 2006). Britain is amongst the countries with highest prevalence of overweight and obesity in Western Europe[1] with 65.7% of British men and 61.9% of British women being categorised as overweight or obese (Figure 1.1). These figures are closely reflected in the Scottish Heath Survey 2003 which demonstrates that 65.4% of Scottish men and 59.7% of Scottish women are either overweight or obese (Bromley *et al.*, 2005). Interestingly, although men in Scotland are more likely to be overweight (43.0% vs. 33.8%), the prevalence of obesity (22.4% vs. 26.0%), morbid obesity (1.6% vs. 3.4%) or raised waist-to-hip ratio (WHR) and WC (28.8% and 28.0% vs. 37.1% and 38.9%) is higher amongst women in Scotland (Bromley *et al.*, 2005). This has several implications since abdominal fat distribution, characterised by raised WC and WHR, is known to be an independent risk factor for obesity-associated co-morbidities and mortality.

[1] Western Europe as defined by UN.

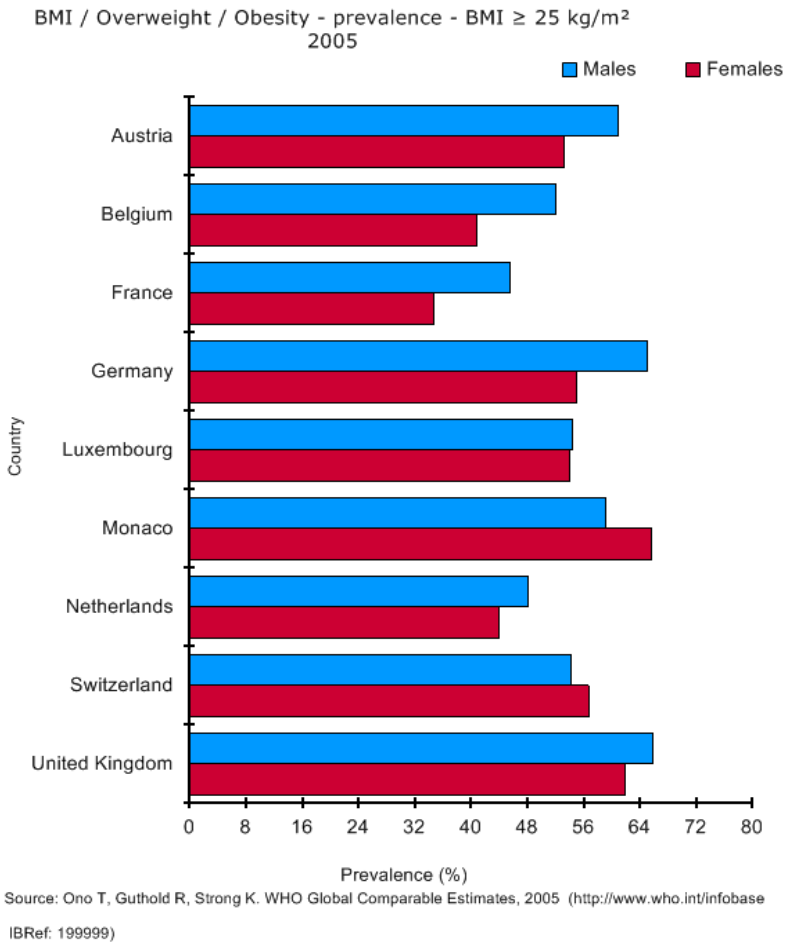


Figure 1.1 Prevalence of overweight and obesity in Western Europe (Ono *et al*., 2005).

1.2.3 Health consequences

Apart from being a major contributor to chronic disease burden and mortality, obesity-particularly abdominal obesity, is also closely related to the metabolic syndrome, a cluster of diseases that encompasses the following conditions (WHO, 2000):

- Dyslipidaemia
- Hypertension
- Glucose intolerance and insulin resistance
- Abdominal obesity

Using analyses from 33 cohort studies and data from a number of international epidemiological studies such as the International study on Salt and Blood Pressure (INTERSALT) and the National Health and Nutrition Examination Survey (NHANES) III, James *et al.* (2004) estimated that the global burden of chronic diseases ascribed to excess

body weight were 58% for type II diabetes, 21% for ischemic heart disease and 39% for hypertension. Based on this data, overweight and obesity were found to be accountable for 2.5 million global annual deaths and 30 million disability-adjusted life years in 2000 (James *et al.*, 2004). In Scotland, obesity accounts for 500,000 cases of hypertension, 50,000 cases of coronary heart disease and over 30,000 cases of type II diabetes, amongst others (Grant *et al.*, 2007) (Table 1.1) with substantial costs incurred by the National Health Service (Bourn, 2001).

Table 1.1 The estimated prevalence of obesity-related diseases and the estimated number of cases attributable to obesity in Scotland in 2003 (Grant *et al.*, 2007).

Disease	Estimated proportion attributable to obesity[71] (%)	Estimated number of prevalent cases/annual incident cases in Scotland[72] (n)	Estimated number of cases in Scotland attributable to obesity (2003) (n)
Cardiovascular			
Hypertension	36%	1,329,696 (p)	478,691
Angina pectoris	15%	250,344 (p)	37,552
Myocardial Infarction	18%	135,432 (p)	24,378
Stroke	6%	92,340 (p)	5,540
Endocrine			
Type 2 diabetes	47%	73,872 (p)	34,720
Neoplastic			
Colon cancer	29%	2,242 (i)	650
Ovarian cancer	13%	616 (i)	80
Prostate cancer	3%	2,318 (i)	70
Endometrial cancer	14%	449 (i)	63
Rectal cancer	1%	1,123 (i)	11
Musculo-skeletal			
Osteoarthritis	12%	118,500 (p)	14,220
Gout	47%	20,150 (p)	9,470
Gastro-intestinal			
Gallstones	15%	11,350 (p)	1,702

Table 1.2 provides estimates of the relative risk of developing chronic diseases as a result of obesity (Grant *et al.*, 2007). Taking in account these figures, the strong association between obesity and diabetes, hypertension and cardiovascular disease (CVD) and the continually

rising obesity epidemic, the need for implementing adequate public health strategies to prevent obesity and its associated complications is becoming ever-more important. Notably, the WHO in its Global Strategy on Diet, Physical Activity and Health emphasises the need to render 'healthier diet options more affordable and accessible' (Samuelson, 2004). This emphasis stems from the recognition that in the absence of a supportive physical and economical environment, nutrition and health messages often fail to influence individuals 'dietary behaviour as a result of the persistent pressure to consume unhealthy diets' (Samuelson, 2004). Wieringa *et al.* (2008) have identified nutritional factors and food product supply, particularly the quality of food products, as 'prominent points of intervention for prevention strategies'. Although, Wieringa *et al.* (2008) argued a limited role for functional foods in preventing overweight and obesity as a result of their inability to tackle the underlying lifestyle risk factors, excess energy intake and physical inactivity, functional food may provide a cost-effective strategy in preventing overweight and obesity-associated co-morbidities and mortalities. This is particularly true when considering that 90% of type II diabetics are obese (Kumanyika *et al.*, 2002) and that in women, obesity is the third most powerful predictor of CVD (after age and blood pressure) and that women who are obese are 12 times more likely to develop type II diabetes than women of a healthy weigh (Bourn, 2001). In order to understand how functional foods and polyphenols can influence overweight and obesity, it is important to explore the underlying pathophysiology of overweight and obesity and apply current knowledge in this field to the chemical and physiological properties of polyphenols.

Table 1.2 Relative risk of chronic diseases in obese women and men (Grant *et al.*, 2007).

Disease	Relative risk in women	Relative risk in men
Type 2 diabetes	12.7	5.2
Hypertension	4.2	2.6
Myocardial infarction	3.2	1.5
Colon cancer	2.7	3.0
Angina	1.8	1.8
Gall bladder diseases	1.8	1.8
Ovarian cancer	1.7	-
Osteoarthritis	1.4	1.9
Stroke	1.3	1.3

1.3. Pathophysiology: Insulin resistance as the centre of obesity-associated complications

Understanding the physiological abnormalities governing the current obesity epidemic is pivotal to the development of effective dietary approaches to deal with this complex multi-factorial disease. This is particularly evident when considering the central role of obesity in the development of CVD and its ability to influence other CVD risk factors (Grundy, 2004) (Figure 1.2). For decades, the association between insulin resistance, type II diabetes, hypertension and dyslipidaemia have been recognised. Reaven (1988) proposed the term Syndrome X as a condition encompassing all of the above metabolic disturbances with insulin resistance forming the centre of these physiological abnormalities. Surprisingly Reaven (1988) did not include obesity in their initial definition of Syndrome X and it was not until 1991 that Ferrannini and colleagues postulated that insulin resistance is the underlying metabolic abnormality linking obesity with type II diabetes, hypertension and dyslipidaemia. The concept of the metabolic syndrome as an insulin resistance syndrome was born.

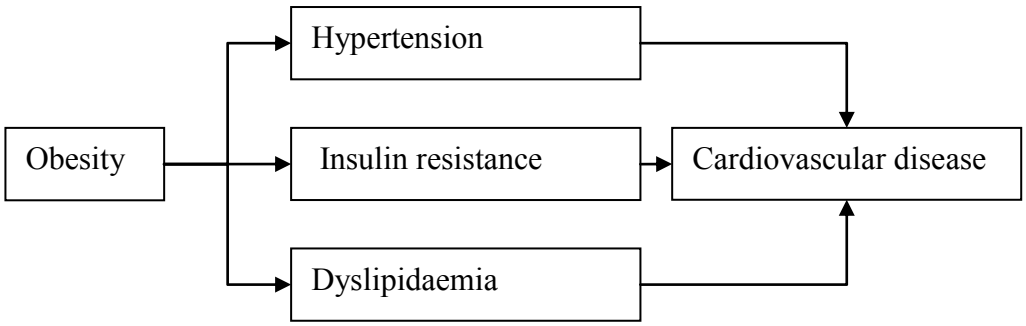


Figure 1.2 Obesity as the underlying risk factor of cardiovascular disease

Insulin resistance is defined as reduced insulin action in peripheral tissues including skeletal muscle, adipose and liver (Caballero, 2003). It is characterised by an exaggerated response to normal circulating levels of fasting glucose (Simoneau and Kelley, 1998) and has been suggested to be the main driving force behind the metabolic syndrome cluster (Reaven, 1988). However, insulin resistance is not a prerequisite feature of obesity since only 26% of the obese population exhibit this condition while the majority demonstrate insulin

hypersecretion (Ferrannini *et al.*, 1997). Similarly, more recent findings suggest that none of insulin resistance, fat mass or fat distribution are sole determinants of the risk of hypertension and CVD in obesity and that instead each factor contributes distinctively via a variety of metabolic pathways to form the metabolic syndrome cluster (Ferrannini *et al.*, 2007). Nonetheless, consistent evidence still exists for the association between insulin resistance, type II diabetes, hypertension, atherosclerosis, and endothelium dysfunction with close associations being reported between insulin resistance and higher FFA, triglycerides (TG), low-density lipoprotein (LDL)-cholesterol and lower high-density lipoprotein (HDL)-cholesterol, and between compensatory hyperinsulinaemia and higher fasting glucose and blood pressure (Ferrannini *et al.*, 2007), as will be discussed in the following sections.

1.4. Insulin and glucose metabolism

Insulin is the chief regulator of glucose metabolism in the body. Its actions are mediated via the insulin receptor (IR) which is widely distributed in both non-insulin sensitive tissues such as the central nervous system, in particular the brain where it helps control glucose and energy metabolism body weight and food intake (Bruning *et al.*, 2000; Bingham *et al.*, 2002; Kyriaki., 2003), and in insulin target tissues such as the liver, skeletal muscle and adipose tissue where it acts to maintain glucose homeostasis in both the fed and fasting state (Saltiel and Khan, 2001). In skeletal muscle and adipose tissue, insulin controls glucose uptake via enhanced translocation, docking and fusion of glucose transporter-4 (GLUT4) into plasma membranes (Saltiel and Khan, 2001). This step is considered the rate-limiting step for glucose utilisation and glycogenolysis (Saltiel and Khan, 2001). Storage of glucose in the form of glycogen is also initiated in skeletal muscle and in the form of TG in adipose tissue during the fasting and fed state (Klover and Mooney, 2003). In the liver, insulin assists in the regulation of numerous genes that control the synthesis of key enzymes involved in gluconeogenesis and glycogenolysis. This ability to regulate gene expression by affecting gene transcription, mRNA stability and mRNA translation has been extensively reviewed and is considered one of the chief functions of insulin with over 100 genes being influenced by this hormone (Sutherland *et al.*, 1998; O'Brien *et al.*, 2001; Foufelle and Ferre, 2002). Insulin can also directly influence the activity of gluconeogenic and glycogenic enzymes by controlling their phosphorylation state (see Saltiel and Khan, 2001). As such, Klover and Mooney (2003) have described insulin's hepatic activities as both acute (post-translational modification of key enzymes) and chronic (gene expression).

Together insulin's actions in skeletal muscle, adipose tissue and liver are directed towards maintaining glucose blood concentrations within a tight physiological range. In obesity, defects in insulin's glucoregulatory functions result in disruption of the above metabolic processes leading to hyperglycaemia, hyperinsulinaemia and insulin resistance. The following sections will focus on describing some of the molecular disturbances in insulin's downstream signalling pathways seen in obesity and insulin resistant states and which help explain the link between insulin resistance and overweight and obesity-related diabetes, hypertension and dyslipidaemia. The role of FFAs in inducing hyperglycaemia and hyperinsulinaemia will also be highlight later in section 1.7.5.

1.4.1 Insulin downstream signalling pathways

As stated earlier, insulin's actions are mediated via the IR. Insulin binding to IR results in tyrosine phosphorylation of the β-subunit of IR which leads to conformational changes in IR and subsequently tyrosine phosphorylation of insulin receptor substrate (IRS) proteins and activation of two main downstream signalling pathways: the phosphatidylinositol 3-kinase (PI3K)–Akt/protein kinase B (PKB) pathway and the Ras–mitogen-activated protein kinase (MAPK) pathway (Saltiel and Khan, 2001; Taniguchi *et al.*, 2006) (see Figure 1.3). The former pathway controls insulin's metabolic functions while the second is involved in gene expression and together both these pathways play a role in cell growth and differentiation (Taniguchi *et al.*, 2006) (Figure 1.3). The activity of these pathways is generally regulated by secondary messengers known as protein kinases. Of relevance to this discussion are the protein kinases responsible for GLUT4 translocation and subsequent glucose uptake which include PI3K and its downstream effectors Akt/PKB and protein kinase C (PKC) (Vollenweider *et al.*, 2002) (Figure 1.3). Akt/PKB and PKC are considered downstream effectors of PI3K because their activity depends on PI3K-induced activation of phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) which then promotes phosphoinositide-dependent protein kinase-1 and -2 (PDK-1 and 2)-dependent Akt/PKB and PKC phosphorylation and activity at the level of plasma membrane (Alessi *et al.*, 1997; Chou *et al.*, 1998; Le Good *et al.*, 1998; Bandyopadhyay *et al.*, 1999a-b; Wick *et al.*, 2000; Bandyopadhyay *et al.*, 2005). The complexity of this insulin signalling pathway has been recently described in an excellent review by Taniguchi *et al.* (2006) but overall, the result of such network of events is increased GLUT4 translocation to plasma membrane and enhanced glucose uptake in skeletal muscle and adipose tissue (Bandyopadhyay *et al.*, 1999a-b; Hill *et al.*, 1999; Wang *et al.*, 1999; Braiman *et al.*, 2001). Undoubtedly any dysfunctions in this

signalling network will result in diminished insulin-induced glucose disposal, hence insulin resistance.

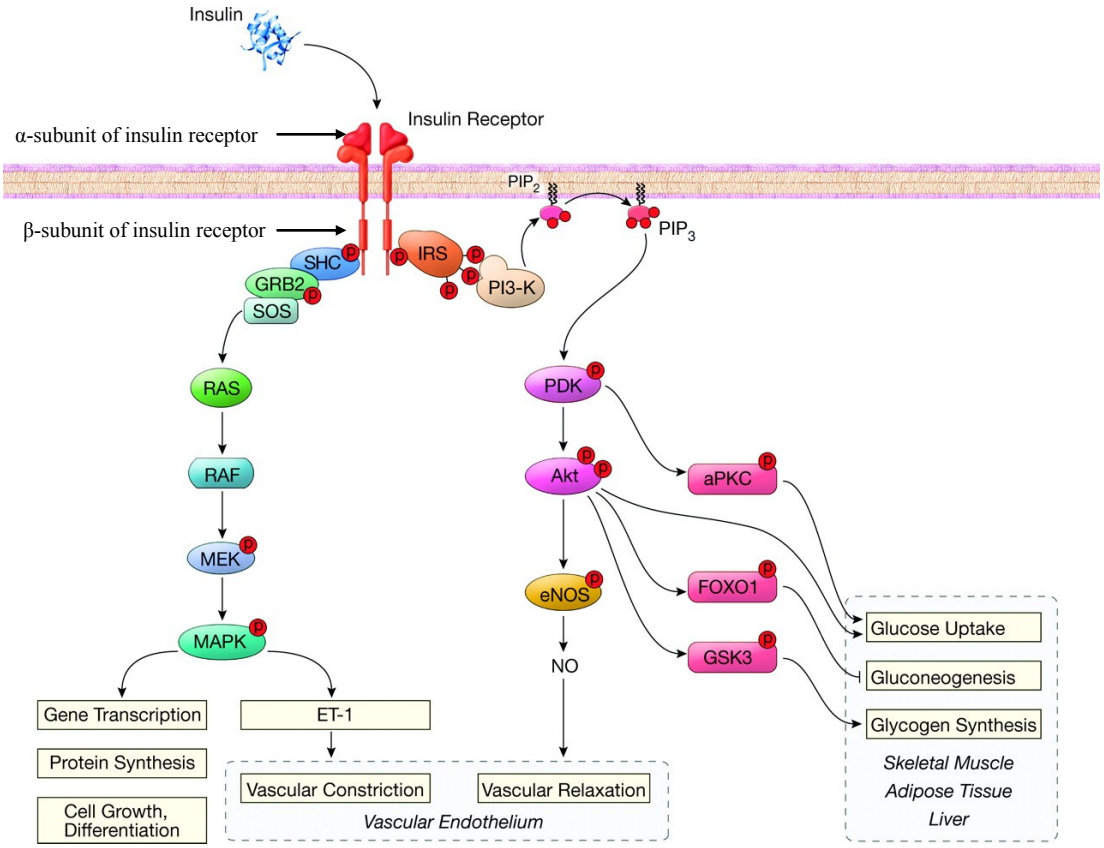


Figure 1.3 Schematic of insulin downstream signalling pathway showing the insulin receptor, the insulin receptor substrate (IRS) and the phosphatidylinositol 3-kinase (PI3K)-AKt/protein kinase B (PKB) pathway and Ras-mitogen activated protein kinase (MAPK) pathway (Muniyappa *et al.*, 2007).

1.4.2 Insulin downstream signalling in obesity

Obesity is known to be associated with various defects in insulin downstream signalling in both skeletal muscle (Caro *et al.*, 1987) and adipose tissue (Goodyear *et al.,* 1995). However, inconsistencies still exist concerning the location of these defects within the insulin signalling pathway. To exemplify, Goodyear *et al.* (1995) has demonstrated that skeletal muscle biopsies from morbidly obese patients express a 35% diminishment in insulin-stimulated receptor tyrosine phosphorylation, a 38% decrement in IRS-1 tyrosine phosphorylation and a 3.5-fold reduction in insulin-dependent IRS-1 PI3K activity compared to lean individuals. The density of IR, IRS-1 and the p85 subunit of PI3K, according to Goodyear *et al.* (1995), is also altered with obese individuals expressing only 55, 54, and 64% of these components relatively to lean individuals. In contrast, skeletal muscle from obese glucose-intolerant patients exhibits impaired insulin-stimulated glucose uptake in the absence of any changes in IRS-1-dependent PI3K activation, or the protein expression of IR, IRS-1, IRS-2, the p85 regulatory subunit of PI3K, Akt, PKC-ζ/λ, GLUT1, or GLUT4 (Vollenweider *et al.*, 2002). In fact, it appears that in obese glucose-intolerant patients, defective insulin-stimulated glucose uptake in skeletal muscle is precipitated by a reduction in insulin-stimulated IRS-2 tyrosine phosphorylation, IRS-2–dependent PI3K and PKC-ζ/λ activation (Vollenweider *et al.*, 2002). To add to these controversies, more recent studies have reported that the activity of PI3K, PDK1 and Akt/PKB is unaltered in obesity and that the levels of PKCλ/ζ are comparable between lean and obese individuals (Kim *et al.*, 1999; Kim *et al.*, 2003). At present, insufficient evidence exists to elaborate on the differences between the afore-mentioned findings. However, it can be speculated that disturbances in insulin-signalling network are likely to differ depending on the degree of obesity or the extent and severity of insulin resistance. Patients from Goodyear *et al.*'s (1995) study had a BMI of 52.9±3.6 kg/m^2 compared to the studies of Vollenweider *et al.* (2002) and Kim *et al.* (1999, 2003) wherein patients had a BMI of 32.5±1.6 kg/m^2, 31±1.3 kg/m^2 and 33.4±1.4 kg/m^2, respectively. Similarly, patients from Vollenweider *et al.* (2002) were glucose-intolerant whereas glucose tolerance was not tested in the other studies. Additional support could be gained from findings that obese patients with established type II diabetes demonstrate more prominent defects in insulin signalling, and subsequently in insulin-stimulated skeletal muscle glucose uptake, than either lean or obese non-type II diabetic patients (Kim *et al.*, 1999). This is further seen in that insulin-stimulated IRS-1 tyrosine phosphorylation and PI3K activity is 40–50% lower in obese type II diabetes patients compared to lean or obese individuals (Kim *et al.*, 2003) and so is insulin induced IRS-2–dependent PI3K activity (Kim *et al.*, 1999). Moreover, obese type II diabetes patients

demonstrate a 46% reduction in the expression of PKCλ/ζ which is then reflected in reduced PKCλ/ζ activity compared to lean and obese subjects (Kim *et al.*, 2003). These effects could be attributed to the fact that insulin resistance and the accompanying compensatory hyperinsulinaemia can impair insulin-stimulated skeletal muscle glucose uptake independently of obesity (Korsheninnikova *et al.*, 2002).

Regardless of these inconsistencies, it is now becoming increasingly recognised that the inability of insulin to stimulate glucose disposal could arise from defects in the expression of IRS protein domains, such as the p85 subunit, or in PKCλ/ζ activation. Accordingly, insulin-induced activation of PKCλ/ζ has been shown to be reduced by as much as 57% in obesity (Kim *et al.*, 1999; Kim *et al.*, 2003). Kim *et al.* (2003) argues that a PI3K-independent mechanism is implicated in the aetiology of impaired insulin-induced PKCλ/ζ activation (Kim *et al.*, 2003). Support for this concept could be derived from observations that the PKCζ isoform provokes GLUT4 translocation and glucose uptake in the absence of insulin (Braiman *et al.*, 2001). Farese *et al.* (2005) has also suggested that the mechanism accounting for impaired insulin-induced atypical protein kinase C (aPKC) activation in skeletal muscle could lie downstream of PI3K; for instance at the level of PIP3 (Sajan *et al.*, 2004; Farese *et al.*, 2005). However, according to Kim *et al.*'s (2003) the defect is possibly situated even further downstream of the insulin signalling network since PDK1 activity is not impaired in obesity.

As described earlier, the p85 subunit is the regulatory subunit of IRS1. It mediates IRS1-dependent stimulation of PI3K via its ability to stabilise and inhibit the catalytic activity of the p110 subunit of IRS1 (Yu *et al.*, 1998a). This effect occurs through the binding of the nSHS-linked iSH2 domain of p85 to p110α (Yu *et al.*, 1998b). In general, the balance between the p85 and the p110 subunits determines the extent of p100 activity (Brachmann *et al.*, 2005). In obesity, there is an excess expression of p85/55/50 (Bandyopadhyay *et al.*, 2005). This enhanced P85/55/50 expression can inhibit p100 catalytic activity on lipid kinase PIP3 (Ueki *et al.*, 2002) resulting in a negative correlation being present between P85/55/50 expression and insulin sensitivity (Bandyopadhyay *et al.*, 2005). Furthermore, p85 can interact with IRS1 in the cytosol producing large p85-IRS complexes that become docked in the cytosol and cannot be translocated to the plasma membrane, the site of PIP3 production (Luo *et al.*, 2005). The consequence of such actions is reduced PI3K signalling even in the absence of a defect in insulin growth factor (IGF)-1 activation (Luo *et al.*, 2005). Similar effects can be induced with overfeeding in women wherein increasing caloric intake

produces an increase in p85α expression in skeletal muscle, a fall in P110, a raised p85α to p110 ratio, and a reduction in PI3K activity, all of which is then reflected as reduced insulin sensitivity (Cornier *et al.*, 2006). Deletion of all p85 isomers, on the other hand, in mice improves insulin sensitivity regardless of the degree of impairment in PI3K activity (Terauchi *et al.*, 1999; Fruman *et al.*, 2000; Mauvais-Jarvis *et al.*, 2002). Overexpression of P85/55/50 is also accompanied with increased activity of the proinflammatory kinases: Jun N-terminal kinase-1 (JNK-1) and PKCϴ, which can act as alternative mechanisms by which p85 isomers impair insulin signalling (Bandyopadhyay *et al.*, 2005). This could be illustrated in that these proinflammatory kinases stimulate serine/threonine phosphorylation of IRS-1/2 while suppressing its tyrosine phosphorylation leading to suppression of IRS1 (Aguirre *et al.*, 2000) and PI3K activity (Bandyopadhyay *et al.*, 2005), hence diminished insulin-stimulated glucose uptake (Itani *et al.*, 2000). In concurrence, endoplasmic reticulum stress, characterised by elevated levels of JNK, have recently emerged as a key pathological feature of impaired IR signalling in obesity (Ozcan *et al.*, 2004).

Defects in adipose tissue insulin regulation are also present in obesity. These defects somehow resemble the abnormalities described in skeletal muscle. This is because compared to lean individuals, insulin-induced glucose disposal and IR-dependent stimulation of IRS-1, PI3K and aPKCs, but not PKB, is reduced in adipose tissue of obese individuals (Sajan *et al.*, 2004). The ability of PIP3 to activate aPKCs is also decreased (Sajan *et al.*, 2004). Once type II diabetes develops further defects in IRS-1 expression and IRS-1–dependent PI3K activity are observed in obesity (Rondinone *et al.*, 1997). However the extent to which abnormalities in adipose tissue insulin signalling can directly contribute to whole-body insulin resistance remains speculative (Kahn and Flier, 2000). To explain, consistent with the importance of insulin signalling in stimulating glucose uptake in adipocytes, in mice overexpression of GLUT4 in adipose tissue but not skeletal muscle is associated with improved fasting glucose and glucose tolerance. Whole-body glucose uptake and adipose tissue glucose disposal is also ameliorated under basal and insulin-stimulated conditions (Shepherd *et al.*, 1993; Gnudi *et al.*, 1996). Paradoxally, knock-out of IR gene produces mice that are equally protected against obesity-induced glucose intolerance (Bluher *et al.*, 2002). Such anomalies could be explained by the fact that overexpression of GLUT4 in adipose tissue, while enhancing glucose uptake into adipocytes, may reduce glucose supply to skeletal muscle forcing the latter to utilise fatty acids as a source of energy (Gnudi *et al.*, 1995). Moreover, according to Gnudi *et al.* (1995-1996), improvements in adipose tissue insulin sensitivity does not necessarily protect against high-fat diet induced insulin

resistance in skeletal muscle or liver (Gnudi *et al.*, 1995; Gnudi *et al.*, 1996). Because of these inconsistencies, it is unsurprising that defects in insulin downstream signalling pathways in skeletal muscle are counted as the main driving force precipitating the development of systemic insulin resistance in obesity, especially when considering that glucose disposal in skeletal muscle accounts for 75% of glucose uptake in the body (Saltiel and Khan, 2001).

In contrast to skeletal muscle and adipose tissue, hepatic insulin-induced aPKC activation appears to be preserved in type II diabetic non-obese Goto-Kakazaki rats and *ob/ob*-diabetic mice (Farese *et al.*, 2005) or upregulated in obese (*fa/fa*) rats (Qu *et al.*, 1999) while PKB activation is either maintained in non-diabetic high-fat fed mice or compromised in type-II diabetic non-obese Goto-Kakazaki rats and *ob/ob*-diabetic mice (Standaert *et al.*, 2004). The cause of this differential aPKC activity between skeletal muscle, adipose tissue and liver is unknown, but could be postulated to involve tissue-specific differences in aPKC regulation by IRSs. Accordingly, IRS1 is seen as the dominant IRS responsible for insulin-induced aPKC activation in skeletal muscle, while in adipose tissue aPKC is regulated by both IRS1 and IRS2 and finally in the liver aPKC is controlled by IRS2 (Kido *et al.*, 2000; Previs *et al.*, 2000; Farese *et al.*, 2005) (Figure 1.4). Regardless of these differences, hepatic changes in aPKC and PKB in combination with skeletal muscle and adipose tissue-induced systemic insulin resistance, are likely to further exacerbate the metabolic situation in obesity by promoting hyperglycaemia, dyslipidaemia and non-alcoholic steatohepatitis (Standaert *et al.*, 2004; Farese *et al.*, 2005). In particular, dyslipidaemia could be induced by the up regulatory effect of the sustained or heightened hepatic PKC activity on sterol regulatory element binding protein-1c (SREBP-1c) expression (see section 1.6.2).

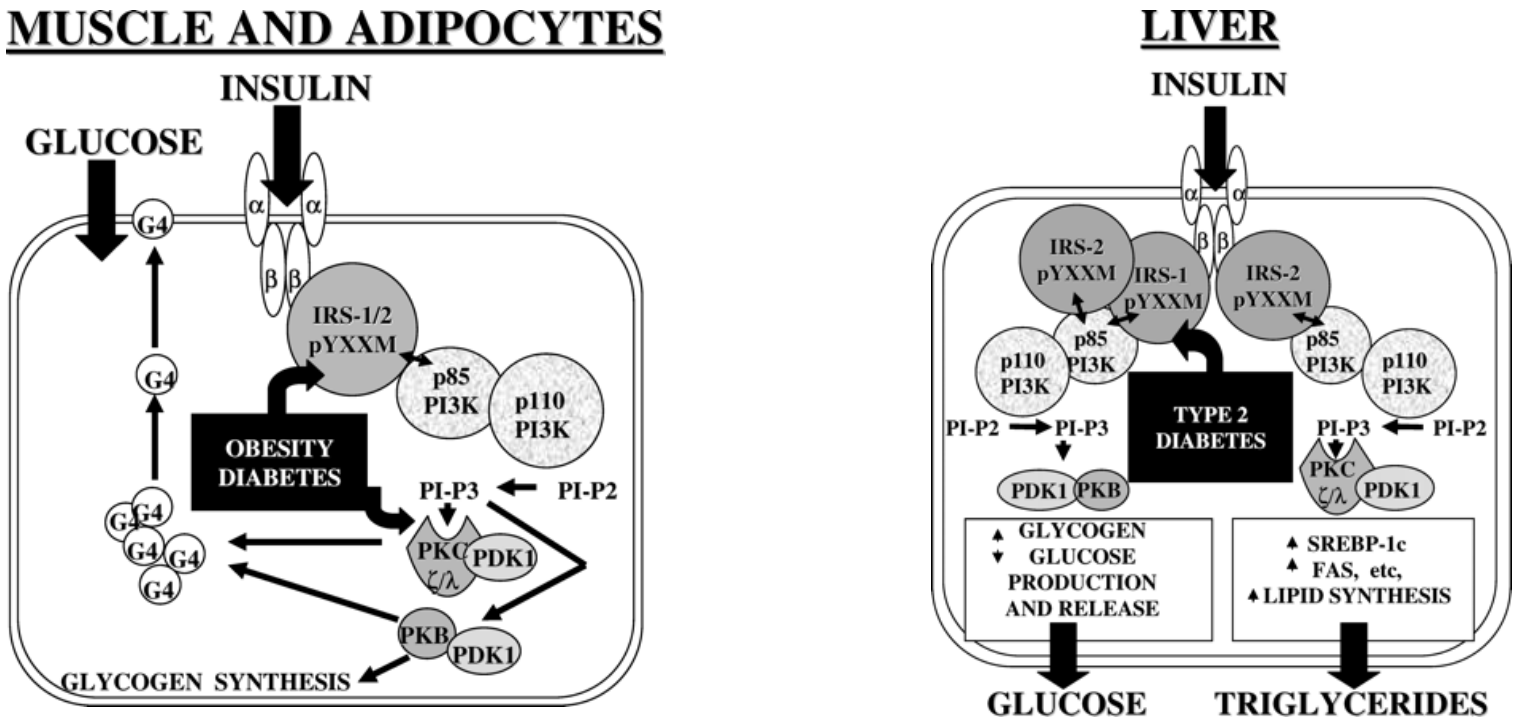


Figure 1.4 Differential regulation of PKC by IRS-1/PI3K in skeletal muscle, by IRS-1/2/PI3K in adipose tissue and by IRS-2/PI3K in liver. Note the role of IRS-2/PI3K in lipogenesis and IRS-1/PI3K in glycogenolysis and gluconeogenesis (Farese *et al.*, 2005).

1.4.3 Insulin's vascular actions: role in glucose metabolism

In addition to regulating IR signalling network, insulin can influence glucose metabolism through its vascular actions (Baron *et al.*, 1995; Baron *et al.*, 1996). The molecular basis of these vasodilatory properties and the association between insulin resistance and endothelium dysfunction are well established and will be discussed in section 1.5. Overall, these vascular actions are directed towards increasing blood flow to target tissues, in particular skeletal muscle, where the rise in surface exchange area permits insulin-dependent stimulation of IR downstream signalling and subsequently glucose uptake (Mather *et al.*, 2000). Consistent with this obese subjects exhibit reduced forearm blood flow rate which is accompanied with delayed delivery of insulin into skeletal muscle interstitial fluid, (Sjostrand *et al.*, 2002; Sjostrand *et al.*, 2005), the site of skeletal muscle IR (Vincent *et al.*, 2005).

However, until recently, inconsistencies still existed concerning the extent to which insulin's vascular actions contribute to insulin-mediated glucose uptake. This is because while some authors reported a significant relation between the rate of insulin-dependent skeletal muscle glucose disposal and the magnitude of flow dependent glucose uptake (Baron *et al.*, 1995), several others failed to perceive any association between bulk flow and glucose uptake (Nuutila *et al.*, 1996; Laine *et al.*, 1998; Natali *et al.*, 1998; Pitkanen *et al.*, 1999). These inconsistencies were reported despite the fact that insulin's vasodilatory actions helped explain one-third of insulin's effect on glucose uptake (Baron *et al.*, 1995). Subsequent investigation revealed that, apart from influencing large vessels, insulin can also affect the microvasculature by selectively redistributing blood flow from non-nutritive to nutritive vessels (Baron and Clark, 1997; Clark *et al.*, 2003; Vincent *et al.*, 2005; Newman *et al.*, 2007). These findings were important because they implied that not all increases in bulk flow are associated with improved insulin-mediated glucose uptake since only nutritive vessels are capable of extracting nutrients such as glucose (Newman *et al.*, 2001). This helped explained the earlier discrepancies as most authors who failed to reach similar conclusion as Baron *et al.* (1995, 1996) used vasodilatory agents that increased blood flow into non-nutritive capillaries (Clark *et al.*, 2003). Importantly, it was observed that insulin-stimulated microvascular recruitment (nutritive flow) and glucose uptake is nitric oxide (NO)-dependent (Vincent *et al.*, 2003; Vincent *et al.*, 2004). This specificity was further reinforced by studies demonstrating that administration of methacholine, a NO vasodilator, improves forearm blood flow, capillary recruitment, glucose uptake and interstitial insulin concentrations in obese individuals (Murdolo *et al.*, 2008) while inhibition of NO by nitro-i.-

arginine methyl ester offsets insulin-stimulated rise in total limb flow and microvascular recruitment resulting in a 40% reduction in glucose uptake (Vincent *et al.*, 2003).

Based on this evidence it could be summarised that insulin can potentially promote skeletal muscle glucose uptake through its ability to increase total limb flow and to selectively regulate microvascular perfusion. However, much remains to be clarified as to the contribution of vasodilatation in each of these compartments in modulating insulin glucoregulatory function. This is because compared to the large resistant vessels responsible for bulk flow, the microvasculature is more sensitive to insulin since infusions of physiological concentrations of this hormone increase microvascular recruitment and skeletal muscle perfusion before any changes in total limb flow are detected (Coggins *et al.*, 2001; Vincent *et al.*, 2002) (see Section 1.5.3). Moreover, it appears that capillary recruitment could occur independently of changes in blood flow (Steinberg *et al.*, 1996; Rattigan *et al.*, 1997) and that total blood flow is only a rate-limiting factor for skeletal muscle glucose uptake in insulin sensitive but not insulin-resistant subjects (Baron *et al.*, 2000). Regardless of this, it remains to be stated that in obesity defects in insulin-mediated vascular function could still contribute to postprandial glucose intolerance (Baron *et al.*, 1990) which is one of the earliest defects observed in normal glucose tolerant patients who subsequently develop hyperglycaemia and type II diabetes (Pratley and Weyer *et al.*, 2002).

1.4.4 Physiological aspects of insulin resistance in obesity

Under normal physiological conditions, 50-55% of glucose production is ascribed to gluconeogenesis following an overnight fast which is raised to 93±2% after 42h of fasting (Landau *et al.*, 1996; Petersen *et al.*, 1996). The remaining whole body glucose release is ascribed to hepatic glycogenolysis (Petersen *et al.*, 1996). Glycogen synthesis in skeletal muscle, on the other hand, accounts for the largest proportion of whole-body glucose disposal and effectively all of non-oxidative glucose metabolism (Shulman *et al.*, 1990). The balance between these processes helps maintain glucose homeostasis

Generally, physiological concentrations of insulin suppress gluconeogenesis by 20% and completely abolish glycogenolysis (Gastaldelli *et al.*, 2001). In obesity, these processes are disturbed resulting in heightened hepatic gluconeogenesis and glycogenolysis (Muller *et al.*, 1997; Gastaldelli *et al.*, 2000, 2004; Basu *et al.*, 2005). Skeletal muscle glycogenolysis is also impaired in obesity (Damsbo *et al.*,1991) and the sum of these anomalies stimulates the

development of fasting hyperglycaemia (Gastaldelli *et al.*, 2000, 2004). In relation to gluconeogenesis, it is estimated that for every unit increase in BMI, a 0.9% rise in gluconeogenesis is produced. The degree of overweight and obesity and visceral obesity can also independently predict gluconeogenic flux (Gastaldelli *et al.*, 2004) and the percentage contribution of gluconeogenesis to whole-body glucose production during fasting (Gastaldelli *et al.*, 2000). This enhanced gluconeogenesis is attributed to enhanced influx of amino acids to the liver due to impaired insulin ability to inhibit post-absorptive protein catabolism (Chevalier *et al.*, 2006). Increased hepatic glycolytic and gluconeogenic enzymes activity is also implicated in enhanced gluconeogenesis and glycogenolysis seen in obesity (Seidman *et al.*,1970; Perez *et al.*, 1998).

In relation to glycogenolysis, impaired glucose storage in skeletal muscle of obese individuals is strongly correlated with defective insulin-stimulated glucose uptake (Hojland *et al.*, 2009). This effect occurs because glucose uptake constitutes the rate limiting step for glucose utilisation and glycogen synthesis, and this process is impaired in obesity as a consequence of the disturbances in insulin-stimulated downstream signalling described earlier. Glycogen synthase activity is also severely compromised in obesity (Kim *et al.*, 1999; Golay *et al.*, 2002; Damsbo *et al.*, 1991) which is reflected in diminished non-oxidative glucose metabolism (Golay *et al.*, 2002; Damsbo *et al.*, 1991). In fact, defects in glycogen synthase constitute one of the most recognised and earliest defects observed in obese individuals (Golay *et al.*, 2002; Damsbo *et al.*,1991). These defects have been recently attributed to failure of insulin to induce glycogen synthase phosphorylation, a process that activates glycogen synthase and promotes glycogen synthesis (Hojland *et al.*, 2009). Although the insulin-dependent Akt/PKB transduction pathway is known to be implicated in the stimulation of glycogen synthase kinase, a serine/threonine kinase that regulates glycogen synthase phosphorylation and activation (Welsch and Proud, 1993; Cross *et al.*, 1995; Cohen, 1999), the extent of involvement of glycogen synthase kinase in obesity-related insulin resistance remains to be clarified, since the activity of the Akt/PKB pathway is preserved in obesity (Kim *et al.*, 1999).

1.4.5 Insulin resistance and type II diabetes

Insulin resistance precedes hyperglycaemia and hence is an important risk factor for the development of type II diabetes. It is one of the cardinal features of obesity and evidence from longitudinal studies suggests that in most cases disturbances in insulin-dependent non-

oxidative glucose disposal, hence storage, and insulin-independent glucose uptake occur early during the transition from normglycaemia to glucose intolerance (Weyer *et al.*, 1999; Martin *et al.*, 1992) and almost a decade before the initial onset of type II diabetes (Martin *et al.*, 1992). In addition to defects in insulin action, abnormalities in insulin secretion and endogenous glucose output are involved in the pathogenesis of type II diabetes (Weyer *et al.*, 1999). Each of these pathogenic factors contributes to the development of type II diabetes at different stages of the disease and is, in turn, influenced by excess adiposity (Figure 1.8; p. 37). In particular, impaired insulin activity, delayed insulin release following a meal and post-prandial hyperglycaemia form one of the earliest defects precipitating the rise in endogenous glucose production and clinical hyperglycaemia (Pratley and Weyer, 2001). Subsequently, identifying nutritional strategies that target these specific disturbances in insulin activity and secretion at early stages of disease development remain imperative to the long-term prevention of obesity-associated type II diabetes (Weyer *et al.*, 1999; Pratley and Weyer, 2001; Pratley and Weyer, 2002), especially when considering that in recent years, the prevalence of type II diabetes has risen in equal proportion to the obesity epidemic with over 190 million of people suffering from this chronic condition (WHO, 2009).

In general, it is well documented that both insulin resistance (Haffner *et al.*, 1990; Lillioja *et al.*, 1993; Haffner *et al.*, 1995) and obesity (Wang *et al.*, 2005; Vazquez *et al.*, 2007) are important predictors of type II diabetes. However, it appears that regardless of the reciprocal relation between insulin action or secretion and obesity (Weyer *et al.*, 2000a), excess adiposity, either characterised as abdominal adiposity or enlarged adipocytes, remains an independent and in conjunction with insulin resistance an additive predictor of type II diabetes (Abbasi *et al.*, 2002; Weyer *et al.*, 2000b; Ferrannini *et al.*, 2004). The molecular basis for such relation could be obtained from the ectopic fat storage theory (see Section 1.7.2) wherein dysfunctional mitochondrial β-oxidation and failure of fat cell to proliferate is closely linked to insulin resistance and type II diabetes (Heilbronn *et al.*, 2004). Importantly, however, it appears that hyperinsulinaemia can predict future weight gain and rise in WHR (Odeleye *et al.*, 1997; Gould *et al.*, 1999;) possibly as a result of insulin's ability to regulate lipolysis (Section 1.6.4). This could be further illustrated in that individuals who progress to type II diabetes experience greater weight gain than non-progressors (Weyer *et al.*, 1999) while treatment with insulin simultaneously improves glycaemia and weight control in obese type II diabetics (Ratner *et al.*, 2002; Hollander *et al.*, 2003; Hollander *et al.*, 2004). Likewise, in a prospective 5-year follow-up study, Chen *et al.* (1995) reported that defects in insulin secretion often precede visceral obesity in some type II diabetes progressors which

may imply that a vicious circle of cause and effect exits that contributes to the sustained glucose intolerance and hyperinsulinaemia that then lead to type II diabetes. The consequence of such association could lie in that dietary strategies aimed at reversing obesity-related insulin resistance and type II diabetes may also be central to managing body weight.

1.5. Insulin resistance, endothelium dysfunction and hypertension

The complex relation between insulin resistance and hypertension is likely to involve several physiological mechanisms including insulin's effect on the sympathetic nervous system, the endocrine system and the vasculature (Figure 1.5).

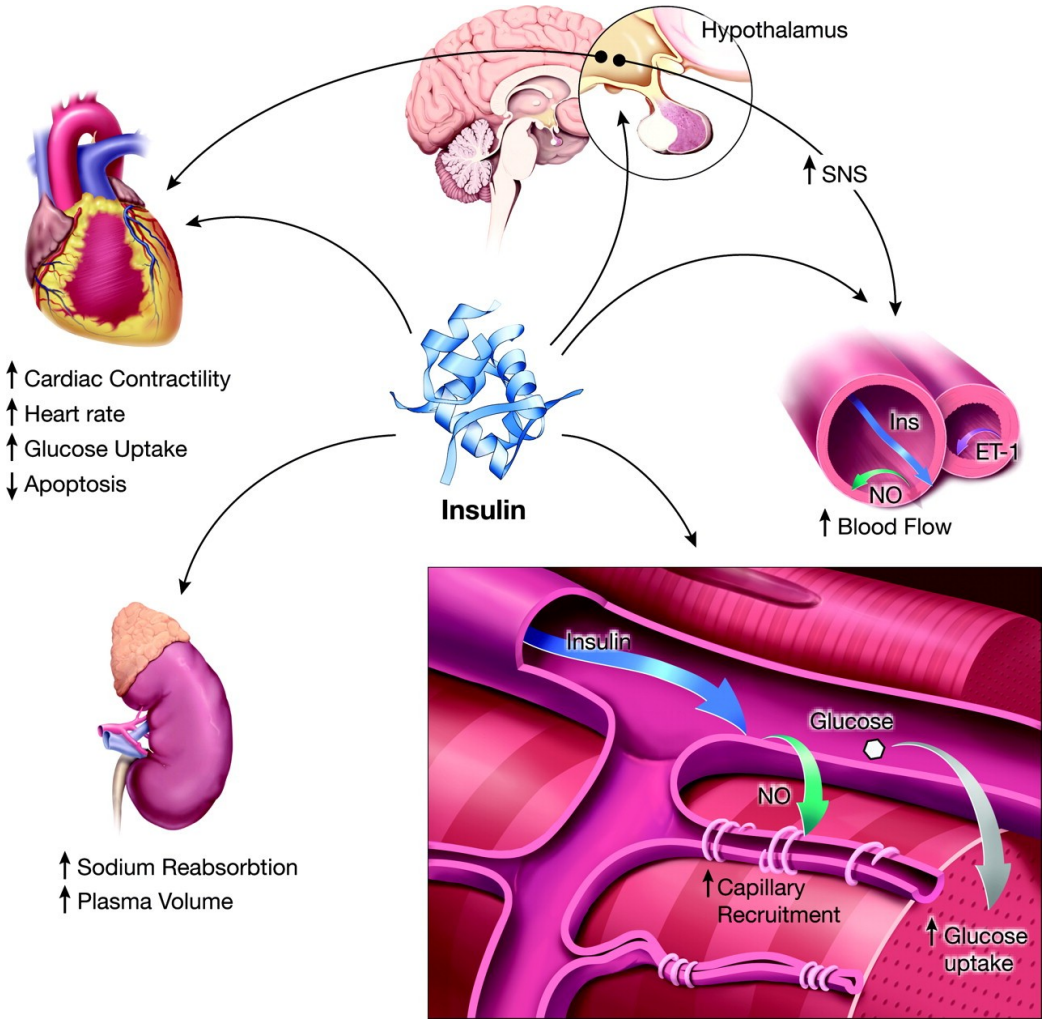


Figure 1.5 Contrasting insulin's actions on cardiovascular system (Muniyappa *et al.*, 2007). Note the effect of insulin on vasculature and hypothalamus wherein increased NO bioavailability and activation of sympathetic nervous system (SNS) lead to vascular relaxation. By contrast, note the effect of insulin on heart and kidney. Herein, enhanced cardiac contractility and sodium reabsorption raise blood pressure. These differential effects of insulin can be explained by the fact that insulin's NO pathway predominates in vasculature while the activity of insulin MAPK pathway predominates in heart and kidney. In obesity and the metabolic syndrome, the balance is shifted towards enhanced activity of MAPK pathway (see text).

In relation to the vasculature, recent studies have identified a selective insulin signalling pathway in the endothelium. This selective pathway bears striking similarity to the insulin-signalling pathways seen in skeletal muscle and adipose tissue, and has been shown, in fact, to express all the necessary components of insulin downstream signalling notably IRS, PI3K and Akt/PKB (Cleland and Connell, 2005; Kim *et al.*, 2006). Interestingly, this pathway appears to play a key regulatory role in maintaining vascular function by controlling the production of NO. This has led several authors to conclude that defects in this signalling pathway could contribute to impairment in NO bioavailability, which is then reflected as abnormalities in vascular function, i.e. impaired capillary recruitment, and diminished limb blood flow (Cleland and Connell, 2005; Kim *et al.*, 2006). The following section describes these defects in relation to obesity-associated hypertension.

1.5.1 Endothelium dysfunction

The vascular endothelium consists of a group of cells that line the capillaries thereby separating the lumen of blood vessels from the vascular smooth muscle. It acts as an endocrine organ, producing a variety of bioactive substances that help regulate vasomotor tone (Kumar and Clark, 2005). Of particular importance is NO, a powerful vasodilator produced in the endothelium through the oxidation of L-arginine via the enzyme endothelial NO-synthase (eNOS) (Kumar and Clark, 2005). In obesity, there is evidence of impaired endothelial dilation (Arkin *et al.*, 2008, DeJongh *et al.*, 2004) and diminished NO bioavailability (Mather *et al.*, 2004). This diminished NO bioavailability and endothelium dysfunction appears to be linked to obesity via an imbalance between insulin's vasodilatory and vasoconstrictive actions (Jonk *et al.,* 2007), as illustrated below.

1.5.2 Insulin's downstream signalling pathways: relevance to vascular endothelium

As part of its glucoregulatory function, insulin helps increase capillary recruitment and blood flow, which as well as dilating the vessels allows for increased glucose uptake by skeletal muscle (Jonk *et al.*, 2007). This effect is achieved through insulin's ability to regulate vasodilation and vasoconstriction through two distinct and independent pathways, the Akt/PKB-dependent pathway and the MAPK-dependent pathway (Scherrer *et al.*, 1994, Potenza *et al.*, 2009) (Figure 1.6). Insulin's net effect on vascular endothelium depends on the balance between these two pathways (Scherrer *et al.*, 1994, Potenza *et al.*, 2009).

In insulin resistance, there is a specific impairment in the Akt-signalling pathway while the activity of the MAPK-pathway remains preserved (Jonk *et al.*, 2007). Coupled with insulin hypersecretion as observed in obesity, this results in enhanced MAPK-signalling and diminished Akt-signalling which leads to an imbalance between the generation of NO and the secretion of endothelin-1, an endothelium-derived factor that opposes NO activity (Jonk *et al.*, 2007). The result is impaired insulin-induced vasodilation and enhanced insulin-induced vasoconstriction. This state is characteristic of endothelium dysfunction and is thought to be one of the earliest underlying pathophysiological abnormalities seen in hypertension.

Much debate remains as to whether insulin resistance precedes and hence causes endothelium dysfunction or whether it is a mere complication of changes in microvascular circulation (Cleland and Connell, 2005). Such uncertainties arise from the complex mechanisms and the common cellular and biochemical pathways shared by insulin resistance and endothelium dysfunction. Nonetheless, what remains clear is that in obesity, a reciprocal relation exits between insulin resistance and endothelium dysfunction, which is eventually expressed in the form of vascular pathologies such as hypertension and atherosclerosis.

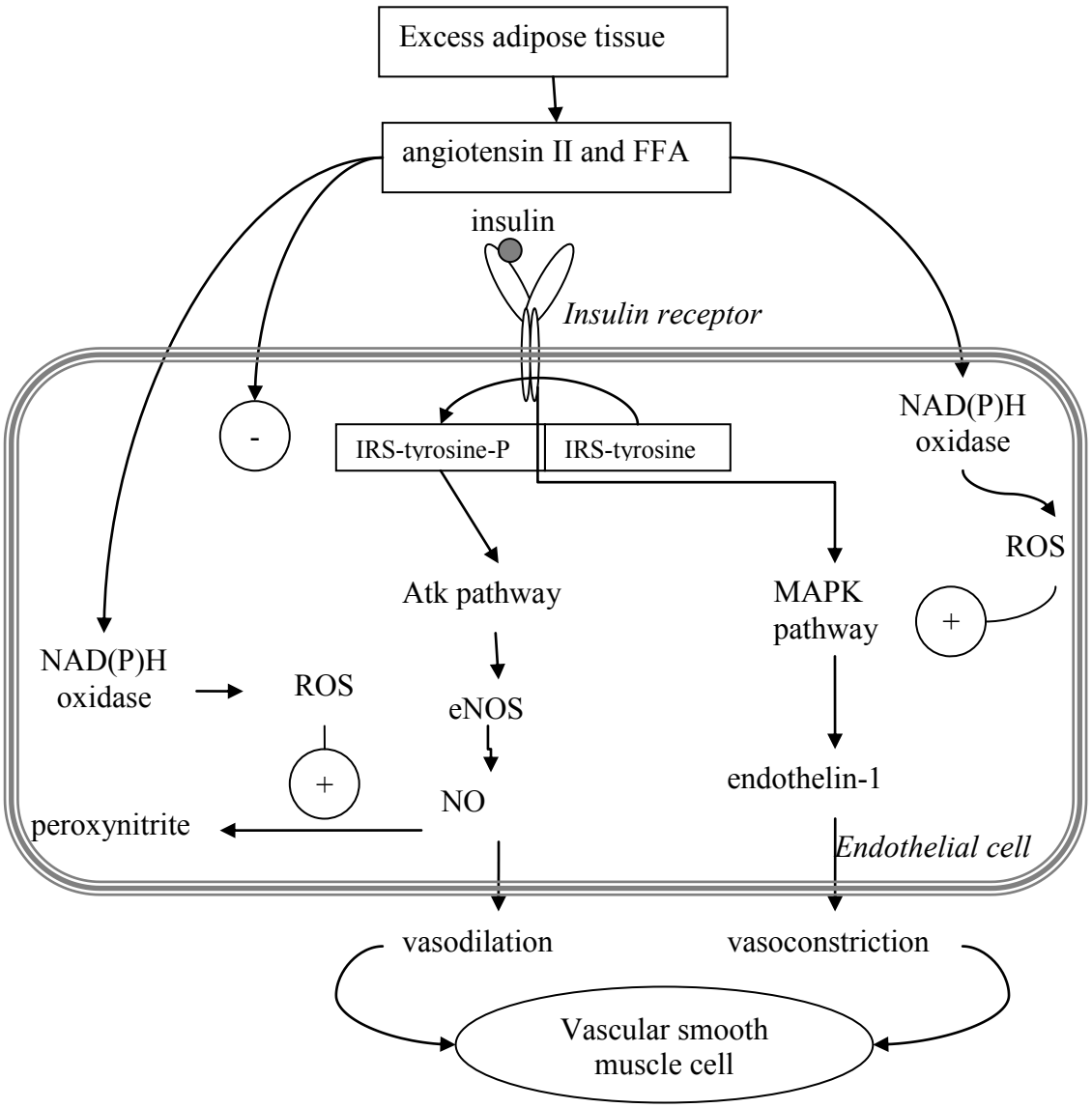


Figure 1.6 Diagram showing effect of increased angiotensin-II and free fatty acid (FFA) production on the balance between insulin's vasoconstrictive and vasodilatory actions (adapted from Jonk *et al.*, 2007). eNOS endothelial nitric oxide synthase, IRS insulin receptor substrate, NO nitric oxide, ROS reactive oxygen species.

In this respect, studies on spontaneously hypertensive rats provide an excellent model on how insulin resistance and endothelium dysfunction act through shared mechanisms to induce hypertension in obesity. Accordingly, these studies demonstrate that in spontaneously hypertensive rats, insulin resistance occurs concurrently with endothelium dysfunction (Potenza *et al.*, 2005). This, as described earlier is expressed in the form of impaired PI3K-dependent NO production and enhanced MAPK-dependent endothelin-1 secretion. Importantly, these dysregulations in insulin's vascular actions and endothelium function become apparent at early stages of hypertension (LeNoble *et al.*, 1990) and are even evident in healthy offspring of patients with hypertension (Antonios *et al.*, 2003).

Interestingly, in spontaneously hypertensive rats, treatment with insulin-sensitizing drugs and anti-hypertensives such as metformin, pioglitazone, enalapril, or rosiglitazone, evokes simultaneous improvements in both plasma insulin levels and systolic blood pressure (SBP) (Verma *et al.*, 1994; Grinsell *et al.*, 2000; Potenza *et al.*, 2006). In the case of rosiglitazone and enalapril, this effect has been shown to occur as a result of re-established balance between insulin's vasodilatory and vasoconstrictive actions (Potenza *et al.*, 2006). Together these findings support the importance of controlling insulin resistance as a means of reducing the risk of hypertension (De Jongh *et al.*, 2006).

In humans, early studies investigating the relation between insulin and hypertension yielded conflicting results. While studies using artificially-induced hyperinsulinaemia showed a beneficial effect on blood pressure (Gans *et al.*, 1992), case-controlled (Welborn *et al.*, 1966; Ferrannini *et al.*, 1987) and prospective cross-sectional studies identified hyperinsulinaemia as an independent risk factor for hypertension (Skarfors *et al.*, 1991; Lissner *et al.*, 1992; Zavaroni *et al*, 1994; Ferrannini *et al.*, 1997) (also refer to review by Cleland and Connell, 2005). These inconsistencies could be explained by the fact that artificially induced hyperinsulinaemia could have promoted insulin's vasodilatory actions (Section 1.5.2). Conversely, in the observational cross-sectional studies, hyperinsulinaemia might have reflected a compensatory rise in insulin in response to insulin resistance, thereby pinpointing insulin resistance as an important contributor to hypertension (Section 1.7.5). Indeed, several studies have shown strong correlation between insulin sensitivity and blood pressure (Welborn *et al.*, 1966; Ferrannini *et al.*, 1987; Serne *et al.*, 1999). This is further reinforced by the fact that hypertensives have lower insulin sensitivity values than normotensives (Serne *et al.*, 1999) and that for every 10-unit increase in insulin resistance a 1.7 mm Hg and 2.3 mm Hg rise in SBP and diastolic blood pressure (DBP) is observed (Ferrannini *et al.*,

1997). Interestingly, the relation between insulin resistance and elevated blood pressure appears to occur independently of BMI (Cleland and Connell, 2005), which is consistent with the concept of insulin resistance being the underlying factor linking obesity with elevated blood pressure. The earlier sections have described the molecular basis of the imbalance in insulin's hemodynamic actions. The next question that arises is how do such defects are then expressed on physiological level?

1.5.3 Insulin-induced capillary recruitment and blood flow

Numerous studies have reported an inverse correlation between microvascular function and blood pressure in lean normotensives with or without familial predisposition to hypertension, in patients with borderline hypertension or hypertension, and in obese individuals (Serne et al, 1999, 2001; De Jongh *et al.*, 2004a-b). In particular, the work by Serne *et al.* (1999) showed that microvascular function is strongly related to both insulin sensitivity and blood pressure, which led the authors to conclude that microvascular dysfunction is the main mechanism linking insulin sensitivity to blood pressure. Such findings were consistent with the concept of microvascular dysfunction as a cause of insulin resistance and hypertension (De Jongh *et al.*, 2004a). However, further investigations by the same group revealed that microvascular function is closely correlated with blood pressure and insulin's metabolic and vasodilatory actions (Serne *et al.*, 2001). In fact, in addition to having impaired insulin sensitivity, hypertensives patients were found to exhibit reduced capillary recruitment, impaired acetylcholine-mediated vasodilatation, and diminished insulin-mediated vasodilatation (Serne *et al.*, 2001). Notably, capillary recruitment after arterial occlusion was shown to correlate negatively with blood pressure but positively with insulin sensitivity and insulin-mediated vasodilatation, this relation persisting from normotensives through to hypertensives (Serne *et al.*, 2001). Similar observations were reported with lean and obese individuals wherein capillary recruitment or NO-dependent vasodilation were found to correlate positively with insulin sensitivity but inversely with blood pressure (De Jongh *et al.*, 2004a). Undoubtedly, such evidence implies that a primary defect in insulin's selective vascular transduction pathway is likely to govern the relation between insulin resistance and hypertension. This could be further reinforced by Serne *et al.* (2002) latest work which showed that hyperinsulinaemia promotes vascular function by inducing both NO/endothelium-dependent and endothelium-independent vasodilation. The former finding is consistent with other studies showing stimulatory effects of insulin on acetylcholine, a known NO-dependent vasodilator (Steinberg *et al.*, 1994, Taddei *et al.*, 1995, Cardillo *et al.*,

1998), although the second finding is in disagreement with De Jongh *et al.* (2004b) who observed no changes in sodium-nitroprusside, an endothelium-independent vasodilator, during hyperinsulinaemia. Despite these inconsistencies, Serne *et al.* (2002) and others (De Jongh *et al.*, 2004b) have repeatedly shown that hyperinsulinaemia can promote capillary recruitment and vasomotion, all of which reflects improved endothelial activity. According to Kim *et al.* (2006), enhanced capillary recruitment is one of the first stages by which insulin improves vascular function and consequently blood pressure. This stage is observed within 10 minutes of administration of intravenous insulin and appears to reach its maximal effect by 30 minutes. Capillary recruitment is thought to be the more critical of the two stages since it permits the shift of blood flow from non-nutritive to nutritive blood vessels (Clark *et al.*, 2003). Moreover, in the absence of an effect on blood flow, increased capillary recruitment following administration of physiological concentrations of insulin could still be observed (Coggins *et al.*, 2001, Clark *et al.*, 2003). The second stage involves increased total limb blood flow which occurs several hours following initial administration of insulin. The stimulatory effect of insulin on blood flow was first documented by Laakso *et al.* (1990, 1992) and their colleagues Baron *et al.* (1993). Accordingly, physiological and supra-physiological levels of insulin were shown to augment overall limb blood flow in a dose-dependent (Laakso, 1990, 1992; Baron *et al.*, 1993; De Jongh *et al.*, 2004b) and NO-dependent manner (Steinberg *et al.*, 1994). Based on this evidence, it could be postulated that insulin's vascular signalling pathway is central to the regulation of capillary recruitment and total blood flow, hence endothelium function, and that disturbances in insulin's hemodynamic actions such as observed in obesity will inevitably result in impaired vascular function and hypertension. In line with this concept, several studies have demonstrated that, in obesity, insulin's ability to evoke skeletal muscle blood flow is impaired (Laakso *et al.*, 1990), and that compared to lean individuals, obese individuals have higher SBP, impaired insulin sensitivity, impaired capillary recruitment and endothelium-mediated vasodilation in both the basal state and during hyperinsulinaemia (De Jongh *et al.*, 2004a).

1.6. Insulin resistance and dyslipidaemia

Physiological relations between insulin resistance and lipid metabolism have been recognised for decades and are thought to play a key role in promoting CVD in insulin-resistant states such as diabetes, obesity and the metabolic syndrome. This could be illustrated by the fact that insulin resistant states often share dyslipidaemia as a common pathological feature (Lamarche and Mauger, 2005). This dyslipidaemia is characterised by elevated plasma TG concentrations and reduced concentrations and size of HDL-cholesterol (Maclean *et al.*, 2000; Garvey *et al.*, 2003; Goff *et al.*, 2005). Notably, this phenotype of elevated plasma TG and reduced HDL appears to be specific to insulin resistant states (Couillard *et al.*, 2000). The concentration of LDL-cholesterol is not necessarily augmented but, the number of the denser, smaller and more atherogenic LDL-cholesterol is increased in insulin-related dyslipidaemia (Maclean *et al.*, 2000; Garvey *et al.*, 2003; Goff *et al.*, 2005). The consequence of such disturbances in lipid metabolism is reflected in the high incidence of CVD and mortality amongst these population subgroups.

Such strong physiological associations between insulin resistance and dyslipidaemia could only arise as a consequence of a close and interlinked biochemical regulation of both glucose and lipid metabolism. Insulin is recognised as one of the primary hormonal regulators of lipid metabolism. It acts on various stages of lipid metabolism. For instance, during the post-prandial state, insulin promotes the uptake of TG into adipose tissue by upregulating the activity of adipose tissue lipoprotein lipase (LPL) (Semenkovich *et al.*, 1989; Panarotto *et al.*, 2002). It also promotes de novo lipogenesis wherein excess glucose is converted to fatty acids for storage as energy reserves in the white adipose tissue (Saltiel and Khan, 2001). Insulin also inhibits lipolysis and gluconeogenesis (Saltiel and Khan, 2001).

1.6.1 Effect on triglyceride uptake

LPL is an enzyme responsible for the hydrolysis of TG-rich lipoproteins postprandially in chylomircons and during fasting in very low density lipoproteins (VLDL) (Lamarche and Mauger, 2005). Defects in its activity are closely linked to a rise in plasma TG levels (Tsutsumi, 2003). In insulin resistant-states, there is a concomitant impairment in plasma LPL activity (Sumner *et al.*, 2005) and adipose tissue LPL expression which together are thought to induce postprandial triglyceridaemia as a result of reduced TG-rich lipoprotein uptake (Panarotto *et al.*, 2002). This is evident in that fasting insulin and insulin-mediated glucose uptake are strongly and inversely correlated with fasting plasma TG and VLDL

concentrations (Maheux *et al.*, 1997; Ambrosch *et al.*, 1998), but inversely with plasma LPL activity and adipose tissue LPL mRNA expression (Maheux *et al.*, 1997). Likewise, defects in LPL activity are also associated with a reduction in plasma HDL in insulin-resistant obese individuals (Tsutsumi, 2003) which explains the high TG/ low HDL dyslipidaemia seen in insulin resistance (James *et al.*, 2003). Recently, De Jongh *et al.* (2006) have also revealed that insulin can influence LPL activity indirectly via its effects on endothelium function. This is exemplified in the negative correlation seen between capillary recruitment and dyslipidaemia (De Jongh *et al.*, 2006; IJzerman *et al.*, 2006). De Jongh *et al.* (2006) argues that such association can result from the loss of contact surface area between LPL and lipoproteins as a result of, reduced capillary recruitment, which then attenuates TG uptake leading to a rise in plasma TG and subsequent changes in HDL and LDL particles, as described later.

1.6.2 Effect on triglyceride clearance and lipogenesis

In addition to regulating TG uptake, insulin controls the clearance of TG-rich chylomicrons and their remnants. In this respect, insulin resistance has been shown to be associated with diminished LDL-receptor activity which is then expressed by the presence of high levels of apolipoprotein B-48 and apolipoprotein B-100 in the circulation (James *et al.*, 2003). Attenuation of insulin resistance in obesity, on the other hand, is accompanied by improved LDL receptor binding and activity (James *et al.*, 2003). Hyperinsulinaemia and insulin resistance have also been documented to stimulate lipogenesis as evident by increased hepatic VLDL synthesis and secretion (Brown and Goldstein, 2008).

The molecular basis of such effect has been hypothesised to include defects in post-receptor insulin signalling (Brown and Goldstein, 2008). These abnormalities somehow resemble the disturbances in insulin's vascular actions (see Section 1.5.2) in that they occur as a consequence of an imbalance between two insulin-dependent pathways. Accordingly hyperinsulinaemia is postulated to selectively downregulate insulin downstream signalling pathways, i.e. IRS/PI3K/protein kinase B/forkhead box O transcription factor 1 (IRS/PI3K/Akt/FOXO1) while activating some poorly defined pathway that regulates hepatic SREBP1c expression and activity (Brown and Goldstein, 2008; Semple *et al.*, 2009). SREBP-1c is a transcription factor found predominantly in the liver which stimulates lipogenesis (Shimano *et al.*, 2007). It is generally activated in the presence of excess energy intake where it acts to promote the storage of energy by the conversion of glucose to TG

(Shimano *et al.,* 2007). Uncontrolled activation of hepatic SREBP-1c can cause hypertriglyceridaemia and synthesis of large TG-rich VLDL in the liver due to direct suppression of insulin signalling pathways and glycogen synthesis (Ide *et al.*, 2004; Shimano *et al.*, 2007) as well as by mediating the effect of insulin resistance on LDL-receptor (Kotzka *et al.*, 2000).

So far, much controversy exists concerning the IRS isomer that controls insulin signalling activation of STREBP1-c. Kohjima *et al.* (2008) has shown that the expression of IRS1 but not IRS2, is positively correlated with the expression of SREBP-1c (Kohjima *et al.*, 2008). However, the latter is in contrast to the findings of Semple *et al.* (2009) of an inverse relation between IRS-2 protein levels and SREBP transcription. Such discrepancy could be explained by the fact that in addition to regulating SREBP1-c, IRS-2 is, in itself, negatively influenced by SREBP1-c (Ide *et al.*, 2004; Taniguchi *et al.*, 2005; Shimano *et al.*, 2007; Kohjima *et al.*, 2008). Regardless of inconsistencies, most authors argue in favour of a primary role of IRS-2/ Akt 1 signalling pathway in modulating insulin's regulation of lipid metabolism (Farese *et al.*, 2005; Taniguchi *et al.*, 2005; Bouzakri *et al.*, 2006). IRS-1/ Akt 2, on the other hand, is thought to be more relevant to glucose metabolism (Taniguchi *et al.*, 2005; Bouzakri *et al.*, 2006). In this respect, Taniguchi *et al.* (2005) has demonstrated that reduced hepatic expression of IRS2 augments SREBP-c levels independently of insulin. Moreover, Kohjima *et al.* (2008) has documented that IRS-2 is negatively correlated with Foxa2, another transcription factor involved in lipid metabolism and which promotes β-oxidation. As such, it may be that increased hepatic expression of IRS1 in conjunction with reduced expression of IRS2 act synergetically and via distinct pathways to augment SREBP-1c expression and consequently lipogenesis. However, in obese insulin-resistant and hyperinsulinaemic states concurrent reductions in hepatic IRS-1 and IRS-2 are observed which argues against such concept (Anai *et al.*, 1998, 1999). In agreement with the specific functions of IRS1 and IRS 2 proposed by Kido *et al.* (2000) and Previs *et al.* (2000), Taniguchi *et al.* (2005) has shown that rats expressing hepatic knock-outs of IRS-1 and IRS-2 exhibit all the salient features of the metabolic syndrome including fasting hyperglycaemia, fasting hyperinsulinaemia, insulin resistance, glucose intolerance, dyslipidaemia amongst others. Such observations undoubtedly mark a central role of defects in the IRS2 pathway in increasing hepatic SREBP1-c expression in insulin-resistant states. Factors such as increased expression and activity of liver X receptor have also been shown to accelerate insulin's stimulatory effect on SREBP-1c transcription (Chen *et al.*, 2004; Taniguchi *et al.*, 2005). Interestingly, Kotzka *et al.* (2000) has identified STREBP-1 as a substrate for the

MAPK signalling pathway, which when considering the unaltered MAPK activity seen in insulin resistance is likely to contribute to the activation of STREBP1-c.

1.6.3 Consequences of impaired triglyceride uptake, clearance and lipogenesis on lipoprotein metabolism

Impaired TG uptake and clearance and enhanced lipogenesis increase plasma TG and total TG pool which precipitates the development of TG-enriched HDL (Rashid *et al.*, 2002a). Enrichment of HDL with TG occurs via upregulation of cholesteryl ester transfer protein (CETP), an enzyme that promotes the uptake of TG from chylomicrons and VLDL to HDL (Matsuura *et al.*, 2006; Yvan-Charvet *et al.*, 2007). A number of cell culture and human studies have demonstrated that insulin can downregulate the activity of CETP (Arii *et al.*, 1997; Kaser *et al.*, 2001) but that in conditions of impaired insulin metabolism, such as type II diabetes, the ability of insulin to suppress CETP activity is hindered (Kaser *et al.*, 2001). This when combined with enhanced hepatic lipase activity under insulin resistant conditions not only alters the composition HDL particles but also enhances the clearance of TG-enriched HDL (Lamarche *et al.*, 1998; Couillard *et al.*, 2000; Rashid *et al.*, 2003a-b). This increased susceptibility of TG-enriched HDL to metabolic degradation, as measured by fractional catabolic rate of apoA-1, a marker of HDL clearance (Nestle *et al.*, 1987), was first documented by Lamarche *et al.* (1999), and subsequently reviewed by Rashid *et al.* (2002b).

Besides generating an atherogenic profile and reducing HDL concentrations, insulin resistance can also alter the size and composition of HDL in a way that leads to the loss of HDL anti-atherogenic properties. The genes that link insulin resistance with altered HDL-particle size have been identified almost over a decade ago by Rainwater *et al.* (1997). Recent studies have elaborated further on the link between insulin resistance and HDL particle size by demonstrating that unregulated CETP activity stimulates the removal of esterified cholesterol from HDL (Matsuura *et al.*, 2006; Yvan-Charvet *et al.*, 2007). This results in the formation of small, dense and dysfunctional HDL. Inhibition of CETP, on the other hand, restores HDL's anti-atherogenic properties by preserving its large cholesterol ester-rich composition, which then reduces the risk of atherosclerosis by accelerating the uptake of free cholesterol from foam cells by HDL (Matsuura *et al.*, 2006; Yvan-Charvet *et al.*, 2007). Likewise, insulin has been shown to regulate the activity of lecithin: cholesterol acyltransferase and phospholipid transfer protein, two enzymes that are involved in promoting HDL's reverse cholesterol transfer activity (Riemens *et al.*, 1999). This reverse

cholesterol transfer mechanism enables HDL to transfer cholesterol from peripheral cells to liver and accounts for HDL's anti-atherogenic effects (Riemens *et al.*, 1999). Although theoretically, enhanced lecithin: cholesterol acyltransferase and phospholipid transfer protein should be associated with increased reverse cholesterol transfer, hence improved anti-atherogenic properties of HDL, in insulin-resistance increased activity of these enzymes is strongly linked to increased plasma TG concentrations (Riemens *et al.*, 1999) and atherosclerosis (Schlitt *et al*., 2003). In respect to phospholipid transfer protein, this effect have been explained by the ability of this enzyme to promote uptake of oxidised phospholipids by HDL, rendering the latter less protective against atherogensis (Schlitt *et al.,* 2003).

It is noteworthy that in contrast to its ability to influence TG, VLDL and HDL, insulin has no direct effect on LDL. Although changes in the size and density of LDL are observed in insulin resistant states, these effects are attributed to hypertriglyceridaemia which has been shown to predict 62% of variance in LDL size (Ambrosch *et al.*, 1998) . As a result, any link between LDL particle size and insulin resistance has been postulated to occur via the effect of insulin resistance on the metabolism of other lipoproteins (Ambrosch *et al.*, 1998; Friedlander *et al.*, 2000).

1.6.4 Effect on lipolysis

Insulin regulates both hormone sensitive lipase (HSL)-dependent and HSL-independent lipolysis. With HSL-dependent lipolysis, insulin activates phosphodiesterase-3B (Rondinone *et al.*, 2000) leading to increased degradation of protein kinase A (PKA)-dependent cAMP (Degerman *et al.*, 1998). Since raised cAMP levels are central to the activation of HSL, a reduction in their levels result in inhibition of HSL activity, hence a reduction in HSL-dependent lipolysis (Stralfors *et al.*, 1984). These molecular mechanisms have been reviewed in detail by Holm (2003) and Jaworski *et al.* (2007). Likewise, insulin inhibits HSL-independent lipolysis, a key process involved in basal lipolysis, by downregulation of a novel lipolytic enzyme known as hepatic triglyceride lipase (Kim *et al.*, 2006). Remarkably, in obese insulin-resistant subjects, the expression of adipose triglyceride lipase and HSL is strongly and inversely correlated with insulin resistance and hyperinsulinaemia (Jocken *et al.*, 2007). This relation occurs independently of fat mass (Jocken *et al.*, 2007).

In addition to these mechanisms, upregulation of STREBP1-c plays a critical role in controlling insulin-mediated suppression of fatty acid oxidation via negative feedback mechanisms involving IRS-2 (Taniguchi *et al.*, 2005; Shimano *et al.*, 2007; Kohjima *et al.*, 2008). Together these disturbances in insulin-mediated lipolysis increase the storage of fat in adipose tissue leading to obesity (Nishino *et al.*, 2007).

1.7. Etiology of insulin resistance in obesity

1.7.1 The role of circulating plasma free fatty acids

Obesity is characterised by elevated plasma FFA levels (Ghanim *et al.*, 2004), which occurs as a consequence of increased adipose tissue mass (Frayn, 2001), impaired post-prandial lipaemia (Frayn, 1998, 2002) and possibly high-fat diets (Schrauwen-Hinderling *et al.*, 2006).

To illustrate, it is generally agreed that fasting insulin levels are negatively correlated with efflux of FFA per unit adipose tissue (Karpe and Tan, 2005). In obesity, although expression of HSL is diminished (Langin *et al.*, 2005) and basal lipolysis is decreased (Steinberg *et al.*, 2007), the sum of FFA released per total adipose tissue mass is greater than in lean individuals (Flatt, 1972, Frayn *et al.*, 2001). When combined with impaired insulin-induced inhibition of lipolysis, this results in a continuous state of elevated circulating FFA (Langin, 2006). This is further exacerbated by the abnormal post-prandial lipaemia observed in obesity, particularly abdominal obesity (Mekki *et al.*, 1999, van Hees *et al.*, 2008). To illustrate, following a meal, TG are transported as chylomicrons to adipose tissue and non-adipose tissue, i.e. liver and skeletal muscle. The proportion of TG deposited in the latter tissues is inversely related to the extent of TG 'trapping' in adipose tissue (Sniderman *et al.*, 1998). In conditions such as obesity, delayed TG uptake from chylomicrons into adipose tissue (Kalant *et al.*, 2000) and impaired chylomicrons remnant clearance (Martins and Redgrave, 2004), increase the availability of FFA to liver and skeletal muscle rendering them susceptible to lipid accumulation (Frayn, 2001, Kotronen and Yki-Järvinen, 2008), hence insulin resistance. Moreover, there appears to be a preferential uptake of chylomicrons-derived FFA compared to circulating FFA (Bickerton *et al.*, 2007). In general it is estimated that LPL releases FFA from TG-chylomicrons into splanchenic bed at a rate of 50-60% in overweight and obesity, a phenomena termed the spill-over effect (Nelson *et al.*, 2007). These TG-rich chylomicrons contribute to 12-13% of systemic FFA and account for one-third of the rise in portal veins FFA following a mixed meal (Nelson *et al.*, 2007).

Regardless of the source of FFA, enhanced availability of plasma circulating FFA can either directly impair insulin-induced capillary recruitment, leading to microvascular dysfunction (De Jongh *et al.*, 2004a), or promote FFA accumulation (Stannard *et al.*, 2002) in skeletal muscle, liver and pancreas and reactive oxygen species (ROS) generation leading to insulin resistance and impaired insulin-signalling (Hulver and Dohm, 2004), as discussed in the following section.

1.7.2 The role of intracellular free fatty acids accumulation

The relation between intracellular FFA accumulation and insulin resistance have been recognized for decades. Randle *et al.* (1963) was the first to hypothesis that enhanced intracellular FFA deposition and oxidation inhibits glucose uptake, phosphorylation and glycolysis. This hypothesis was based on the substrate competition between FFA and glucose for mitochondrial oxidation. As summarised in Figure 1.7, enhanced FFA oxidation increases acetyl-CoA/CoA, NADH/NAD and ATP/ADP ratios. This metabolic milieu favours β-oxidation and inhibits the activity of several glycolytic enzymes leading to enhanced gluconeogenesis and reduced peripheral glucose uptake, hence insulin resistance. However, the initial Randle's hypothesis possessed several limitations mainly because it was based on studies on rat diaphragm and cardiac muscle (Randle *et al.*, 1963). Moreover observations that in obesity, FFA oxidation is decreased, as demonstrated by enhanced respiratory quotient, led most authors to argue against Randle's hypothesis (Kelley *et al.*, 1999; Kim *et al.*, 2000). Since then numerous other paradigms have emerged each attempting to explore the relation between intracellular FFA accumulation and insulin resistance in line with their own strengths and weaknesses. These hypotheses have been extensively reviewed and include the portal hypothesis (Bjorntorp, 1990; Bergman, 2000; Bergman and Ader, 2000), ectopic fat storage syndrome (Montani, 2004; Rasouli *et al.*, 2007; Weiss, 2007; Lara-Castro and Garvey, 2008 ; Blüher, 2009; Szendroedi and Roden, 2009) and adipocytes as an endocrine organ (Hutley and Prins, 2005; Fonseca-Alaniz *et al.*, 2007). The upcoming sections will not attempt to deal with these hypotheses in detail although reference will be made to them when appropriate.

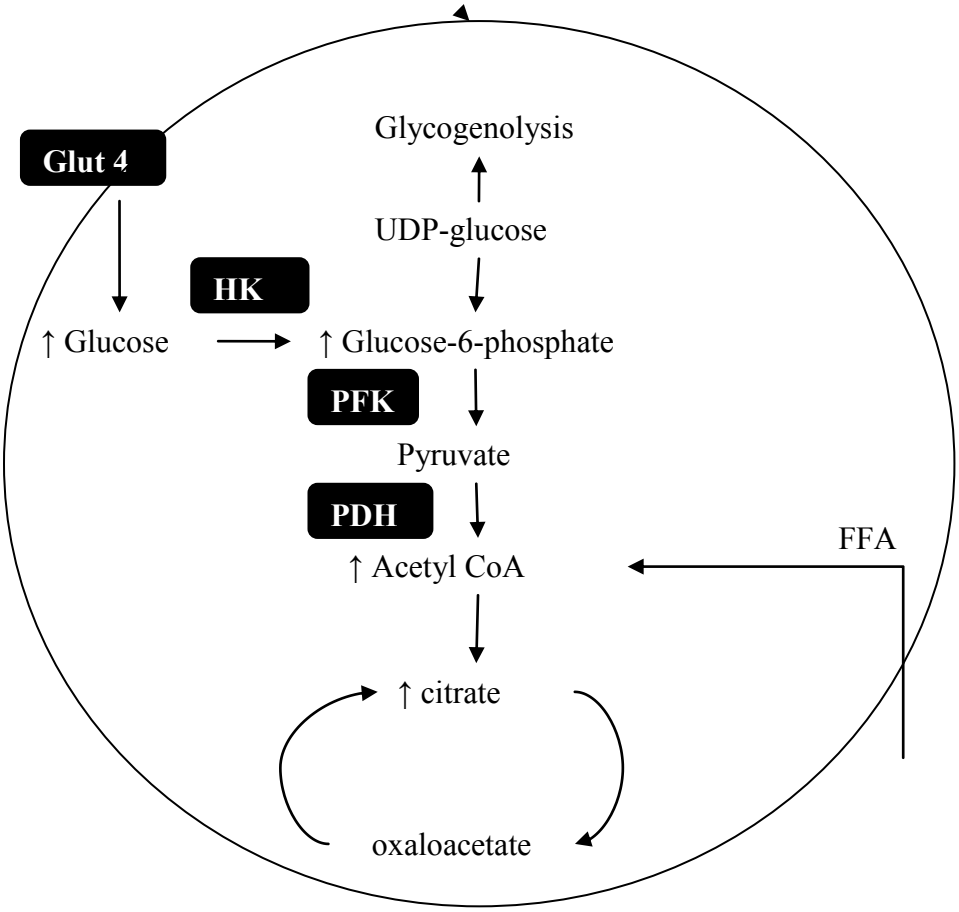


Figure 1.7 Molecular basis of Randle's hypothesis (Randle *et al.*, 1963). Enhanced free fatty acids (FFA)s oxidation creates a metabolic milieu of raised Acetyl-CoA, NADH and ATP. This metabolic milieu leads to inhibition of pyruvate dehydrogenase (PDH), phosphofructokinase (PFK) and hexokinase (HK) causing reduced peripheral glucose uptake.

In general deposition of FFA or so-called ectopic FFA storage could occur in a variety of insulin-sensitive tissue such as liver, skeletal muscle and pancreas (Jensen, 2006). The resulting biochemical disturbances contribute via different mechanisms to systemic insulin resistance (Figure 1.8). For instance, in the pancreas, in which FFA account for 30-50% of insulin secretion, FFA stimulate β-cells to secrete insulin leading to hyperinsulinaemia (Boden, 1998; Jensen, 2006), while in the liver FFA induce hyperglycaemia, hyperinsulinaemia and triglyceridaemia by increasing rate of gluconeogenesis and β-oxidation and by attenuating glycogenesis and insulin clearance (Jensen, 2006). By contrast, in skeletal muscle, enhanced FFA deposition diminishes glucose uptake and IR phosphorylation via mechanisms detailed in Section 1.7.5.

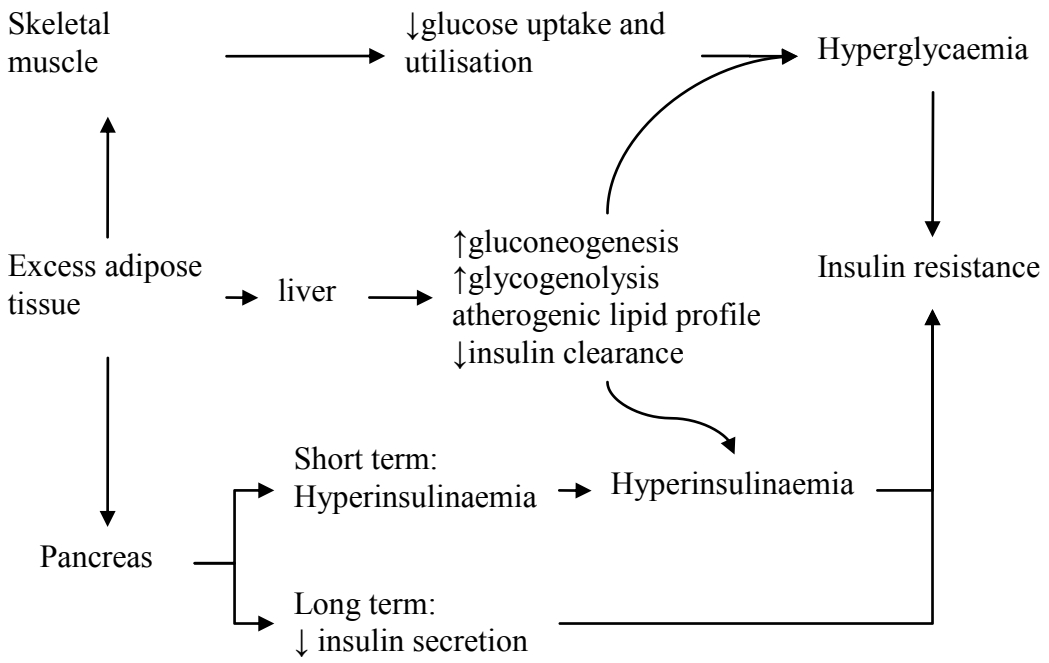


Figure 1.8 Effect of increased adipose tissue mass and FFA accumulation on skeletal muscle, liver and pancreas (adapted from McTernan and Kumar, see Barnett and Kumar, 2004)

1.7.3 Intra-myocellular free fatty acids accumulation

Much debate exits as to the contribution of FFA accumulation in each of the afore-mentioned tissues to systemic insulin resistance. Some authors argue that skeletal muscle is the chief contributor to systemic insulin resistance because it is the major site of substrate oxidation (Jensen, 2006). Others argue in favour of disturbances in FFA metabolism in the liver because the liver is the primary site of glucose and lipid metabolism (Yki-Jarvinen, 2002). Regardless of this, recent evidence has provided some explanation to the molecular basis of enhanced FFA storage in skeletal muscle as well as in the liver. In relation to skeletal muscle, Simoneau and Kelley (1998) have argued that enhanced glucose oxidation in overweight and obese individuals, possibly as a result of hyperglycaemia, may decrease LPL activity leading to diminished TG uptake and oxidation in skeletal muscle. The reduction in β-oxidation implies that TG that do enter skeletal muscle are diverted into storage (Simoneau and Kelley, 1998). As with Randle's hypothesis, Simoneau and Kelley's explanation is based on the competition between FFA and glucose for mitochondrial oxidation. However, unlike Randle's hypothesis, Simoneau and Kelley's concept agrees with studies demonstrating raised respiratory quotient in overweight and obese individuals

(Kelley *et al.*, 1999; Kim *et al.*, 2000). Furthermore, it is consistent with Nisoli *et al.* (2003) who have observed that eNOS knock-out mice have defective biogenesis, possibly as a result of fewer and smaller mitochondria being expressed in insulin target tissues (Le Gouill *et al.*, 2007). The latter is then reflected as diminished energy expenditure and defective mitochondrial ß-oxidation, which in turn is associated with increased TG accumulation in muscle (Le Gouill *et al.*, 2007). Since NO bioavailability is known to be reduced in overweight and obese individuals (Williams *et al.*, 2002; Mather *et al.*, 2004), it is likely that defects in mitochondrial biogenesis contribute to the rise in skeletal muscle TG accumulation in overweight and obesity.

1.7.4 Intra-hepatic free fatty acids accumulation

The liver constitutes one of the major sites of insulin's actions. This suggests that deposition of FFA in the liver could have major implications on the activity of insulin, notably to its effect on glucose and lipid metabolism. This is evident in the positive correlation between hepatic FFA deposition, hyperinsulinaemia, hypertriglyceridaemia, blood pressure and the inverse association between hepatic FFA and HDL (Seppälä-Lindroos *et al.*, 2002)

In obesity, particularly abdominal obesity, there is enhanced deposition of FFA in the liver, a condition now termed non-alcoholic steatohepatitis. In fact according to Kotronen and Yki-Järvinen (2008), non-alcoholic steatohepatitis is considered a novel component of the metabolic syndrome. In general, the liver is exposed to higher levels of FFA than other tissues. This occurs chiefly as a result of the anatomical arrangement of hepatic circulation wherein dietary and adipose-derived FFA are delivered to the liver through both the portal and hepatic circulations (Bradbury, 2006). In relation to the portal circulation, the strong positive association between visceral adipose tissue and hepatic FFA delivery, which is particularly evident in women (Nielsen *et al.*, 2004), led most earlier studies to hypothesise that visceral adipose tissue lipolysis accounts for the largest proportion of FFA delivered to the liver via the portal vein (Bjorntrop, 1990; Rebuffé-Scrive *et al.*, 1990). This Portal hypothesis was based on the higher metabolic activity of visceral adipose tissue as compared to subcutaneous adipose tissue (see review by Wajchenberg, 2000) and the resistance of visceral depots to insulin's antilipolytic actions (Endresen *et al.*, 1994). However, more recent studies suggest that visceral adipose tissue lipolysis contributes to only one-third or one-fifth of upper-body FFA release (Jensen *et al.*, 2003). Conversely, subcutaneous (non-visceral) adipose tissue supplies 70% of systemic FFA under basal and post-prandial conditions (Nielsen *et al.*, 2004) and contributes to 75% of hepatic FFA delivery (Delarue

and Magnan, 2007). Although much debate remains as to the source of FFA in non-alcoholic steatohepatitis, it is generally agreed that when hepatic FFA influx exceeds the processing capacity of the liver and is combined with enhanced hepatic FFA extraction, the result is increased hepatic FFA storage with subsequent disturbances in insulin-mediated glucose and lipid metabolism (Pardina *et al.*, 2009).

1.7.5 Free fatty acids: effect on insulin and glucose metabolism

It is often presumed that hyperinsulinaemia is ensued primarily by compensatory secretion of insulin by pancreatic β-cells in response to peripheral insulin resistance (Reaven, 1988; DeFronzo, 1992; Kahn *et al.*, 2006). However, the liver can also contribute to hyperinsulinaemia by decreasing first-pass hepatic insulin clearance (Duckworth and Kitabch, 1981; Kotronene *et al.*, 2007). In this respect, Svedberg *et al.* (1991) demonstrated that in an *in situ* perfused rat liver model, portal FFAs act as important mediators of hepatic insulin clearance. Accordingly, a dose-dependent association between FFA and hepatic insulin clearance is observed in both the post-absorptive and post-prandial states with higher FFA levels inhibiting insulin clearance to a greater extent, thereby triggering systemic insulin resistance via a rise in peripheral insulin concentrations (Svedberg *et al.*, 1991). Indeed, reduced hepatic insulin extraction under basal conditions have been reported in conjunction with obesity and elevated plasma insulin levels in both humans (Rossell *et al.*, 1983) and animals (Hansen *et al.*, 1993) and is thought to contribute to hyperinsulinaemia, independently of pancreatic β-cells, in obese glucose-intolerant subjects (Bonora *et al.*, 1983). Hepatic insulin clearance have also been documented to be negatively correlated with hepatic fat content (Kotronen *et al.*, 2007, 2008)

In relation to glucose metabolism, it is well recognised that under normal physiological conditions, insulin suppresses glucose production in the post-prandial state while eliciting glycogenolysis and gluconeogenesis in the absorptive state. This ensures sufficient levels of glucose are available as an energy substrate for tissues, particularly the brain and red blood cells, under all physiological states while maintaining euglycaemia (Champe and Harvey, 1994). In obesity, there is diminished insulin-induced suppression of glucose production, probably as a result of elevated glucose-6-phophatase activity (Paquot *et al.*, 2002). The latter is likely to occur due to increased hepatic FFA influx that stimulates β-oxidation leading to the generation of acetyl-CoA, citrate and glucose-6-phosphate, substrates that downregulate insulin-induced inhibition of gluconeogenesis (Randle's effect) (Seppälä-Lindroos *et al.*, 2002, Beck-Nielsen *et al.*, 2005) and glycogenolysis in the liver (Boden *et*

al., 2002). This relation between elevated rates of gluconeogenesis, total body fat, abdominal obesity, plasma circulating FFA and glucose has equally been described by Gastaldelli *et al.* (2000).

1.7.6 Free fatty acids: effect on insulin-mediated regulation of endothelium function and blood pressure

FFA can influence insulin-mediated regulation of endothelium function and blood pressure homeostasis by affecting insulin's vascular actions (see Section 1.5.2; p. 22 and Figure 1.6; p. 24). Herein, FFA promote serine phosphorylation of IR which leads to decreased tyrosine phosphorylation of IR by insulin (Morino *et al.*, 2005) and subsequently reduced Akt and eNOS activation. FFA also activate the renin-angiotensin-aldosterone system which increases angiotensin-II production (Jonk *et al.*, 2007). The latter induces a reciprocal rise in FFA while promoting serine phosphorylation of IR, independently of FFA, resulting again in decreased Akt and eNOS activation (Andreozzi *et al.*, 2004). While decreased Akt activation leads to a decline in GLUT4 translocation, hence reduced glucose uptake and glycogen storage and subsequently insulin resistance (Morino *et al.*, 2005), impaired eNOS activation, hence reduced NO production, induces endothelium dysfunction. Activation of renin-angiotensin-aldosterone system and angiotensin-II further exacerbate endothelium function by activating NADPH oxidase and ROS formation which then promote NO degradation to peroxynitrate (Doughan *et al.*, 2007). Angiotensin-II also activates MAPK pathway which results in raised endothelin-1 production (Mather *et al.*, 2004). This diminished NO bioavailability then leads to endothelium dysfunction and subsequently hypertension (Cleland and Connell, 2005).

1.7.7 Free fatty acids: effect on insulin-induced lipid metabolism

In the case of dyslipidaemia, it is well recognised that the liver is the primary site of lipogenesis in the body and one of the main sources of lipids for adipocytes alongside dietary lipids (McTernan and Kumar, 2004). Enhanced hepatic delivery of FFA results in increased generation of VLDL (Jensen, 2006). This situation is further exacerbated by excess synthesis of apolipoprotein B-100 in the liver, which stimulates the transfer of TG in exchange for cholesterol esters resulting in smaller and less dense HDL particles being produced (Lamarche *et al.*, 1999; Rashid *et al.*, 2002a; Jensen, 2006). These HDL particles are rapidly metabolised leading to a drop in circulating HDL levels (Lamarche *et al.*, 1999; Rashid *et al.*, 2002b). Together these disturbances in lipid metabolism lead to the development of

dyslipidaemia characterised by elevated VLDL, LDL, and apolipoprotein B-100 and reduced HDL, and which acts as a major risk factor for development of CVD in overweight and obesity.

1.8. Adipose tissue as an endocrine organ

The concept of adipose tissue as a passive energy repertoire has long been debated (Siiteri, 1987) and it is now become increasingly recognised that adipose tissue is an active endocrine organ, that produces, and secrets a wide array of bioactive substances including inflammatory mediators (Tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6)) and adipokines (FFA, leptin, resistin, angiotensinogen, adiponectin) which act as major pathophysiological determinants of insulin resistance (Trayhurn and Beattie, 2001; Kershaw and Flier, 2004; Trayhurn and Wood, 2004; Clarke and Mohamed-Ali, 2005; Trayhurn, 2005; Trayhurn and Wood, 2005; Bastard *et al.*, 2006). The role of FFA in obesity-induced insulin resistance has already been discussed while the association between TNF-α, IL-6, adiponectin, leptin and insulin resistance has been extensively reviewed (Trayhurn and Beattie, 2001; Kershaw and Flier, 2004; Trayhurn and Wood, 2004; Clarke and Mohamed-Ali, 2005; Trayhurn, 2005; Trayhurn and Wood, 2005; Bastard *et al.*, 2006). Thus the present section focuses on evaluating the relation between cortisol metabolism and insulin resistance. The focus on cortisol stems from the critical role played by this hormone in controlling short-term and long-term glucose metabolism, as well as in its potential role in regulating other inflammatory cytokines (DeRijk *et al.*, 1997) and adipokines (Fallo *et al.*, 2004). *In vitro*, cortisol has been shown to reduce adipose tissue IL-6 release (Fried *et al.*, 1998), diminish adiponectin gene expression (Degawa-Yamauchi *et al.*, 2005) and secretion (Halleux *et al.*, 2001) and, in conjunction with insulin, stimulate leptin release from adipocytes (Halleux *et al.*, 1998; Kanu *et al.*, 2003). Similar changes in circulating adiponectin levels have also been reported *in vivo* with endogenous cortisol overproduction or exogenous cortisol administration (Fallo *et al.*, 2004). However, whether this effect occurs as a direct action of cortisol on adiponectin secretion remains to be clarified (Gavrila *et al.*, 2003). Cortisol is also known to inhibit TNF-α release from macrophages (Swantek *et al.*, 1997) but not from adipose tissue (Sewter *et al.*, 1999). The latter could carry important implications to obesity considering that TNF-α is overexpressed in adipose tissue of obese insulin resistant subjects (Kern *et al.*, 2001) and is associated, alongside and other inflammatory mediators, with enhanced adipose tissue inflammation (Wellen and Hotamisligil, 2003).

In recent years, the similarities between Cushing disease and abdominal obesity has opted several researchers to consider a role for excessive cortisol production in the pathogenesis of obesity and the metabolic syndrome (Andrews and Walker, 1999; Darmon *et al.*, 2006). This subchapter summarises some of the key findings and provides evidence for considering attenuation of cortisol metabolism as a therapeutic target for treating obesity-related complications.

1.8.1 Cortisol: role in insulin resistance and obesity related-complications

Cortisol is a hormone that is generally secreted from the adrenal cortex in response to hypothalamus-pituitary-adrenal axis (HPA) activation by inflammation, pain, infection and stress (Newton, 2000). It is essential in the long-term maintenance of blood glucose, which is why it is often referred to as a glucocorticoid (Champe and Harvey, 1994). Cortisol is also considered a counter-regulatory hormone because it opposes insulin's metabolic functions by decreasing glucose uptake and utilisation, reducing insulin secretion by β-cells, promoting gluconeogenesis in liver and increasing mobilisation of amino acids and fatty acids from skeletal muscle and adipose tissue (Rizza *et al.*, 1982; Andrew and Walker, 1999; Newton, 2000). These counter-regulatory effects are very much characteristic of the changes in glucose metabolism observed in insulin-resistant states such as obesity (see Figure 1.8, p. 37 and Section 1.4.4, p. 17) and which may explain why excess plasma cortisol is often associated with hyperinsulinaemia and hyperglycaemia (Rizza *et al.*, 1982).

In general, cortisol can induce insulin resistance by influencing both peripheral glucose metabolism and hepatic glucose production (Rizza *et al.*, 1982) whereas withdrawal of cortisol, in humans, improves insulin sensitivity by raising glucose oxidation and reducing endogenous glucose production (Christiansen *et al.*, 2007). In relation to peripheral glucose metabolism, cortisol can affect several stages of insulin-stimulated glucose uptake, including IR binding (Olefsky *et al.*, 1975; Buren *et al.*, 2002). In this respect, acute administration of dexamethasone, a cortisol analogue (Gotelli *et al.*, 1981), in rats has been shown to attenuate insulin binding to its receptor in adipocytes and hepatocytes, with this inhibitory effect being maintained in hepatocytes but not in adipocytes with chronic dexamethasone administration (Olefsky *et al.*, 1975). However, according to Olefsky (1975), decreased IR binding is likely to occur secondary to corticosteroid-induced hyperinsulinaemia rather than as a direct consequence of corticosteroid administration. Consistent with this, several authors have demonstrated that defects in post-receptor insulin signalling and GLUT4 translocation, and

not IR binding, constitute the most recognised abnormalities associated with excess cortisol (Rizza *et al.*, 1982; Oda *et al.*, 1995; Venkatesan *et al.*, 1996). Consequently, in the hippocampus, cortisol prevents insulin-stimulated phosphorylation of the IR and reduces the expression of Akt and GLUT4 (Piroli *et al.*, 2007). Likewise, in adipose tissue, dexamethasone impairs glucose uptake (Czech and Fain, 1972; Buren *et al.*, 2002; Kawai *et al.*, 2002; Ngo *et al.*, 2009) possibly by reducing the levels of insulin signalling substrates IRS-1 and PKB and insulin-induced PKB activation (Buren *et al.*, 2002; Buren *et al.*, 2008). A 50% reduction in the phosphorylation of Akt substrate (AS160), responsible for 14-3-3 recruitment and subsequent GLUT4 translocation, has also been reported with dexamethasone treatment of murine and human adipocytes (Ngo *et al.*, 2009). This reduction occurs under both basal and insulin-stimulated conditions (Ngo *et al.*, 2009) and is further reinforced by the observed decrement in the concentration of GLUT4 on plasma membrane of adipocytes following chronic dexamethasone administration (Oda *et al.*, 1995). In contrast, administration of a glucocorticoid receptor antagonist, reverses dexamethasone-induced alterations in AS160-T642 phosphorylation and restores glucose disposal by as much as 80% (Ngo *et al.*, 2009). Pre-incubation of adipocytes with dexamethasone can also promote the activity and membrane translocation of conventional PKC isozymes, PKCα and PKCβ, as well as the atypical PKC isozyme, PKCζ in adipose tissue (Ishizuka *et al.*, 1995; Ishizuka *et al.*, 1997; Kajita *et al.*, 2000-2001; Kawai *et al.*, 2002), all of which can promote ROS production which then enhances serine/threonine phosphorylation of IR leading to inactivation of IR (see Section 1.8.3 and Section 1.9.3). Importantly, parallel changes in PKC activity and translocation have been reported in skeletal muscle (Ishizuka *et al.*, 1995; Ishizuka *et al.*, 1997; Kajita *et al.*, 2000-2001 ; Kawai *et al.*, 2002) wherein dexamethasone have been shown to suppress basal, insulin- and IGF-stimulated glucose transport (Weinstein *et al.*, 1995), despite unaltered (Oda *et al.*, 1995; Venkatesan *et al.*, 1996) or increased muscle GLUT4 concentrations (Coderre *et al.*, 1996; Weinstein *et al.*, 1998). The consequence of such actions is then reflected in reduced "sensitivity" of glycogenesis and glucose oxidation, but not glycolysis, to insulin (Dimitriadis *et al.*, 1997). Consistent with the latter, Buren *et al.* (2008) recently demonstrated that dexamethasone, in addition to reducing PKB expression and insulin-stimulated glucose uptake, can inhibit glycogenesis via downregulation of glycogen synthase activity.

In the liver, excess cortisol abrogates insulin-stimulated suppression of glucose production (Rizza *et al.*, 1982) possibly through upregulation of the activity of the rate limiting gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEP-CK). Cortisol's lipolytic

(Buren *et al.*, 2008) and proteolytic actions may also contribute to enhanced gluconeogenesis by increasing the supply of gluconeogenic substrates such as amino acids, non-esterified fatty acids (NEFA) and glycerol to the liver. In this respect, cortisol has been shown to raise the concentration (Khani and Tayek, 2001) and plasma rate appearance of the amino acids: alanine, leucine and phenylalanine (Simmons *et al.*, 1984; Brillon *et al.*, 1995; Berneis *et al.*, 1997). Likewise administration of cortisol have been reported to increase serum NEFA and glycerol concentrations in humans (Samra *et al.*, 1998; Khani and Tayek, 2001) with NEFA levels being positively correlated with the rate of gluconeogenesis in healthy subjects (Tayek and Katz, 1997; Katz and Tayek, 1998; Khani and Tayek, 2001) and in diabetics (Tayek and Katz, 1996).

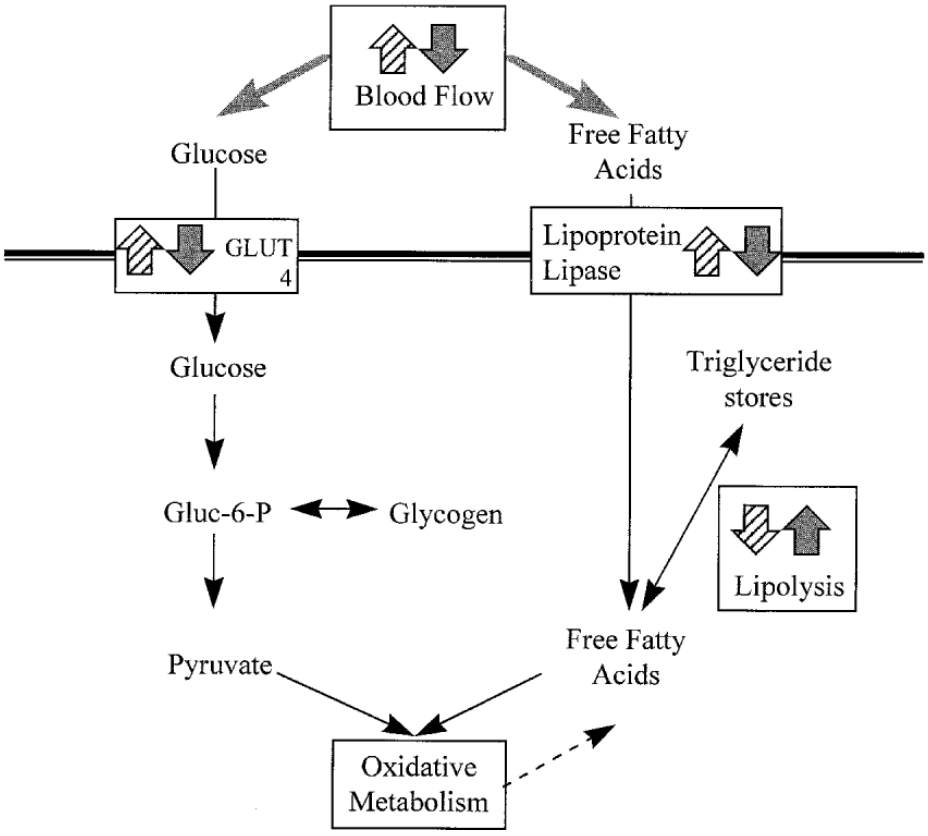


Figure 1.9 Effects of glucocorticoids on peripheral glucose uptake and lipid metabolism (adapted from Andrews and Walker, 1999). Note that cortisol increases lipolysis leading to enhanced availability of free fatty acids which then compete with pyruvate for mitochondrial oxidative metabolism (Randle's effect). Counter-regulatory actions of glucocorticoids are shown by grey arrows while insulin actions are shown by striped arrows.

In addition to the above short-term regulatory functions of cortisol, cortisol can contribute to insulin resistance through its long-term regulation of energy intake and body weight.

Support for this concept could be derived from studies on adrenalectomized rats wherein, in contrast to insulin, glucocorticoids enhance the mRNA expression of neuropeptide Y in the hippocampus causing a subsequent rise in food intake and weight gain (Strack *et al.*, 1995). In fact, the reciprocal actions of glucocorticoids and insulin were found to account for 50% of the variance in food intake and body weight (Strack *et al.*, 1995). Chavez *et al.* (1997) have also observed that, in adrenalectomized rats, the absence of circulating glucocorticoids increases the brain's sensitivity to insulin, and that insulin in the brain acts to lower food intake and body weight via a glucocorticoid receptor-dependent mechanism. Similarly, Santana *et al.* (1995) documented that corticosteroids act to enhance energy acquisition through direct interaction with mineralocorticoid receptors and glucocorticoid receptors, and that insulin prevents this interaction. Notably, dexamethasone or dexamethasone-stimulated insulin were shown to selectively maintain or increase body fat stores (Santana *et al.*, 1995). This enhanced fat accumulation appears to affect visceral fat depots to a greater extent than peripheral fat depots (Burt *et al.*, 2007; Seckl *et al.*, 2004) which when considering the role of visceral fat in supplying hepatic FFA via the portal vein could have important implications to obesity-associated insulin resistance (Section 1.7.3). Cortisol also increases protein catabolism and oxidation (Burt *et al.*, 2007) which can result in an unfavourable body composition such as is observed in Cushing's disease (Burt *et al.*, 2006) while treatment of cortisol excess reduces energy intake and reverses changes in body composition (Pirlich *et al.*, 2002; Wang *et al.*, 2006).

1.8.2 Regulation of cortisol activity: role of 11β-hydroxysteroid dehydrogenase type-1 and type II

Several enzymes are involved in the metabolism of cortisol (Figure 1.10). In particular, the interconversion of active cortisol to inactive cortisone is regulated by two isozymes: 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and type 2 (11βHSD2). These isozymes are products of distinct genes and consequently they differ in their physiological functions and tissue distribution which has important implications to determining tissue cortisol metabolism and activity (Stewart and Krozowski, 1999). 11βHSD1 was the first of the two isoforms to be discovered by Amelung *et al.* (1953) who demonstrated that incubation of cortisone with liver homogenates, particularly liver microsomes, yields cortisol. Interestingly, Amelung *et al.* (1953) also observed that addition of cortisone to kidney produced negative values for cortisol. This observation went on to be explained by the presence of another 11βHSD isozyme in the kidney: 11βHSD2. This isozyme was first purified by Brown *et al.* (1993) from human placenta and subsequently its gene coding was isolated from kidney, placenta and other aldosterone target tissues including salivary glands and reproductive tissues (Agarwal *et al.*, 1994; Albiston *et al.*, 1994).

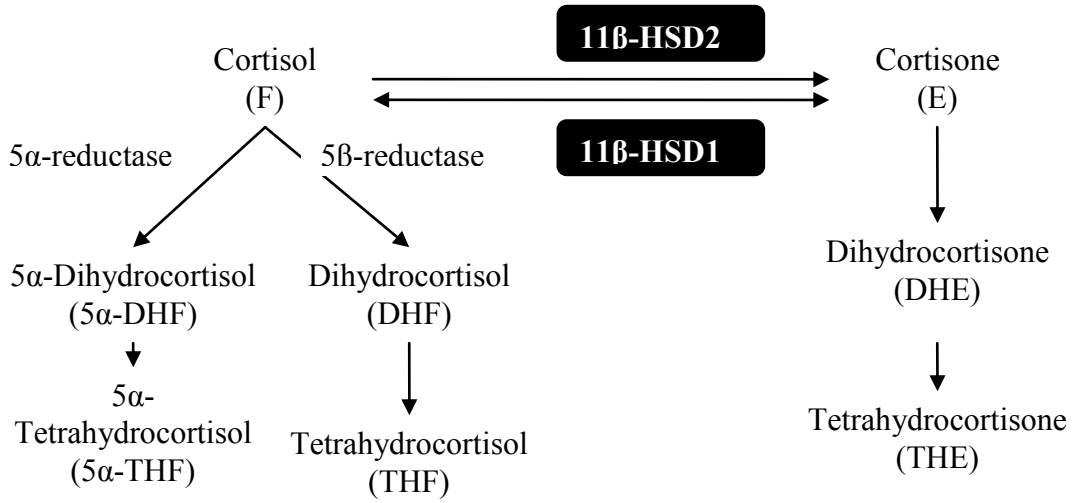


Figure 1.10 Regulation of cortisol metabolism by various reductases and dehydrogenases (N'Gankam *et al.*, 2002).

As shown in Figure 1.10, 11βHSD1 is a bidirectional enzyme that acts predominantly as a reductase converting inactive cortisone to active cortisol. It is found in the liver and adipose tissue although recent studies have also identified its presence in the endothelium, brain and

central nervous system (Seckl and Walker, 2001). Selective inhibition of 11βHSD1 produces knock-out mice that have reduced mRNA expression of PEP-CK and glucose-6-phosphate and are subsequently protected against obesity- or stress-induced hyperglycaemia and hyperinsulinaemia (Kotelevtsev *et al.*, 1997; Holmes *et al.*, 2001; Alberts *et al.*, 2002; Alberts *et al.*, 2003). These 11βHSD1 knock-out mice also show improved lipid and lipoprotein profile (Morton *et al.*, 2001). In general, the activity of 11βHSD1 is determined by the availability of the substrate: cortisone (Seckl and Walker, 2001; Seckl *et al.*, 2004), which is supplied by the activity of 11βHSD2 in the kidney (Whitworth *et al.*, 1989) (see Figure 1.11). Unlike cortisol, cortisone does not show a diurnal rhythm and is mainly found in its free form (Dunn *et al.*, 1981) in concentrations as high as 50-100nmol/l (Walker *et al.*, 1992; Seckl and Walker, 2001; Seckl *et al.*, 2004). In contrast, plasma cortisol concentrations fluctuate between 1nmol/l and 100nmol/l through the day (Seckl and Walker, 2001) and although concentrations could reach 400-600nmol/l during diurnal peak (Seckl *et al.*, 2004), this cortisol is physiologically inactive since 90-95% of it is bound to corticosteroid-binding globulin (Dunn *et al.*, 1981; Mendel, 1989).

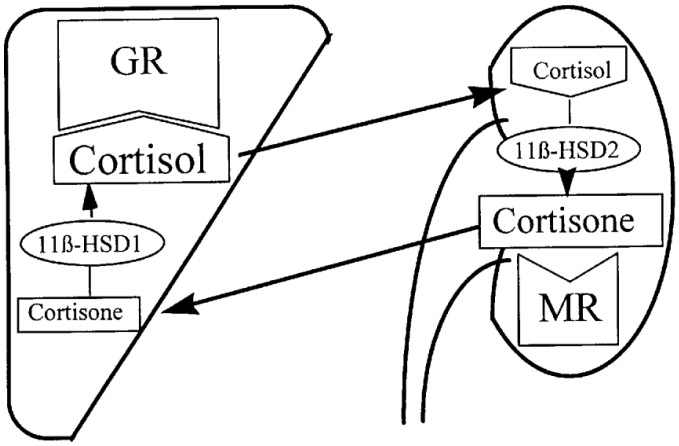


Figure 1.11 Contrasting influence of 11β-hydorxysteroid dehydrogenase type 1 and type 2 (11βHSD1 and 11βHSD2) on cortisol sensitivity in liver and kidney (Andrews and Walker, 1999). Note how the activity of 11βHSD2 in the kidney protects against the mineralocorticoid effects of cortisol and supplies cortisone for 11βHSD1 in the liver.

In contrast, 11βHSD2 is a unidirectional enzyme that acts solely as a dehydrogenase. It is mainly found in the placenta and kidney where it confers aldosterone-specificity to mineralocorticoid receptor by reducing intracellular cortisol concentrations (Funder, 2005) (Figure 1.11). This inhibitory action is important when considering that cortisol has a higher

affinity to mineralocorticoid receptor than aldosterone (Funder, 2005) and that even following 90% inhibition by 11βHSD2, cortisol levels remain 10-fold higher than those of aldosterone, while mineralocorticoid receptor continues to be occupied but not necessarily activated by cortisol (Funder and Myles, 1996; Funder, 2005). In general defects in 11βHSD2 activity promote the formation of cortisol- mineralocorticoid receptor complexes which produces symptoms of mineralocorticoid excess characterised by hypertension, sodium retention and enhanced urinary potassium excretion (Holmes *et al.*, 2001; Seckl *et al.*, 2004).

1.8.3 Cortisol metabolism in obesity

In obesity, particularly abdominal obesity, several alterations in cortisol metabolism are observed (see Table 1.3). For instance, abdominally obese women have lower awakening salivary cortisol compared to peripherally obese women (Duclos *et al.*, 2005). This association between morning salivary cortisol and WHR is solely attributed to fat distribution and not BMI or total fat mass (Duclos *et al.*, 2005). There is also evidence of increased peripheral metabolism of cortisol, as evident by increased urinary cortisone-to-cortisol ratio in abdominally obese women (Duclos *et al.*, 2005). Moreover, obese women show greater postprandial hypercortisolism which according to Duclos *et al.* (2005) may exacerbate insulin resistance due to glucocorticoids' counter-regulatory actions. This is evident in that obese women, particularly those with abdominal fat distribution, show higher fasting insulin and homeostasis-model assessment of insulin resistance (HOMA-IR) than lean women, with a significant correlation being present between $cortisol_{AUC}$, fasting insulin and HOMA-IR (Vicennati and Pasquali, 2000).

Although the basis of hypothalamic-pituitary-adrenal axis hyperactivity in obesity remains unknown (Walker, 2006), some evidence exists from cross-sectional studies conducted by Barker and colleagues which suggest that low birth weight and intrauterine programming of HPA can predict fasting cortisol concentrations in adults (Phillips et al, 2000). In fact, intrauterine programming have been hypothesised to be the underlying mechanism linking low birth weight with the insulin resistance syndrome (Phillips et al, 1998). This is evident in that variations in fasting cortisol concentrations even within the normal range can strongly predict changes in blood pressure, fasting glucose, glucose tolerance, insulin resistance and TG levels (Phillips et al, 1998).

Numerous changes in the gene expression and activity of enzymes that control the inter-conversion of active cortisol to inactive cortisone have also been reported in obesity. These changes are tissue-specific and differ between adipose, renal and liver tissues. For instance, 11βHSD1 gene expression is increased in obese men and women's adipose tissue, suggesting increased conversion of cortisone to cortisol (Paulsen *et al.*, 2007). This abnormality is more prominent in women than in men indicating that obese women are at a higher risk of developing cardiovascular-related diseases than obese men (Paulsen *et al.*, 2007). Subcutaneous adipose tissue 11βHSD1 mRNA levels are also significantly higher in subjects with metabolic syndrome and type II diabetes compared with non-obese subjects or those with impaired or normal glucose tolerance, this being related to increased fasting glucose, urinary free cortisol, adiponectin, TNF-α and decreased serum adiponectin levels (Alberti *et al.*, 2007). Increased 11βHSD1 activity is known to impair glucose-stimulated insulin secretion (Ortsater *et al.*, 2005), all of which confirms cortisol's central role in the pathophysiology of insulin resistance in obesity. It is noteworthy, however, that insulin, itself, could influence cortisol by inhibiting 11βHSD1 activity (Jamieson *et al.*, 1995) at the level of 11βHSD1 gene transcription or by inhibiting TNF-α's stimulatory effects on 11βHSD1 (Handoko *et al.*, 2000). Moreover, several factors that are implicated in impairing insulin's metabolic functions in obesity are also involved in regulating cortisol actions. This is evident in the afore-mentioned Randle's hypothesis wherein excess FFA and hyperglycaemia raise NADPH/NADP ratio and glucose-6-phosphate which can then stimulate 11βHSD1 activity via mechanism that will be described later (see Section 1.11.7, p. 98 and Figure 1.20, p. 101).

Hypercortisolaemia carries several implications to obesity. First, hypercortisolaemia in the presence of insulin resistance can promote TG accumulation in adipose tissue via enhancement of the expression of LPL which consequently leads to weight gain (Ottosson *et al.*, 1994). Second, cortisol can modulate the activity of several enzymes involved in lipid metabolism. As such, increased postprandial cortisol excretion in obesity is inversely associated with HDL (Duclos *et al.*, 2005) whereas 11βHSD1's absence, hence reduced cortisol, is linked to increased HDL and reduced TG, an effect mediated by increased hepatic expression of carnitine palmitoyltransferase and acetyl-CoA (Morton *et al.*, 2001). Third, excess cortisol also influences blood pressure and obese hypertensives are often characterised by raised late-night and early morning salivary cortisol (Kidambi *et al.*, 2007). Last, and as mentioned earlier, cortisol regulates glucose metabolism. It is, thus, unsurprising why obesity and the metabolic syndrome are often associated with

hypercortisolaemia and hyperinsulinaemia (Bjorntorp, 1996; Björntorp and Rosmond, 2000; Ferrannini et al, 2007) and why several authors have placed defects in cortisol metabolism at the centre of obesity-related complications (Phillips et al, 1998, 2000). This could be further exemplified when considering that some of the disturbances in lipid metabolism induced by cortisol form the basis for excess FFA generation which then affects insulin-mediated regulation of blood pressure, glucose and lipid profile. Additional support could be gained from observations that demonstrate that when present in relatively high concentrations, cortisol induces hypertension, atherosclerosis and diabetes mellitus via enhanced ROS production (see Figure 1.12, p 54). The excess ROS could be generated as a result of cortisol's ability to reduce the expression of guanidine triphosphate-cyclohydrolase, the rate limiting enzyme regulating tetrahydrobiopterin (BH4) production (Johns *et al.*, 2001). This results in diminished BH4 levels (Bjelaković *et al.*, 2007) which causes a rise in superoxide generation by eNOS (Kawashima, 2004). Increased ROS production then leads to impaired endothelium function and subsequent deterioration in blood pressure (Johns *et al.*, 2001) and insulin sensitivity (Houstis et al, 2006). Glucocorticoids can also affect endothelium function via modulation of the bioavailability of NO. Herein, glucocorticoids have been shown to influence the activity of eNOS, the availability of the NO substrate, L-arginine, and the co-factor NADP(H) (Whitworth *et al.*, 2002). Both glucocorticoid receptor protein and mRNA as well as the two 11βHSD(1-2) isozymes have been identified in endothelial and smooth muscle cells (Yang and Zhang, 2004) where they have been shown to regulate the degree of decrement in eNOS expression induced by cortisol (Liu *et al.*, 2009).

Table 1.3 Summary of endocrinological disturbances in overweight and obesity

Study	Study population	Disturbances in cortisol metabolism
Pasquali 1993	Abdominally obese vs. Peripherally obese	↑24-h urinary free cortisol
Andrews et al 1998	Obese men and women	↑24-h cortisol ↑11βHSD1 ↑5α-reductase ↑THF/THE in obese men vs lean men and in women vs men =11βHSD2
Rosmond 1998	Abdominally obese men	↓ diurnal cortisol variation
Duclos 1999	Abdominally obese vs. Peripherally obese women	=plasma cortisol ↓salivary post- overnight dexamethasone cortisol =24-h urinary free cortisol ↑Overnight urinary free cortisol
Stewart 1999	Abdominally obese vs. Peripherally obese	=morning cortisol and cortisone ↓11 βHSD1 ↑5ß-reductase =11ßHSD2 = urinary free cortisol-to-cortisone ratio = urinary free cortisol ↑urinary cortisol metabolites ↑cortisol clearance Impaired cortisol→cortiosne conversion
Rask 2002	Overweight and obese vs. Lean females	↑11βHSD1 in adipose tissue ↓11βHSD1 in liver ↑urinary cortisol metabolites ↑ cortisol clearance
Purnell 2004	Healthy men and women with a wide range of BMI	↑24-h cortisol production ↑24-h plasma free cortisol in obesity
Duclos 2005	Abdominally obese vs. Peripherally obese	↓morning salivary cortisol ↑ 24-h plasma free cortisol

1.9. Oxidative stress, insulin resistance and obesity

1.9.1 Obesity and oxidative stress

Obesity is characterised by systemic oxidative stress (Keaney *et al.*, 2003) and increased plasma (Olusi, 2002) and intramuscular lipid peroxidation (Russell *et al.*, 2003) with a direct correlation being present between excess body fat, as determined by BMI, WHR and WC, and markers of oxidative stress including plasma thio-barbituric acid-reactive substances (TBARS) and urinary 8-epi-prostaglandin F2α (Keaney *et al.*, 2003; Furukawa *et al.*, 2004). Since oxidative stress is also commonly associated with other pathologies such as insulin resistance, hypertension and CVD, several researchers have hypothesised that oxidative stress might be the underlying mechanism linking obesity with insulin resistance and the cardinal features of the metabolic syndrome (Keaney *et al.*, 2003; Furukawa *et al.*, 2004). However, before proceeding to discussing the relation between obesity and oxidative stress, it is necessary to define oxidative stress.

Oxidative stress is a condition arising from an imbalance between oxidant and antioxidants leading to excess production of ROS. ROS are natural by-products of mitochondrial electron-transport chain reactions. They can be classified into six species: Superoxide anion (O_2.-), hydrogen peroxide (H_2O_2), peroxyl radical (ROO.), hydroxyl radical (HO.), singlet oxygen ($1O_2$) and peroxynitrite (ONOO-) (Huang *et al.*, 2005). The presence of an unpaired electron in the structure of ROS renders them highly unstable (Baskin and Salem, 1997). In an attempt to stabilise this odd electron, ROS react readily with other adjoining substances such as nucleic acids, proteins and lipids, by abstracting a hydrogen atom from these molecules (Baskin and Salem, 1997). ROS can also react with oxygen to form a peroxyl radical (Baskin and Salem, 1997). In the case of membrane lipids, ROS can abstract a proton from a polyunsaturated fatty acid molecule which produces an unstable lipid radical that reacts with oxygen forming a lipid peroxyl radical (Biesalski and Grimm, 2005). Because this radical is equally unstable, it can propagate a chain of reactions which if not terminated can lead to cellular damage (Biesalski and Grimm, 2005). Under normal physiological conditions, the body can control the production of ROS through a variety of mechanisms that involve both endogenous and exogenous antioxidants (Biesalski and Grimm, 2005). However under pathological conditions, excess production of ROS overcomes the body's natural defence mechanisms leading to oxidative stress.

Much remains to be elucidated as to whether obesity *per se* causes oxidative stress. Although the work of Keaney *et al.* (2003) and others demonstrates a strong independent correlation between markers of adiposity, BMI and WHR and oxidative stress, Higdon and Frei (2003) argue that the presence of other metabolic risk factors alongside obesity can confound any true effect of obesity. Similar inferences could be made in relation to the association between obesity, oxidative stress and insulin resistance. Herein, oxidative stress has been found to predict insulin resistance independently of BMI, suggesting that oxidative stress might act through mechanisms other than those related to excess body fat to promote insulin resistance (Meigs *et al.*, 2007). Nonetheless, the combination of obesity and oxidative stress was found to correlate with the highest levels of insulin resistance implying that certain features of the obese state could have a cumulative effect on ROS-induced insulin resistance (Meigs *et al.*, 2007). Indeed in obesity several factors could interact to promote ROS overproduction. For instance, angiotensin-II and TNF-α stimulate the activity of NADPH oxidase in the vascular endothelium which can enhance the formation of superoxide anion and H_2O_2 (Griendling *et al.*, 1994; Frey *et al.*, 2002; Li and Shah, 2003). Angiotensin-II can also promote lipid peroxidation within macrophages by increasing oxidised-LDL uptake by macrophages (Keidar *et al.*, 2001). Likewise, hyperinsulinaemia which is characteristic of the first stages of insulin resistance can increase H_2O_2 generation in adipocytes via a G-protein coupled mechanism involving NADPH oxidase (Krieger-Bauer *et al.*, 1997), while hyperglycaemia which precedes the pre-diabetic state upregulates NADPH-dependent H_2O_2 production in adipocytes via a PKC dependent mechanism (Talior *et al.*, 2005). The impact of FFAs and cortisol on ROS production has already been discussed in Sections1.7.6 and 1.8.3.

1.9.2 Oxidative stress as the cause of insulin resistance

Each of the above factors can, in turn, induce insulin resistance via excessive ROS production as summarised in Figure 1.12. It could thus be postulated that oxidative stress is one of the underlying and unifying pathologies linking obesity with insulin resistance and other metabolic risk factors. This hypothesis has been proposed by numerous reviewers including Eriksson (2007) and Grattagliano *et al.* (2008) who have argued that hyperglycaemia, inflammation and other endocrine determinants such as angiotensin-II and adipokines share ROS production as a common pathway propelling insulin resistance. However, what could be the primary source of oxidative stress in obesity?

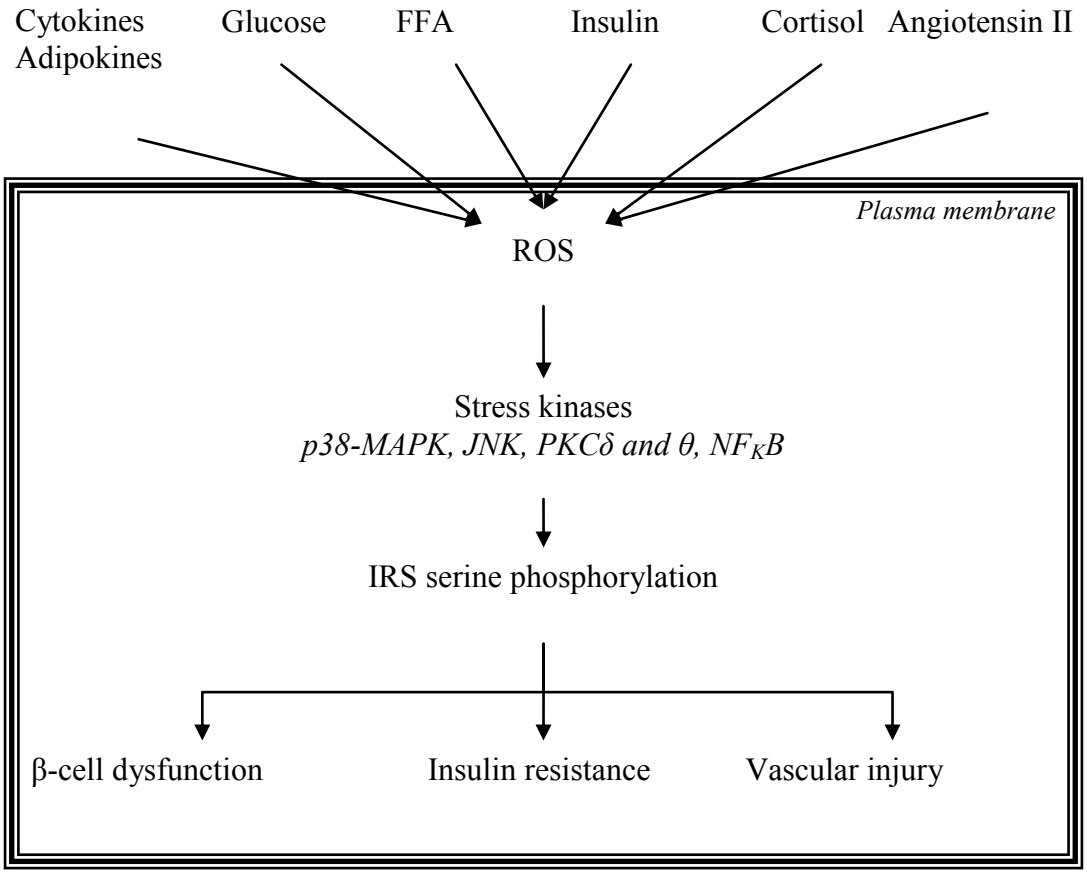


Figure 1.12 Oxidative stress as a unifying pathway leading to insulin resistance and endothelium dysfunction (Eriksson, 2007).

Some evidence exists to suggest that in obesity dysregulation of the redox state at the level of adipocytes lies at the heart of systemic oxidative stress. This entails that local impairments in adipose tissue can progress to cause systemic effects. Support for this hypothesis stems from findings showing that overexpression of NADPH oxidase in adipocytes is accompanied with elevated plasma markers of lipid peroxidation (Furukawa *et al.*, 2004). Furukawa *et al.* (2004) explained this link by suggesting that excess ROS from fat cells can be secreted into blood circulation triggering a systemic rise in ROS levels which, in turn, induces insulin resistance in skeletal muscle, liver, pancreas and adipose tissue itself. These organ-specific dysregulations in insulin reactivity then manifest as systemic insulin resistance, a mechanism consistent with the well-established effects of increased adipose tissue mass, summarised earlier in Figure 1.8. Interestingly, it is this exact increase in adipose tissue mass that is thought to activate the over-expressed NADPH oxidase causing ROS overproduction (Furukawa *et al.*, 2004). The latter could, in turn, promote NADPH oxidase expression through a positive feedback mechanism (Dana *et al.*, 1998; Levy *et al.*, 2000). In addition to

NADPH expression, macrophage infiltration into the stromavascular region of white adipose tissue has been implicated in the loss of redox balance in adipocytes (Weisberg *et al.*, 2003, Xu *et al.*, 2003). Here, accumulated macrophages have been shown to activate a number of inflammatory pathways in adipose tissue leading to excess ROS production and the loss of adipose tissue endocrine function (Weisberg *et al.*, 2003). Importantly, the loss of endocrine function presented as adipokine dysregulation and reduced adiponectin expression in adipocytes, appears to, in turn, promote macrophage infiltration, thereby creating a vicious cycle of raised ROS production (Xu *et al.*, 2003; Cancello *et al.*, 2006). It is important to note that macrophage infiltration occurs only after the expansion of adipose tissue suggesting that adipose tissue mass is an important determinant of the degree of macrophage influx (Weisberg *et al.*, 2003). Accordingly, adipose tissue from obese humans has been estimated to contain up to 5 times more macrophages than adipose tissue from lean individuals (Weisberg *et al.*, 2003). This might also imply that an initial trigger in adipose tissue might induce ROS production which in themselves can attract macrophage into the adipocytes (Furukawa *et al.*, 2004). In this perspective, overfeeding and excess influx of energy substrates such as glucose and FFA might play an important role since both these mechanisms are known to activate NADPH oxidase and increase ROS via the mitochondria chain reaction (Ceriello and Motz, 2004; Ceriello, 2006). The molecular mechanism by which glucose in particular upregulates NADPH oxidase has been identified and appears to involve a PKCδ-dependent pathway (Talior *et al.*, 2005). Regardless, of the cause of oxidative stress in adipose tissue, consistent evidence from cross-sectional studies is emerging which suggests a strong correlation being present between excess fat mass, especially visceral fat, and systemic oxidative stress as measured by plasma and urinary isoprostane concentrations, as well as systemic stress and insulin resistance(Keaney *et al.*, 2003, Urakawa *et al.*, 2003, Pou *et al.*, 2007).

1.9.3 Mechanisms of oxidative stress-induced insulin resistance and obesity-related complications

Eriksson (2007) summarises the mechanism by which ROS induces insulin resistance to include: activation of stress kinases, injury to cellular proteins, lipid membranes, endoplasmic reticulum and DNA. With the former, serine/threonine phosphorylation of IRS proteins is increased leading to diminished insulin signalling (Xu *et al.*, 2003, Eriksson, 2007). When this effect occurs at the level of adipose tissue, lipolysis could be induced resulting in excess FFA efflux into the circulation (Xu *et al.*, 2003). The latter could then trigger systemic insulin resistance through mechanism described in Section 1.7.2.

Importantly, in obesity adipose tissue appear to be more susceptible to H_2O_2-induced impairment in insulin signalling compared to skeletal muscle, an effect attributed to the rapid removal of ADP from adipocytes, which seems to amplify the effect of H_2O_2- (Schmitt *et al.*, 2005). Interestingly, the loss of adipose tissue redox balance and endocrine function as a result of macrophages infiltration could also lead to a similar impairment in insulin signalling in adipocytes (Xu *et al.*, 2003) implying again that dysregulation at the level of adipocytes could induce systemic insulin resistance, which in turn explains why Xu *et al.* (2003) observed that macrophage infiltration is closely correlated with insulin resistance. Since adiponectin also controls some of the gene involved in FFA oxidation (Yamauchi *et al.*, 2001, Thamer *et al.*, 2002), dysregulation of adipose tissue endocrine function by oxidative stress can promote intramyocellular lipid accumulation and subsequent insulin resistance as well as promote macrophage-to-foam cell transformation, hence atherosclerosis (Ouchi *et al.*, 2001). These direct and indirect mechanisms of ROS action make it unsurprising why ROS have been implicated in the development of multiple forms of insulin resistance (Houstis *et al.*, 2006) and in the pathogenesis of hypertension and CVD (Ohara *et al.*, 1993; Ceriello and Motz, 2004; De Champlain *et al.*, 2004).

In addition to this, ROS have been implicated in impaired insulin secretion from pancreatic β-cells (Piro *et al.*, 2002; Newsholme *et al.*, 2009) and glucose transport and uptake into skeletal muscle (Bitar *et al.*, 2005) and adipose tissue (Rudich *et al.*, 1998; Tirosh *et al.*, 1999). ROS are also though to induce vascular damage via increased lipid peroxidation in macrophages, lipoproteins and cell walls leading to endothelium dysfunction, hypertension and CVD (see Keidar *et al.*, 2004), a mechanism that could in turn affect insulin's glucoregulatory functions by opposing the latter's vascular action. This latter mechanism, in particular, has led to the development of the common soil hypothesis which places oxidative stress at the centre of the insulin resistance-endothelium dysfunction link (Ceriello and Motz, 2004).

Oxidative stress can also influence insulin actions indirectly via its effects on lipid metabolism. To illustrate, apolipoprotein-E is a protein expressed in a variety of tissues involved in lipid metabolism such as macrophages, liver, muscle and adipose tissue. Its function consists of promoting the uptake of lipids from TG-rich lipoproteins and their subsequent influx into the above mentioned cells. In obesity, there appears to be a reduction in adipocytes apolipoprotein-E expression (Espiritu and Mazzone, 2008). This reduction hinders the adipocytes' buffering capacity leading to a shift in lipid storage from adipocytes

to the liver and skeletal muscle. Interestingly, antioxidants have been shown to reverse this effect by upregulating adipocyte apolipoprotein-E expression (Espiritu and Mazzne, 2008). Moreover, antioxidants that act via blockade of angiotensin-II and NADPH oxidase in adipose tissue have been shown to reduce ROS generation from fat-overloaded adipose tissue, implying an important role for antioxidants and angiotensin-II inhibitors in the prevention of atherosclerosis and CVD in the metabolic syndrome (Kurata *et al.*, 2006). Likewise, treatment with NADPH oxidase inhibitors has been demonstrated to diminish TNFα expression, improve adiponectin expression in white adipose tissue, and reduce subsequent hyperinsulinaemia, hyperglycaemia, hypertriglyceridaemia and hepatic steatosis (Furukawa *et al.*, 2004). Dietary approaches aimed at increasing antioxidant intake in the overweight and obese population have also been shown to successfully lower blood pressure by counteracting dyslipidaemia –induced oxidative stress (Lopes *et al.*, 2003). Together the above evidence, confirms the importance of maintaining redox balance in overweight and obesity and the need for adequate antioxidant intake to prevent oxidative stress-induced complications.

1.10. Polyphenols

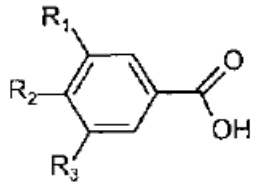
1.10.1 Definition

Polyphenols are a group of antioxidants characterised by the presence of several hydroxyl groups on an aromatic ring (Manach *et al.*, 2004). Their importance stems from their abundance in the diet, their antioxidant properties and ability to regulate various biological/biochemical processes (Manach *et al.*, 2004). Over the last decade, polyphenols have been implicated in the prevention of a number of oxidative-related diseases including CVD, hypertension and diabetes. These antioxidants occur largely in plant and plant-derived products and are extensively involved in plant defence mechanisms (Manach *et al.*, 2004). They are often categorized into 4 groups depending on the number of phenol rings embodied in their structure and the elements that bind these rings together (Manach *et al.*, 2004). Distinction is hence made between phenolic acids, flavonoids, stilbenes and lignans (Scalbert and Williamson, 2000, Manach *et al.*, 2004). So far, thousands of polyphenols have been identified in the diet (DeLogeril and Salen, 2007) and the average dietary intake of these plant-products is estimated to be in the range of 1g/ d (Scalbert and Williamson, 2000; Saura-Calixto and Goñi, 2006). Nevertheless, the maximum plasma concentration of polyphenols rarely exceeds 1μM following ingestion of 10-100 mg of a single phenolic compound (Scalbert and Williamson, 2000). As a consequence, only a minority of these compounds are of therapeutical value. In fact, the structural diversity of polyphenols implies differences in bioavailability and subsequent biological activity of these compounds which means that the most abundant polyphenols in the diet are not in effect the most biologically active (Scalbert and Williamson, 2000, Manach e al, 2004). It is, thus, empirical to differentiate between the different structures, bioavailabilities and consequent biological activities of these compounds in order to identify the most relevant polyphenols to health.

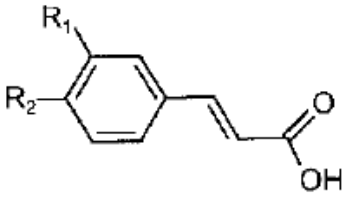
1.10.2 Main classes

1.10.2.a Phenolic acid

Phenolic acids are deemed to be the most basic class of polyphenols because they only contain one phenol ring (Bagchi and Preuss, 2007). Phenolic acids could be classified into derivatives of hydroxybenzoic acid and hydroxycinnamic acids (Manach *et al.*, 2004) as a function of the degree of hydroxylation and methylation of the phenol ring (Bagchi and Preuss, 2007) (see, Figure 1.13).



Hydroxybenzoic acids



Hydroxycinnamic acids

Figure 1.13 Basic chemical structure of hydroxybenzoic and hydoxycinnamic acids showing the single phenol ring and different degrees of methylation (Manach *et al.*, 2004).

Hydroxybenzoic acids are less commonly found in the diet than hydroxycinnamic acids, although both phenolic acids can exist in a free or an esterified form in the diet (Manach *et al.*, 2004). The most representative monomer of the hydroxybenzoic acid class is gallic acid (GA) (Manach *et al.*, 2004). Other examples include ellagic acid, ellagitannins; which are dimers or polymers of GA, respectively (Manach *et al.*, 2004), and gallotannins. Ellagitannins and gallotannins are also called hydrolysable tannins (Manach *et al.*, 2004). GA is found predominantly in tea (Tomas-Barberan and Clifford, 2000) while ellagic acid is present in pomegranate and certain varieties of berries (Amakura *et al.*, 2000). Cloudberry, raspberry, rose hip, strawberry, and sea buckthorn, on the other hand, are important dietary sources of ellagitannins (Koponen *et al.*, 2007). In general, hydroxybenzoic acids, with the exception of GA and ellagic acid (Tomas-Barberan and Clifford, 2000), do not contribute largely to total polyphenol intake and their dietary burden does not exceed 5mg/d (Clifford and Scalbert, 2000).

Hydroxycinnamic acids include caffeic, ferulic, sinapic and p-coumaric acids (Clifford, 2000). These hydroxycinnamic acids rarely exist in their free form and are almost always esterified with other compounds yielding a range of caffeoyl, feruloyl and other trans-cinnamic esters (Scalbert and Williamson, 2000). When the latter esters combine with quinic acids, they produce caffeoylquinic (CQA), feruloylquinic or coumaroylquinic acids, which collectively are known as chlorogenic acids (Clifford, 1999). 5-O-CQA is the most renowned caffeoylquinic ester found in the diet (Clifford, 1999). It is present in high concentrations in green coffee beans (Suzuki *et al.*, 2002) and other food products (see Table 7.6, p. 305) and is thus often referred to in the literature as chlorogenic acid (CGA). To avoid confusion, the term chlorogenic acid will be used in the present thesis as synonymous

to the total CGA isomers found in a food product while the most predominant CGA will be referred to by its chemical name; 5-O-caffeoylquinic acid. CGA, particularly 5-CQAs, are predominantly present in coffee, blueberries, apples and ciders (Clifford, 1999) with coffee being the chief dietary source of 5-CQA supplying 50-150mg 5-CQA per 200ml instant brew (2% w/v) (Clifford, 1999). Based on the dietary consumption patterns from British National Diet and Nutrition Survey (NDNS) (2002), coffee alongside apples and pears could be considered amongst the most important sources of hydroxycinnamates in the average British diet.

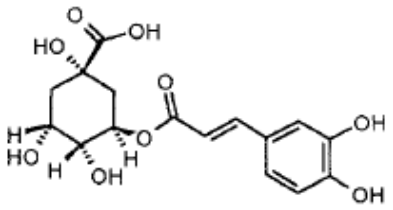
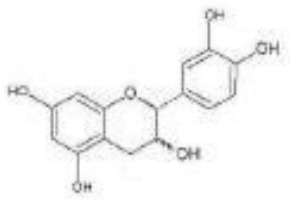


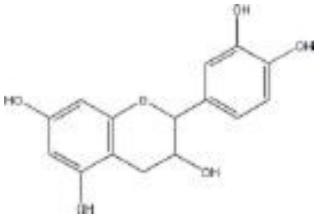
Figure 1.14 Chemical structure of 5-O-caffeoylquinic acid (Scalbert and Williamson, 2000).

1.10.2.b Flavonoids

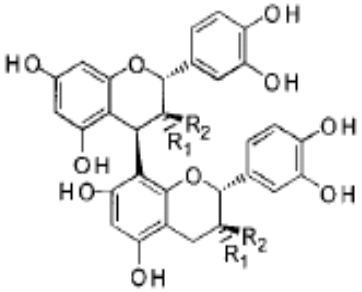
Flavonoids are phenolic compounds with 2 aromatic rings (A and B) that are linked together by an oxygenated heterocycle consisting of 3 carbon atoms (ring C) (Manach *et al.*, 2004). They could be classified into 6 subclasses; flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (catechins and proanthocyanidins), depending on the characteristics of ring C (Manach *et al.*, 2004). Flavan-3-ols are found primarily in cocoa, dark chocolate, green tea, apricots, black and red grapes and their products (USDA, 2007a). They exist as monomers (Catechin, epicatechin, epicatechin gallate, gallocatechin and epigallocatechin) or proanthocyanidins (Figure 1.15). Catechin and epicatechin are the main monomers found in cocoa and dark chocolate, while epicatechin gallate, gallocatechin, epigallocatechin are the main monomers in grapes and tea (Arts *et al.*, 2000).



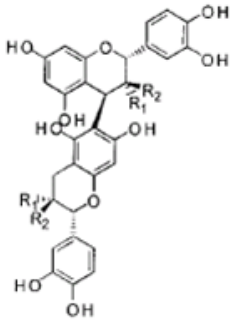
epicatechin



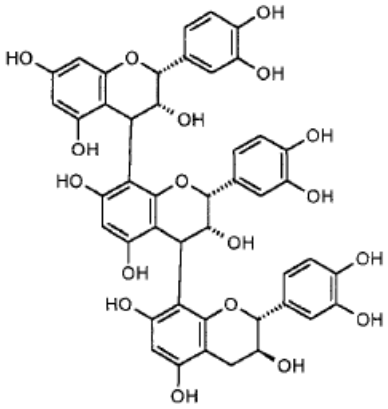
catechin



Procyanidin dimer (4β-6)



Procyanidin dimer (4β-6)



Procyanidin trimer (4β-8)

Figure 1.15 Chemical structure of epicatechin, catechin and procyanidin dimers and trimers (Hammerstone, 2000; Scalbert and Williamson, 2000; Ariefdjohan and Savaiano, 2005).

Proanthocyanidins are dimers, oligomers and polymers of catechins (Bagchi and Preuss, 2007) bound together by links between C4 and C8 (or C6) (Hammerstone *et al.*, 2000, Manach *et al.*, 2004) (Figure 1.15). They are also referred to as condensed tannins because they possess the ability to precipitate proteins from aqueous solution (Bagchi and Preuss, 2007). More precisely, proanthocyanidins form complexes with salivary protein which then account for the astringent taste of chocolate and certain fruits (grapes, peaches, kakis, apples,

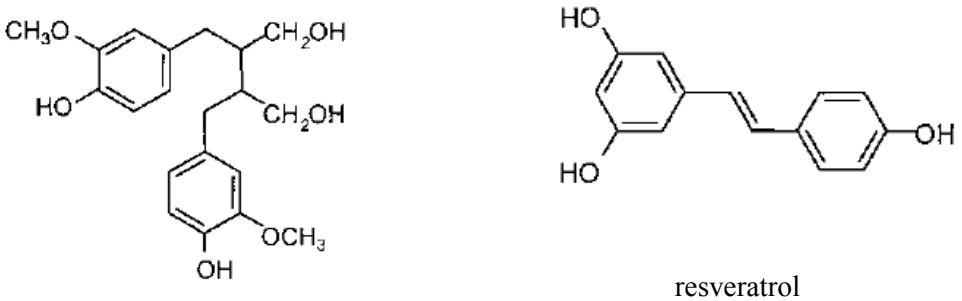
pears, berries) and beverages (Manach *et al.*, 2004). Chocolate constitutes the major source of proanthocyanidins, although the proanthocyanidins content of certain varieties of apples, including Granny Smith and Red Delicious apples could exceed that of chocolate on mg per portion basis (Hammerstone *et al.*, 2000).

1.10.2.c Lignans

Lignans are dimeric compounds consisting of two phenylpropane units (Manach *et al.*, 2004, D'Archivo *et al.*, 2007) bound by the central carbon (C8) of their side chains (Umezawa, 2003) (Figure 1.16). They are found in lignified tissues, seeds and roots (Lamblin *et al.*, 2008). The main source includes flaxseed and flaxseed oils, although other dietary sources such as cereals, oilseeds and nuts have recently been identified (Smeds *et al.*, 2007). They are transformed in the intestines to mammalian lignans (Smeds *et al.*, 2007) which posses oestrogen agonist and antagonist properties (Scalbert and Williamson, 2000). Lignans have thus recently been implicated in cancer prevention (Lamblin *et al.*, 2008).

1.10.2.d Stilbenes

Stilbenes are the least common polyphenols in the diet. The main representative is resveratrol, a stilbene found in wine (Figure 1.16).



lignan

resveratrol

Figure 1.16 Chemical structure of lignans showing the two phenylpropane units and the main stilbene, resveratrol (Manach *et al.*, 2004).

1.10.3 Importance of studying hydroxycinnamic acid and flavonoids

The nutritional/biological importance of hydroxybenzoic acids, lignans and stilbenes is restricted by the relatively low prevalence of these compounds in the diet. Consequently protective effects cannot be achieved though dietary intake (Manach *et al.*, 2004). Phenolic acids (hydroxycinnamic acids) (Manach *et al.*, 2004) and flavonoids, on the other hand, are widely distributed in the diet (Scalbert and Williamson, 2000) and could, hence, play an important role in preventive nutrition which aims at promoting health and preventing diseases.

1.10.3.a Chocolate and coffee: Contribution to polyphenol and antioxidant intake in the western diet

Cocoa and coffee are examples of food products rich in flavonoids and hydroxycinnamic acids, respectively. They are also amongst the most widely consumed food commodities worldwide. In the past few years, world cocoa consumption has witnessed a continuous annual growth of 2.9% (ICCO, 2008), while the average per capita world consumption of coffee has increased from 66153 in 1980s to 81003 in 2000-2003 (ICO, 2004) with more than two-thirds of the world's population consuming coffee (ICO, 2006). Because of their popularity, both cocoa and coffee possess the potential to play a central role in promoting health. Consequently minor changes in their processing methods could have major health implications.

Chocolate is an important purveyor of dietary antioxidants both in Europe and US. In the US, chocolate is estimated to be the third dietary source of polyphenols after coffee and tea providing on average 100mg phenolics per day (Vinson *et al.*, 2006). In the Dutch population, on the other hand, chocolate has been shown to supply 20% of total catechin intake compared to tea which provides 55% of dietary catechins (Arts *et al.*, 1999). In the UK, no studies have as yet estimated the average polyphenol intake in the British diet making it difficult to elaborate on the potential contribution of these products to polyphenol intake. Nonetheless, as the annual UK and world consumption of chocolate continues to rise steadily (DEFRA, 2010, ICCO, 2007) and with increased consumer concern over health and nutrition and higher demand for high quality chocolate (ICCO, 2007), the need for improving current commercial processing methods is becoming ever more important. In a survey commissioned by Barry Callebaut and conducted by IPSOS in 2006, 1 in 3 European consumers expressed their wishes for healthy chocolate to become commercially more available (Barry Callebaut, 2007). This when combined with chocolate's potential effects on

health highlights a wide market for functional chocolate products with important implications to public health.

Depending on consumption levels, coffee could also act as a key contributor to total antioxidant intake. In fact, in the Spanish and Mediterranean diet, coffee has been estimated to provide up to 66% of total antioxidants intake, followed by red wine (16%), fruit juices (6%), beer (4%) and finally tea (3%) (Pulido *et al.*, 2003, Svilaas *et al.*, 2004). In the UK, coffee is the second most widely consumed beverage after tea. Coffee is produced from green coffee beans that are subjected to different degrees of roasting. Since green coffee beans are rich in CGA, suggestions have been made to extend the use of GCBE from the more traditional cosmetic products market (Farah *et al.*, 2008a) to new coffee formulations with functional properties (Oka, 2007). In fact, Nestlé have recently launched a new coffee formulation, the Nescafé Green Blend, which is made out of 35% unroasted coffee beans and which is claimed to provide 160mg polyphenols per 200ml.

1.10.4 Polyphenols in cocoa and dark chocolate

The most ubiquitous subclass of polyphenols found in cocoa and DC are the flavonoids. These compounds are present in cocoa and chocolate in a unique combination of monomeric catechins and epicatechins, on the one hand, and polymeric procyanidins, on the other hand (Hammerstone *et al.*, 1999). This combination of low and high molecular weight polyphenols confers several advantages to cocoa and DC. These advantages arise from the varying degree of absorption and metabolism, hence bioavailability, of these flavonoids which subsequently determines their *in vivo* biological activities, as will be discussed later.

Overall, the most prevalent low molecular weight flavonoids in cocoa and DC are (-)-epicatechin and (+)-catechin (Osakabe *et al.*, 1998) which alongside other low molecular dimers and trimers form 30% of total procyanidins in chocolate (Gu *et al.*, 2006). Cocoa and DC also contain high levels of dimer to hexamer procyanidins, these procyanidins being characteristic of cocoa and chocolate products (Adamson *et al.*, 1999, Natsume *et al.*, 2000). Surprisingly, cocoa have also been shown to contain more polyphenols and flavonoids per serving than red wine, green tea or black tea (see Figure 1.17).

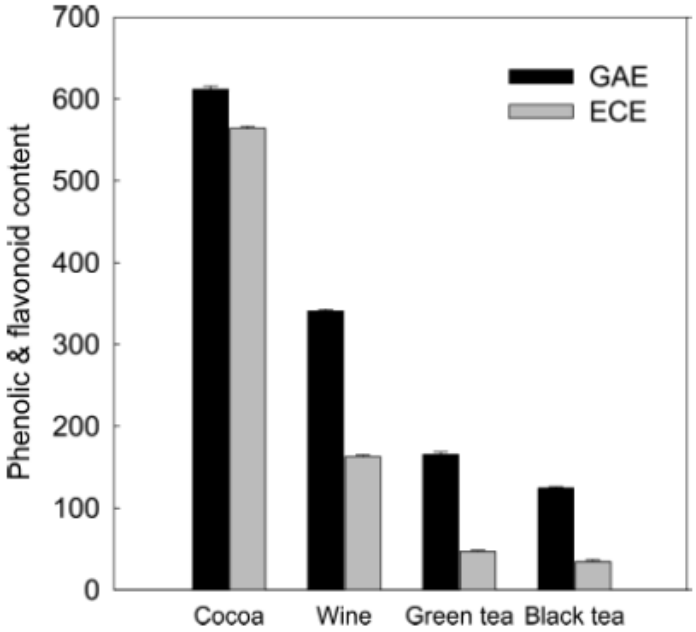


Figure 1.17 Total phenolic content and flavonoid content of cocoa, red wine, green and black tea expressed as milligrams of gallic acid equivalents and epicatechin equivalents per serving, respectively. (Lee *et al.*, 2003). GAE, gallic acid equivalents; ECE, epicatechin equivalents.

1.10.4.a Effect of processing on polyphenol content

Generally, the polyphenol content of cocoa or DC differs depending on environmental factors and processing methods. For instance, the procyanidin content of cacao liquors depends largely on cacao liquor variety and tends to decrease with increased fermentation time (Counet *et al.*, 2004). The degree of fermentation also appears to be an important determinant of procyanidin content, with fine varieties of cocoa beans with short fermentation time exhibiting the highest procyanidin content (Counet *et al.*, 2004). This is in contrast to the aromatic compounds which tend to correlate positively with the length of bean fermentation and are thus inversely related to procyanidin content (Counet *et al.*, 2004). In addition to fermentation, roasting have been implicated in the epimerization of (-)-epicatechin to (+)-catechin (Caligiani *et al.*, 2007) and the loss of up to 90% of phenolic content (Jalil and Ismail, 2008).

Differences in the proportion of polyphenols between various cocoa products have also been attributed to variation in the manufacturing processes of these products. Chocolate is generally produced from a combination of cocoa liquor, sugar and cocoa butter. Consequently, chocolate has similar proportions of catechins and proanthocyanidins to cacao liquor (Natsume *et al.*, 2000). Conversely, cocoa powder undergoes alkalisation or dutching,

a process known to alter the antioxidant properties and procyanidin content of cocoa (Gu *et al.*, 2006). As a result cocoa powder differs in its composition to chocolate and cocoa liquor, with the former containing a higher ratio of flavan-3-ols to total monomer and oligomer polyphenols (Natsume *et al.*, 2000). The implications of such differences in the quantity and proportion of polyphenols amongst these products remains elusive. Nevertheless, it is generally agreed that the quantity of procyanidins is directly related to the non-fat cocoa solid content, antioxidant activity and total polyphenol content (Adamson *et al.*, 1999, Gu *et al.*, 2006, Miller *et al.*, 2006). As a result, products which contain the highest non-fat cocoa solids like cocoa powder have the highest procyanidin content and total antioxidant capacity while products with the lowest non-fat cocoa solids like milk chocolate demonstrate the lowest procyanidin content and total antioxidant capacity (Gu *et al.*, 2006). In fact, according to Arts *et al.* (2000) milk chocolate contains negligible quantities of catechin, hence the escalating interest in investigating the health properties of solid DC which contains more non-fat cocoa solids than other varieties of chocolate (Jalil and Ismail, 2008).

1.10.5 Polyphenols in green coffee beans

Green coffee beans contain 6-10% CGA on a dry matter basis (Clifford, 1999) with CQA, dicaffeoylquinic and feruloylquinic acids forming 98% of total CGA content of green coffee beans (Clifford and Staniforth, 1977 see Noirot *et al.*, 2003). Of these, the 5-CQA isomer contributes to 85% of total CGA content of green coffee beans (Clifford *et al.*, 1989, see Noirot *et al.*, 2003). The 5-CQA isomer is also the component with the highest *in vitro* and *ex vivo* anti-hydroxyl radical activity in both green and roasted coffee (Daglia *et al.*, 2004). In general, CGA content varies between and within coffee species (Noirot *et al.*, 2003). This is then reflected in the antioxidant activity of coffee species with *Robusta* green coffee beans having twice the antioxidant activity of *Arabica* green coffee beans (Richelle *et al.*, 2001).

1.10.5.a Effect of manufacturing processes on chlorogenic acid content

Roasting results in gradual reduction in CGA (DelCastillo *et al.*, 2002) and formation of chlorogenic lactones (Clifford, 2000) alongside other Maillard reaction compounds (Daglia *et al.*, 2004). In fact, for every 1% reduction in dry matter, 8-10% of CGA is lost (Clifford, 2000). Subsequently, green coffee beans have a higher CGA content than roasted beans which is then reflected in higher antioxidant activity of green coffee beans as compared to roasted coffee beans (Daglia *et al.*, 2000).

1.10.6 Bioavailability

Bioavailability is key to understanding the functions and actions of biological compounds as chemical compounds may have potent antioxidant properties *in vitro* but their biological effects *in vivo* may be restricted by poor bioavailability, including poor gut absorption or rapid metabolism (Manach *et al.*, 2004).

1.10.6.a Bioavailability of cocoa polyphenols

Cocoa and DC are rich in low molecular weight phenols like (-)-epicatechin and (+)-catechin, compounds that are considered to be highly bioactive because of their high bioavailability. The latter could be illustrated by the kinetics of (-)-epicatechin which is considered the main predictor of total polyphenol content and especially the content of procyanidin B2 and C1 (Cooper *et al.*, 2007). Epicatechin diffuses rapidly through intestinal epithelium where it forms several conjugates (Baba *et al.*, 2000) and reaches its maximum concentration in blood 2h post DC consumption (Richelle *et al.*, 1999, Rein *et al.*, 2000, Wang *et al.*, 2000) where it increases plasma antioxidant capacity (Wang *et al.*, 2000) and decreases plasma TBARS (Rein *et al.*, 2000). However, because of its basic structure, epicatechin is also rapidly eliminated and by 6h, its concentration returns to baseline alongside plasma antioxidant capacity (Wang *et al.*, 2000). Subsequently, this compound produces only a transient effect on plasma total antioxidant capacity and repeated ingestions are required to maintain its plasma levels.

Conversely, the large polymeric procyanidins are more resistant to metabolic degradation than the simpler phenols. Although the long polymeric structure and high molecular weight of procyanidins generally compromises their intestinal absorption and bioavailability (Scalbert and Williamson, 2000), cocoa and DC are unique in that they contain high levels of dimer to hexamer procyanidins (Adamson *et al.*, 1999, Natsume *et al.*, 2000). These procyanidins are more resistant to metabolic degradation and excretion than epicatechin (Rios *et al.*, 2002), but are nonetheless more readily absorbed than the high polymers of 10 catechin units (Weisburger, 2005). Subsequently, high levels of these compounds reach the intestines where they are converted by microbial flora to various metabolites including m-hydroxyphenylpropionic acid, ferulic acid, 3,4-dihydroxyphenylacetic acid, m-hydroxyphenylacetic acid, vanillic acid, and m-hydroxybenzoic acid (Rios *et al.*, 2003). Although, the exact advantage of these metabolite formation have not yet been identified, Rios *et al.* (2003) argues that they are likely to protect the gastrointestinal tract from oxidative damage and thus contribute to the antioxidant properties of cocoa and DC.

In addition to molecular weight and structure, bioavailability is also influenced by other macronutrients and food products. To illustrate, simultaneous carbohydrate ingestion has been shown to increase the bioavailability of cocoa flavanols (Schramm *et al.*, 2003). Conversely, certain studies indicate that the addition of milk to chocolate reduces its antioxidant capacity compared to DC (Serafini *et al.*, 2003), others, however, do not confirm these findings (Schroeter *et al.*, 2003). In their review, Keen *et al.* (2005) explained the latter controversies by arguing that the reduction in antioxidant capacity seen in Serafini *et al.*'s study could be due to the overall food matrix, rather than milk proteins alone.

Regardless of the role of macronutrients and milk as determinants of the bioavailability of DC polyphenols, ingestion of 80g of polyphenol-rich DC containing 164 mg epicatechin have been reported to increase plasma epicatechin concentrations to 0.7mmol/L (Richelle *et al.*, 1999) which according to Richelle *et al.* (1999) and Rice-Evans (1996) is close to the 1mmol/L range required to produce biological effects *in vivo*.

Table 1.4 provides a summary of peak plasma epicatechin concentrations achieved 2h following ingestion of different doses of polyphenol-rich DC. As observed from this table, there are large variations in maximum plasma epicatechin concentrations between the different studies. These variations possibly reflect large inter-subject variations in the metabolism of DC polyphenols.

Table 1.4 Peak plasma concentrations of epicatechin at 2h following ingestion of polyphenol-rich dark chocolate or cocoa.

Study	Phenolic compound	Number of participants	Ingested dose	Baseline epicatechin	Maximum concentration
Richelle 1999	epicatechin	8	40g DC (892mg polyphenols 82mg epicatechin)	undetectable	300nmol/L
			80g DC (1783 mg polyphenols 164 mg epicatechin)		700nmol/L
Rein 2000	epicatechin	10 (4 males and 6 females)	80g DC 557 mg procyanidins, 137 mg epicatechin	22± 4 nmol/L)	257 ± 66 nmol/L
Wang 2000	epicatechin	13	27g DC (186 mg procyanidins 46 mg epicatechin)	2±2	133 ± 27
		13	53g DC	4±2	258 ± 29
		10	80g DC	4±3	355 ± 49
Wan 2001	epicatechin	6 (3 males and 3 females	22 g cocoa powder and 16 g dark chocolate 466 mg procyanidins	3.3 ± 0.1 nmol/L	36.2 ± 8.2 nmol/L
Engler 2004	epicatechin	11	46g DC	undetectable	200- 227 nmol/L
		2	120g DC		

1.10.6.b Bioavailability of green coffee beans polyphenols

The majority of the studies relating to CGA metabolism have examined the bioavailability of CGA from pure CGA supplements or coffee. These studies however do not provide a true reflection of the bioavailability of CGA from GCBE, since they do not take in account the effect of food matrices or differences in composition between GCBE and roasted coffee (Olthof *et al.*, 2001a-b). Only two studies have directly examined the bioavailability of CGA from GCBE. According to these studies, CGA is highly bioavailable with 24-33% of CGA or its related metabolites being recovered in plasma following ingestion of 0.4g GCBE (170mg (451µmol) CGAs) (Farah *et al.*, 2008a-b). CGA was also found to persist in plasma for up to 8h following ingestion of GCBE (Farah *et al.* 2008a). In fact, CGA has even been identified intact in urine, saliva, digestive fluids and plasma of fasting subjects (Farah *et al.*, 2006). All of this evidence is consistent with a slow elimination of CGA and could also possibly imply that storage and recycling of CGA might occur through digestive fluids (Farah *et al.*, 2008a-b). This could be further illustrated in the unique pharmacokinetic properties of CGA. Compared to other polyphenols, consumption of CGA from GCBE has been shown to produce two chromatogram peaks, one achieved between 0.5-1h and the other at 8h (Farah *et al.*, 2008b). Farah *et al.* (2008a) explained the presence of these two maximum concentration values by arguing that the first peak is an indication of early absorption of CGA from the stomach and jejunum while the second peak suggest a late absorption through the colon. Farah *et al.* (2008a) also observed a preferential absorption of 5-CQA as reflected by the higher plasma levels of this isomer compared to other CGA isomers. In fact, 5-CQA exhibited an apparent bioavailability of 33±17% which is similar to the bioavailability of 5-CQA from pure 5-CQA supplements (Olthof *et al.*, 2001a). In addition to this, Farah *et al.* (2008a) detected a number of metabolites that were unique to the consumption of GCBE but not roasted coffee and which include: ferulic, isoferulic and p-coumaric (Figure 1.18). A large inter-subject variability in the bioavailability of CGA was also observed, with plasma CGA recovery ranging from 7.8-72.1% (Farah *et al.*, 2008a). According to Farah *et al.* (2008a), such differences in bioavailability are likely to be the product of genetic polymorphisms in CGA metabolism and may subsequently result in differences in biological outcome of GCBE consumption.

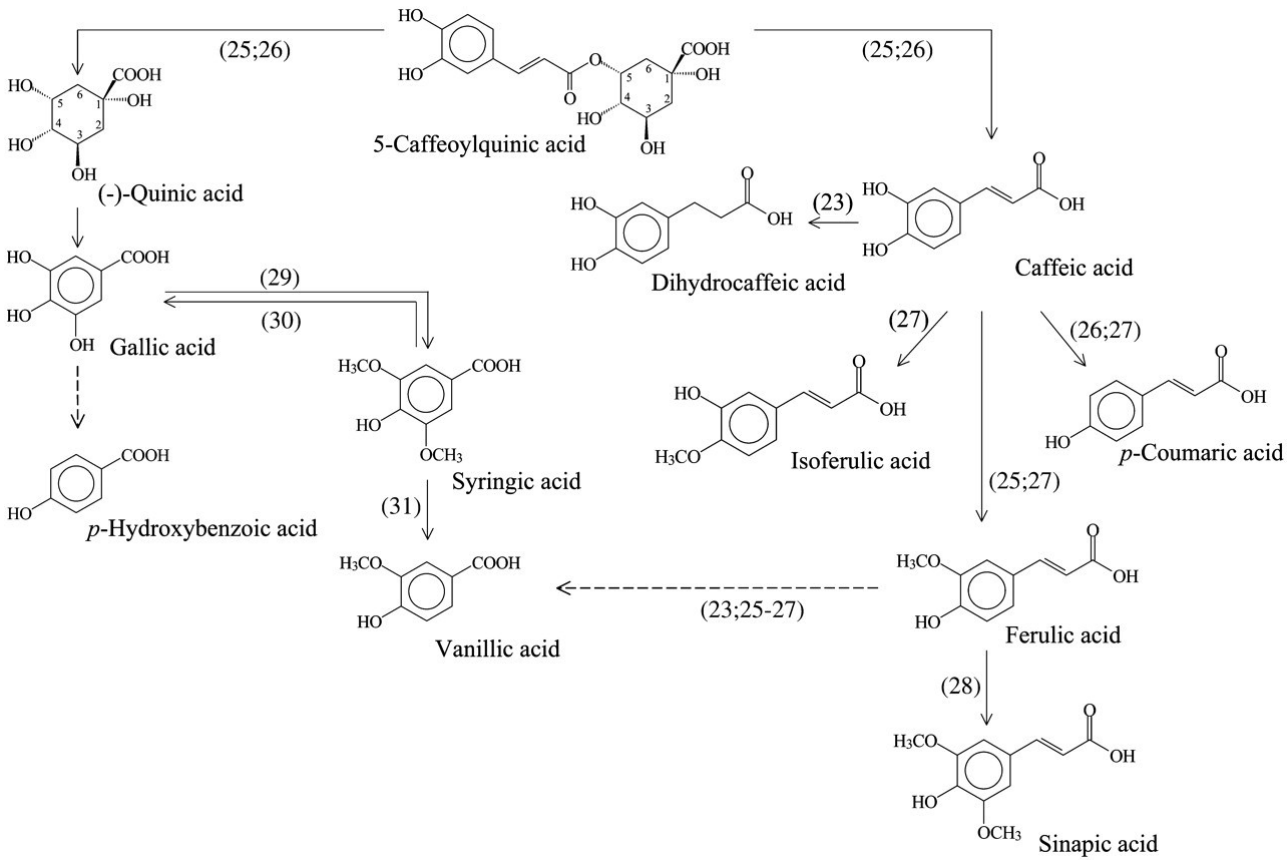


Figure 1.18 The metabolism of 5-Caffeoylquinic acid (Farah *et al.*, 2008a). Note the metabolites: ferulic, isoferulic and p-coumaric which are specific to chlorogenic acid-rich green coffee bean extract. Also note the metabolites: ferulic acid, isoferulic acid and gallic acid which potentially can contribute to the antioxidant properties of green coffee bean extract

1.11. Actions of polyphenols

1.11.1 Polyphenols as antioxidants

1.11.1.a Antioxidants properties of cocoa and dark chocolate

Cocoa was first shown to produce less H_2O_2 than tea and coffee (Long *et al.*, 1999). This ability to resist oxidation led some other to suggest that cocoa and DC may contain certain compounds that prevented them from being oxidised (Osakabe *et al.*, 1998, long *et al.*, 1999). As mentioned earlier, cocoa contains more polyphenols and flavonoids per serving than red wine, green tea or black tea (Lee *et al.*, 2003). These polyphenols confer potent antioxidant properties to cocoa and DC with cocoa possessing the highest antioxidant activity amongst red wine, green, black (Lee *et al.*, 2003) and herbal tea (Richelle *et al.*, 2001). In fact to quote Lee *et al.* (2003): " on a per-serving basis, [the antioxidant capacity of cocoa is] 4-5 times stronger than that of black tea, 2-3 times stronger than green tea, and almost 2 times stronger than red wine".

In a study conducted by Record *et al.* (2003), consumption of high procyanidin chocolate containing 200mg of flavanols and related procyanidins was shown to reduce ROS production in faecal water. However, faecal water antioxidant capacity as measured by Trolox equivalent antioxidant capacity (TOC) and ferric-reducing capacity of plasma (FRAP) was not altered significantly (Record *et al.*, 2003). Moreover, Record *et al.* (2003) observed a reduction in free radical production in faecal water even with the low-procyanidin chocolate (<10mg polyphenols), which possibly indicated that components, other than chocolate procyanidins accounted for chocolate's inhibitory effect on ROS generation. Despite these findings, several researchers who have used more robust methods for measuring antioxidant capacity, i.e oxygen radical absorbance capacity (ORAC) and total antioxidant capacity assays (AOC), have demonstrated that the procyanidin content of cocoa and chocolate is strongly and positively correlated with total antioxidant capacity of these products (Adamson *et al.*, 1999, Gu *et al.*, 2006, Miller *et al.*, 2006). In particular, epicatechin oligomers, especially tetramers, have been shown to be more powerful inhibitors of oxidation and nitration reactions than the monomeric epicatechin (Arteel and Sies, 1999, Arteel *et al.*, 2000). In fact even when expressed in relative monomeric efficiency units, oligomers, especially those with three or more epicatechin or catechin units, appear to inhibit oxidation reactions to a greater extent than monomers (Counet and Collin, 2003).

In addition to inhibiting peroxynitrite reactions, cocoa and cocoa liquor can enhance antioxidant capacity by inhibiting H_2O_2 and O_2. production (Sanbongi *et al.*, 1997) possibly via upregulation of the activity of superoxide dismutase and catalase (Ramiro-Puig *et al.*, 2007). Cacao liquor polyphenols have also been reported to inhibit the formation of lipid peroxides in plasma independently of their effect on α-tocopherol levels (Yamagishi *et al.*, 2001), although a sparring effect on α-tocopherol as well as β-carotene have also been reported (Baba *et al.*, 2000; Lotito and Fraga, 2000).

In humans, consumption of polyphenol-rich cocoa and chocolate raises plasma antioxidant capacity, as measured by ORAC (Wan *et al.*, 2001), antioxidant chain-breaking (TRAP) and FRAP assays (Flammer *et al.*, 2007). Prevention of lipid peroxidation, as evident by the fall in plasma TBARS and isoprostanes levels and urinary isoprostanes, have also been observed following ingestion of cocoa and DC by some authors (Wang *et al.*,2000, Flammer *et al.*, 2007) but not others (Engler *et al.*, 2004). It, thus, follows that cocoa and DC possess powerful antioxidants properties *in vitro* and *in vivo*. These antioxidants properties have been attributed mainly to the procyanidins content of cocoa and DC (Counet *et al.*, 2004). However, recent evidence appears to suggest that as well as possessing antioxidant properties, procyanidins could equally act as pro-oxidants when present in their purified form. Accordingly, procyanidin B2 (epicatechin-(4β8)-epicatechin), have been shown to protect DNA against oxidative damage caused by H_2O_2 but to induce H_2O_2 production at high concentrations (Sakano *et al.*, 2005). Similar findings have been made in relation to cocoa butter (Vinson *et al.*, 2006). When found on its own, cocoa butter acts as a mild pro-oxidant both *ex vivo* and *in vivo* because of its high unsaturated fatty acids content (Vinson *et al.*, 2006). However, once combined with polyphenols, as is the case in a chocolate bar, this pro-oxidant effect appears to be attenuated to a certain extent by the presence of polyphenols (Vinson *et al.*, 2006). This explains why when comparing defatted cocoa to non-defatted cocoa with a similar polyphenol content, defatted cocoa presents with a higher antioxidant activity because it lacks the pro-oxidant properties of cocoa butter (Vinson *et al.*, 2006). Since cocoa also contains compounds other than polyphenols which express antioxidant properties including: N-phenylpropenoyl-L-amino acids; N-[3'4-dihydroxy-(E)-cinnamoyl]-L-tryptophan, N-[4-hydroxy-(E)-cinnamoyl]-L-tryptophan and N-[4'hydroxy-3'-methoxy-(E)-cinnamoyl]-L-tyrosine (Stark *et al.*, 2006), it could be postulated that the antioxidant properties of cocoa and DC arise as a result of a complex interplay between the various constituents of these food products. Consequently, consumption of food products naturally rich in polyphenols, such as cocoa or DC, is likely to confer greater health benefits than the

use of purified procyanidin supplements, whose metabolic efficacy and safety remains questionable.

1.11.1.b Antioxidants properties of chlorogenic acid and green coffee beans

Several studies have reported *in vitro* antioxidant activity of CGA. Accordingly, CGA and its precursors, caffeic and ferulic acid, have been shown to increase LDL resistance to lipid peroxidation (Castelluccio *et al.*, 1995; Chen and Ho, 1997) and to prevent oxidative damage of apolipoprotein-B100 (Castelluccio *et al.*, 1995). CGA has also been reported to possess greater 1,1 –diphenyl-2-picilhydrazyl (DPPH) scavenging activity than α-tocopherol (Chen and Ho, 1997). However, as yet, no study has reported *in vivo* antioxidant activity of CGA from CGBE. According to Olthof *et al.* (2003), *in vivo* antioxidant activity of CGA could be restricted by the relative high metabolic degradation of this compound in liver and colon. Furthermore, hippuric acid, the main metabolite of CGA, lacks antioxidant activity because of the absence of a hydroxyl group in its structure (Olthof *et al.*, 2003). However, absorption of intact CGA has been reported by Olthof *et al.* (2001a), Monteiro *et al.* (2007) and Farah *et al.* (2008a-b). In fact according to Olthof *et al.* (2001a) 33% of CGA is absorbed while only a minor fraction is hydrolysed in the digestive tract to caffeic acid, the latter being a potent antioxidant. The remaining two-thirds reaches the colon where it is extensively metabolised by the microflora (Olthof *et al.*, 2003) to yield different microbial metabolites such as: m-coumaric acid and derivatives of phenylpropionic, benzoic and hippuric acids, which together total for over 57% of CGA intake (Gonthier *et al.*, 2003). Generally, the advantage of metabolite formation lays in their long half-lives (Setchell *et al.*, 2002), hence their ability to maintain plasma antioxidant capacity over prolonged periods. Moreover, certain metabolites exert more potent biological effects compared to their parent compounds, as observed with equol, the main microbial metabolite formed from the isoflavones diadzin and diadzein (Setchell *et al.*, 2002). Although the antioxidant contribution of CGA metabolites remains to be elucidated, it could be postulated that the antioxidant properties of GCBE rich in CGA are likely to arise from a combination of the pharmacokinetical properties of CGA (Section 1.10.6.b) and possibly its microbial metabolites (refer again to Figure 1.18).

1.11.2 Polyphenols, carbohydrate metabolism and diabetes

1.11.2.a Effect of cocoa and dark chocolate

Cocoa and DC flavonoids act on different stages of carbohydrate metabolism. For example in the gastrointestinal tract, cocoa polyphenols have been shown to downregulate α-amylase,

an enzyme involved in the breakdown of carbohydrate, with the larger polymers being more potent than the simpler phenols (Quesada *et al.*, 1996). These findings are consistent with the findings of McDougall *et al.* (2005) who identified proanthocyanidins as the strongest *in vitro* inhibitors of salivary and pancreatic α-amylase activity. Other mechanisms that affect carbohydrate metabolism involve inhibition of facilitated intestinal glucose transporter, GLUT2 (Chen *et al.*, 2007) and sodium-dependent glucose transporters namely GLUT1 and sodium-dependent glucose transporter-1 (Cermak *et al.*, 2004). Both these inhibitory actions occur via direct interactions between polyphenols such as epicatechin gallate or quercetin-3-O-glucoside and the respective transporters, which results in slower carbohydrate breakdown and delayed absorption, hence improved glycaemic response (Cermak *et al.*, 2004; Chen *et al.*, 2007).

In diabetic rats, consumption of a cocoa extract, containing 285.6mg polyphenols per gram, has been shown to reduce glucose concentrations in blood (Ruzaidi *et al.*, 2005) while consumption of cacao liquor procyanidins have been reported to reduce both blood glucose and fructosamine levels in diabetic obese mice (Tomaru *et al.*, 2007). In humans, consumption of DC improves insulin resistance, insulin sensitivity and fasting glucose levels in healthy (Grassi *et al.*, 2005a), hypertensives (Grassi *et al.*, 2005b), glucose-intolerant hypertensives (Grassi *et al.*, 2008), and obese subjects (Davison *et al.*, 2008). In particular, Grassi *et al.* (2005a) reported a significant improvement in HOMA-IR and quantitative insulin sensitivity check index (QUICKI) following consumption of 100g of DC containing 500mg polyphenols by healthy normotensive volunteers for 15days, but not following intake of 100g of polyphenol-deficient white chocolate. In a later study, Grassi *et al.* (2005b) observed a significant improvement in HOMA-IR, QUICKI, oral glucose tolerance test in patients with essential hypertension following 15days of 100g DC. Most recently, Grassi *et al.* (2008) reported improvement in HOMA-IR, QUICKI, insulin-sensitivity index (ISI) as well as β-cell function as calculated by corrected insulin response (CIR120) in hypertensive subjects with impaired glucose tolerance following 15days of 100g DC. Most importantly in this study, changes in ISI, QUICKI, CIR120 were correlated with improvement in flow-mediated dilation, thereby confirming previous observation that a relation exists between insulin resistance and endothelium function (see section 1.5). Changes in ISI were also correlated with changes in blood pressure.

Such improvements in glucose metabolism could be partially attributed to decreased carbohydrate digestion and delayed absorption. However, improved vascular function is

possibly by far the main mechanism by which DC flavonoids exert their beneficial effect on glucose metabolism (Grassi *et al.*, 2008), particularly when considering that improved endothelium function may increase substrate delivery to target tissue (Section 1.5.2). Flavonoids could also improve insulin resistance by preventing serine-phosphorylation associated with increased circulating FFA levels as discussed earlier.

Currently, inconsistencies still exist regarding the treatment duration and dose required to achieve a glucose-lowering effect (see Table 1.5). For example in their pilot study Stote and colleagues (2007) failed to show any significant improvement in glucose, HOMA-IR and ISI following 5days of twice daily consumption of procyanidin-rich cocoa beverage containing 22 to 900mg procyanidins by insulin-resistant men and women. In contrast, Davison *et al.* (2008) showed reduced insulin resistance following consumption of a cocoa beverage containing 902mg flavanols twice daily for 12 weeks in overweight and obese subjects. Taubert *et al.* (2007), on the other hand, failed to show any improvement in glucose or insulin levels following 18 weeks of daily ingestion of 6.3g of DC with 30mg polyphenols. Together these studies might suggest that a longer duration and a higher dose of polyphenols could be required to achieve a significant reduction in glucose levels. It can also be postulated that improvement in glycaemic regulation can occur without a detectable reduction in fasting glucose. This is evident in a study by Balzer *et al.* (2008) that investigated the effect of regular cocoa intake on diabetics. This study demonstrated that despite not having any effect on fasting glucose, consumption of cocoa containing 963mg and 75mg flavonols for 30 days significantly reduces the levels of haemoglobin A_{lc}, implying improved glycaemic control (Balzer *et al.*, 2008). In the latter study, flavonoid-rich cocoa was given to patients undergoing either oral or insulin anti-diabetic therapy (Balzer *et al.*, 2008). As such, it might be possible that the effect of cocoa on fasting glucose would not be detected due to the effect of treatment on fasting glucose. It could also imply that cocoa intake might be more essential in terms of reducing diabetes-related complication in this population subgroup, as evident by the improvement in endothelium function following cocoa consumption (Balzer *et al.*, 2008) and the reduction in cataract formation in diabetic rats (Osakabe *et al.*, 2004). Additionally it could be argued that Balzer *et al.* (2008) used a cocoa beverage containing 27g of carbohydrates of which 15g was sugar. Since the latter is known to induce hyperglycaemic, replacing sugared-cocoa with sugar-free cocoa might have yielded more prominent results.

Table 1.5 Summary of studies investigating the effect of polyphenol-rich dark chocolate and cocoa on glucoregulatory biomarkers. Note the overall large quantities of DC used and the great variation in polyphenols doses and treatment duration.

Study	Study Design	Population	Number of participants	Dose	Placebo	Glucoregulatory parameter	Change significant?
Mathur 2002	Non-randomised trial, subjects studied at the end of 6 weeks DC and at the end of 6weeks follow-up period	Healthy	25 (12 females and 13 males)	36.9 g DC and 30.95 g cocoa drink 651mg procyanidins	Habitual diet	Fasting glucose	No
Grassi 2005a	Randomised, crossover, 15 days	Healthy	15 (7 males and 8 females	100g DC 500mg polyphenols 88mg flavanols	90g white chocolate	OGTT, HOMA-IR, QUICKI, fasting insulin	Yes
Grassi 2005b	Randomised, crossover, 15 days	never-treated, grade I patients with essential hypertension	20 (10 males and 10 females)	100g DC 500mg polyphenols 88mg flavanols	90g white chocolate	OGTT, HOMA-IR, QUICKI, ISI, fasting insulin	Yes
Stote 2007	Randomised, parallel-group, 5 days	12 insulin resistant, 1 diabetic, 7 healthy	20 subjects (10 men, 10 women)	3 cocoa procyanidin doses 900, 400 and 200mg procyanidins taken twice daily	Control beverage 22mg procyanidins	OGTT, HOMA-IR	No
Taubert 2007	Randomised, parallel control, 18 weeks	upper-range prehypertension or stage 1 hypertension	44 (24 females, 20 males)	6.3g DC 30mg polyphenols	30g white chocolate	Fasting glucose	No
Balzer 2008	Randomised, parallel control, 30 days	Type-II diabetics	44 of which 3 dropped-out (29 females and 12 males)	18g cocoa 321 mg flavanols taken thrice daily (Total 963mg flavanols)	18g cocoa 25 mg flavanols taken thrice daily (Total 75mg flavanols)	Fasting glucose , haemoglobin A_{1C}	Significant reduction in HBA_{1C} after both doses
Davison 2008	Randomised, parallel control, 12 weeks	Overweight and obese	49 (31 females and 18 males)	cocoa drink 902 mg flavanols	Low flavanol cocoa drink	HOMA-IR	No
Grassi 2008	Randomised, crossover, 15 days	hypertensives with impaired glucose tolerance	19 (11 males, 8 females)	100g DC 500mg polyphenols	90g white chocolate	OGTT, HOMA-IR, QUICKI, ISI, CIR_{120}, fasting insulin	Yes

1.11.2.b Effect of green coffee bean extract

CGA is unique in its ability to regulate glucose homeostasis since it acts on different stages of glucose metabolism (Figure 1.19). In the intestine, CGA can either delay (Johnston *et al.*, 2003, Bassoli *et al.*, 2007) or inhibit (Welsch *et al.*, 1989) intestinal glucose uptake. In the first instance, CGA has been shown to modulate the secretion of gastrointestinal hormones such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-I (GLP-1) secretion (Johnston *et al.*, 2003). GIP is generally secreted in response to active glucose absorption through sodium-dependent glucose transporter-1 (Sirinek *et al.*, 1983). A reduction in its level implies reduced sodium-dependent glucose uptake (Sirinek *et al.*, 1983). GLP-1, on the other hand, is secreted in response to carbohydrates' presence in the distal portion of the small intestine (Enc *et al.*, 2001). It, thus, acts as an indicator of delayed carbohydrate and glucose uptake (Enc *et al.*, 2001). GLP-1 also functions as a stimulator of β-cell insulin secretion, in response to increased plasma glucose (McCullough *et al.*, 1983, McCarty, 2005) and a rise in its production levels have been postulated to counteract the adverse effect of chronic FFA exposure on β-cells in overweight and insulin resistant subjects (McCarty, 2005).

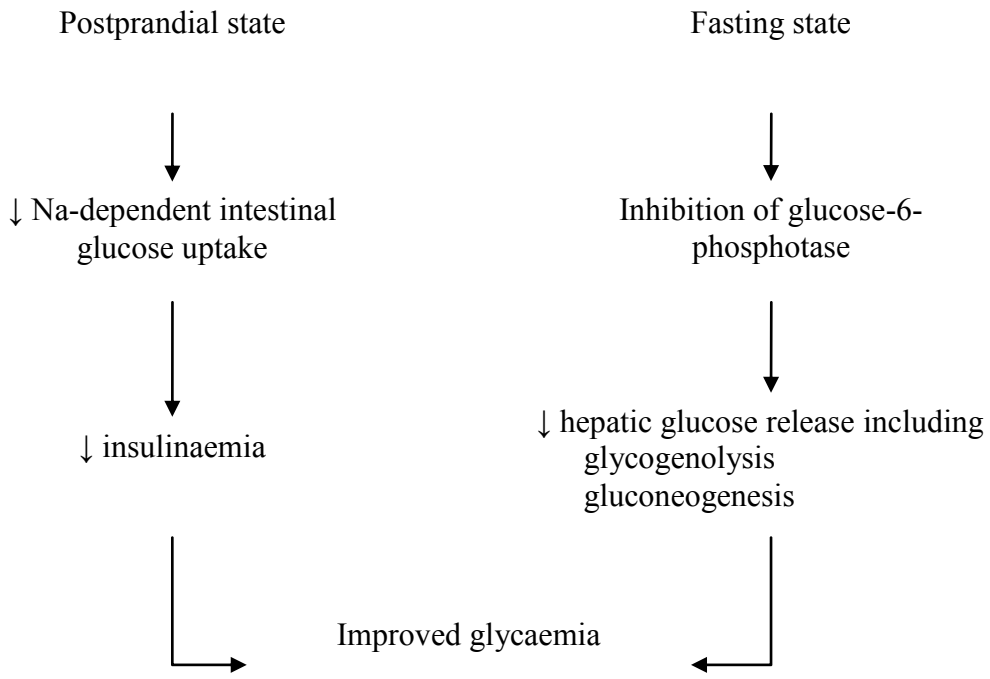


Figure 1.19 A summary of the mechanisms by which GCBE improves glycaemia (Blum *et al.*, 2007)

As for inhibition of glucose absorption, CGA have been demonstrated to reduce sodium-dependent glucose transport by 80% (Welsch *et al.*, 1989). This effect being induced by evoking the dissipation of the Na+ electrochemical gradient, which provides the driving force for active glucose uptake (Welsch *et al.*, 1989). According to McCarty (2005), these combined mechanisms of delayed and reduced glucose absorption serve to preserve pancreatic β-cells function by reducing postprandial hyperglycaemia.

In addition to its actions on the intestine, CGA can also dose-dependently inhibit gluconeogenesis and glycogenolysis in the liver by inhibiting the glucose-6-phosphate translocase system (Hemmerle *et al.*,1997; Bassoli *et al.*, 2008). Finally, in the pancreas, CGA diminishes insulin secretion from pancreatic β-cells, as demonstrated by a reduction in plasma C-peptide following consumption of CGA-rich coffee (Wu *et al.*, 2005). According to Wu *et al.* (2005), changes in C-peptide are a better indicator of insulin secretion because as well as being released from proinsulin into blood in equimolar amounts as insulin (Wahren *et al.*, 2000), C-peptide has a longer half-life than insulin (Hovorka *et al.*, 1998). This, in conjunction with the afore-mentioned evidence, confirms CGA's ability to influence glucose metabolism and improve glycaemia.

1.11.3 Polyphenols and blood pressure

1.11.3.a Effect of cocoa and dark chocolate

The first observations of an association between cocoa consumption and blood pressure came from studies on the Kuna population of Panama. The Kuna Indians were found to have low rates of CVD and hypertension despite consuming large quantities of salt (McCullough *et al.*, 2006), an effect that was explained by the large quantities of cocoa, 5 cups per day, consumed by this population (Hollenberg, 2006) and which provide over 900mg polyphenols (Bayard *et al.*, 2007). This high cocoa consumption was found to be associated with a 3-fold higher urinary nitrate: nitrite excretion in Kuna Indians compared to mainland dwellers who did not consume flavonoid-rich cocoa (Hollenberg, 2006). This led authors to suggest that activation of NO system was the underlying mechanism by which cocoa consumption reduced blood pressure (Hollenberg, 2006). This was confirmed by a later observational study that showed low mortality rates from NO-dependent conditions like CVD, cancer and diabetes among the Kuna Indians (Bayard *et al.*, 2007).

Reductions in blood pressure were also reported by short-term randomised-controlled trials. Here, Taubert *et al.* (2003) reported a 5.1mmHg and a 1.8mmHg reduction in both SBP and DBP, respectively, following consumption of 100g DC containing 500mg polyphenols by elderly patients with untreated stage-1 mild isolated systolic hypertension. This effect was achieved within 10days of DC consumption but was not sustained 2days after discontinuation (Taubert *et al.*, 2003). Reductions in SBP (Grassi *et al.*, 2005a) and DBP (Grassi *et al.*, 2005b, Grassi *et al.*, 2008, Faridi *et al.*, 2008) have also been demonstrated by other short-term studies using similar research protocol. However, these reductions differed depending on the instruments used to monitor blood pressure and the population studied. For instance, in their first study, Grassi *et al.* (2005a) observed a significant 6.4mmHg fall in SBP, but not DBP, after ingestion of 100g DC by healthy volunteers. In a later study on patients with essential hypertension, Grassi *et al.* (2005b) used a 24h-ambulatory blood pressure monitor, a more robust method of measuring blood pressure. Here both 24h SBP and DBP were reduced by –11.9±7.7 mm Hg and –8.5±5.0 mm Hg, respectively. In their latest study, Grassi *et al.* (2008) also reported a reduction in 24h SBP and DBP in glucose-intolerant hypertensive patients. However, in this study the reduction was lower than the one reported with patients suffering from hypertension only (24-h SBP, –4.52mm Hg; 24-h DBP, –4.17 mm Hg) (Grassi *et al.*, 2008). This may suggest that DC varies in its effect on different populations probably due to differences in the severity of disease and possibly due to the involvement of other pathophysiological factors in these conditions.

Differences between the effect of solid versus liquid cocoa and sugared versus sugar-free cocoa have also been reported. For instance, acute consumption of DC and sugar-free cocoa by overweight and obese individuals have been shown to reduce SBP and DBP by 3.2mmHg or 1.2mmHg and 1.4mmHg or 1.2mmHg, respectively (Faridi *et al.*, 2008). In contrast, sugared-cocoa has not been shown to reduce blood pressure (Faridi *et al.*, 2008).

Long-term randomised-controlled trials have also reported improvement in blood pressure following flavonoid-rich cocoa or DC. Here, reductions of 1.6 and 1.2mmHg in DBP and arterial blood pressure, respectively, have been observed in overweight and obese subjects following 12 weeks of twice daily consumption of cocoa containing 902mg flavanols (Davison *et al.*, 2008). The reduction in DBP (-1.3mmHg) is similar to the one seen in Taubert *et al.*'s (2007) study following 12 weeks ingestion of 6.3g of DC with 30mg polyphenols by patients with untreated upper-range pre-hypertension and stage-1 hypertension. However, in the latter study, SBP and DBP continued to decrease further to -

2.9mmHg and -1.9mmHg following 18 weeks of DC consumption (Taubert *et al.*, 2007). This effect was correlated with an increase in S-nitrosoglutathione, thereby confirming previous observational studies' findings of the relation between cocoa consumption, NO system and blood pressure.

It is worthy of attention that Taubert *et al.* (2007) stated that although the reduction in blood pressure was significant, none of their participants attained lower-range pre-hypertension (130/85) or an optimal blood pressure (120-80). This statement is important since it suggests that statistical significance does not necessarily imply clinical relevance. Nonetheless and as cited by Taubert *et al.* (2007), in the Zutphen Elderly Study, similar reductions in blood pressure related to cocoa ingestion have been correlated with a 50% reduction in cardiovascular and all-cause mortality (Buijsse *et al.*, 2006). This suggests that introducing cocoa or DC to the diet of hypertensive subjects or those at risk of hypertension such as the overweight and obese population may be more important in the long-term reduction of hypertension-related complications rather than in controlling hypertension itself. This is similar to what have been discussed earlier in relation to treated diabetics, where cocoa and DC might be more essential in terms of preventing diabetes-associated complication rather than in improving glycaemic control. Another question that arises is whether recommending DC and cocoa to patients who are already on antihypertensive treatment is of any benefit to them. Taubert *et al.* (2007) has also reported that the reduction in blood pressure seen in their long-term study is similar to the reduction seen in their short-term study using a larger dose of DC. This led Taubert *et al.* (2007) to state that 'the cumulative phenol dose may determine the magnitude of transcriptional NO synthase activation and subsequent fall in blood pressure'. This statement is important since most studies which have observed significant reduction in blood pressure have focused on providing DC in the form of a 100g bar which in the long-term might adversely affect body weight given the high-fat content of DC (Desch *et al.*, 2009).

Table 1.6 Changes in systolic blood pressure (SBP) and diastolic blood pressure (DBP) reported by previous studies (adapted from Desch *et al.*, 2009).

Study	Study Design	Population	Number of participants	Dose	Reduction in SBP	Reduction in DBP
Taubert 2003	Crossover, 1 week	Healthy elderly	13	100g DC 500mg polyphenols	**-5.1[-6.40, -3.80]**	**-1.80[-2.89, -0.71]**
Murphy 2003	Parallel-group, 28days	Healthy	Cocoa: 13; Control: 15	Tablets 234 mg cocoa flavanols and procyanidins	1.00 [-6.43, 8.43]	-1.00 [-6.94, 4.94]
Engler 2004	Parallel-group, 2 weeks	Healthy	Cocoa: 11; Control: 10	46g DC 213 mg procyanidins, 46 mg epicatechin	-1.80[-4.37, 0.77]	1.00[-0.71, 2.71]
Grassi 2005a	Crossover, 15 days	Healthy	15	100g DC 500mg polyphenols	-6.20[-7.41, -4.99]	-3.50[-4.51, -2.49]
Fraga 2005	Crossover, 2 weeks	Soccer players	27	105g DC 168mg of flavanols; 39 mg catechin and epicatechin	-4.00[-4.59, -3.41]	-4.00[-4.59, -3.41]
Grassi 2005b	Crossover, 15 days	never-treated, grade I patients with essential hypertension	20	100g DC 500mg polyphenols	-11.00[-13.02, -8.98]	-8.30[-10.10, -6.50]
Taubert 2007	Parallel-group, 18 weeks	upper-range prehypertension or	Cocoa: 22; Control: 22	6.3g DC 30mg polyphenols	-3.00[-3.50, -2.50]	-1.90[-2.70, -1.10]
Grassi 2008	Crossover, 15 days	hypertensives with impaired glucose tolerance	19	100g DC 500mg polyphenols	-4.60[-6.26, -2.94]	-4.20[-6.00, -2.40]
Crews 2008	Parallel-group, 6 weeks	Healthy elderly	Cocoa: 45; Control: 45	37g DC and 237 ml cocoa 754.71mg procyanidins	-0.53[-5.69, 4.63]	0.07[-1.72, 3.72]
Muniyappa 2008	Crossover, 2 weeks	Essential hypertension	20	150mg cocoa 900mg flavanols	-1.00[-6.82, 4.82]	1.00[-1.72, 3.72]

It is important to note that several studies have failed to report any significant changes in blood pressure following cocoa or DC ingestion (Fisher *et al.*, 2003, Murphy *et al.*, 2003, Heiss *et al.*, 2007). For instance, no significant change in blood pressure was observed following 2 weeks of 46g DC (213mg procyanidins) in healthy subjects (Engler *et al.*, 2004). This occurred irrespective of the improvement in endothelium function (Engler *et al.*, 2004). Similar findings were reported by Crews *et al.* (2008). In both these studies, participants had normal baseline blood pressure levels. Crews *et al.* (2008) thus attributed the lack of significant findings to these normal baseline blood pressure levels. However, in Grassi *et al.*'s (2005a) study, subjects also had a mean SBP of 113.9mmHg yet a significant 6.4mmHg reduction in SBP levels was observed. This suggests that differences in these studies could be related to other factors like the duration of intervention, time point at which blood pressure was measured, dose of flavonoids used and the instruments used to monitor blood pressure. Baseline differences in blood pressure levels among participants should also be taken in account, as those with lower baseline blood pressure levels may experience an increase in blood pressure leading to insignificant findings.

1.11.3.b Effect of green coffee bean extract

The ability of GCBE and CGA to improve blood pressure have been demonstrated in both spontaneously hypertensive rats and in humans (Kozuma *et al.*, 2005; Suzuki *et al.* 2002; Chikama *et al.*, 2006; Suzuki *et al.*, 2006; Watanabe *et al.*, 2006). In humans, CGA-rich GCBE has been documented to reduce blood pressure in patients with mild hypertension in small scale (Watanabe *et al.*, 2006) as well as large-scale interventions (Kozuma *et al.*, 2005) (Table 1.7). These hypotensive properties were shown to be dose-dependent with GCBE containing 93mg and 185mg CGA being more effective than GCBE with a 46mg CGA content (Kozuma *et al.*, 2005). Moreover, the antioxidant properties of GCBE and its ability to modulate endothelium function appear to lie at the centre of GCBE blood pressure-lowering effects.

It is important to note that so far the effect of GCBE on blood pressure is consistent in the literature whilst the role of coffee remains unconceivable (Suzuki *et al.*, 2008). It seems that in relation to blood pressure, consumption of GCBE has certain advantages over ingestion of coffee. This is because GCBE contains high levels of CGA whilst in coffee the presence of some pro-oxidant compounds such as benzenetriols, including hydroxyhydroquinone can interfere with CGA's hypotensive properties by counteracting CGA's actions on ROS generation, NO bioavailability and subsequently endothelium function and blood pressure

(Suzuki *et al.*, 2008). This together suggests that the composition of GCBE and the balance between the pro-oxidant and oxidant compounds can have a profound effect on determining the biological actions of this polyphenol-rich product.

Table 1.7 Summary of studies investigating the blood-pressure lowering effects of green coffee bean extract. Note the limited amount of research.

Study	Study Design	Population	Number of participants	Dose	Change from baseline (mmHg)
Kozuma 2005	Randomised placebo-controlled, 28days	Mild hypertension	117 males	**Three GCBE doses** **46 mg GCBE 25mg CGA** **93 mg GCBE 50mg CGA** **185 mg GCBE 100mg CGA**	 **-3.2±4.6** **-4.7±4.5** **-5.6±4.2**
Watanabe 2006	Randomised placebo-controlled, 15 days	Mild hypertension	28	GCBE 140mg CGA	-4

1.11.4 Polyphenols, nitric oxide and endothelium function

1.11.4.a Effect of cocoa and dark chocolate

DC flavonoids have been shown to improve NO bioavailability through a variety of mechanisms. To illustrate, quercetin increases eNOS activity, while epicatechin decreases superoxide levels by directly scavenging it (Sanchez *et al.*, 2006). Conversely, epicatechin metabolites reduce superoxide generation by inhibiting NADPH oxidase (Sanchez *et al.*, 2006, Steffen *et al.*, 2007) while the dimer procyanidin B2 and (-)-epicatechin glucuronide could both scavenge superoxide and inhibit NADPH oxidase (Steffen *et al.*, 2008). In relation to eNOS, polyphenols have also been shown to prevent eNOS uncoupling due to decreased BH4, an eNOS co-factor (Kawashima *et al.*, 2004). The clinical relevance of this mechanism could be viewed in that under pathological conditions when BH4 levels are reduced and dihydrobiopterin (BH2) levels are increased, eNOS becomes dysfunctional and produces superoxide rather than NO (Kawashima *et al.*, 2004). As for NADPH oxidase, its inhibition is of particular relevance to diabetes, since increased glucose levels have been reported to increase NADPH oxidase activity leading to increased ROS production (Akbari *et al.*, 1998, Title *et al.*, 2000). It is also of relevance to the metabolic syndrome where upregulation of NADPH oxidase and downregulation of dismutase, glutathione, peroxidase, heme-oxygenase has been reported, resulting in increased ROS production and oxidative stress (Roberts *et al.*, 2006).

DC flavonoids could also improve NO bioavailability by modulating the activity of arginase, an enzyme in the urea cycle. Here, (-)-epicatechin and its metabolites have been shown to lower arginase-2 mRNA expression and activity in human endothelial cells *in vitro* (Schnorr *et al.*, 2008). This is essential since arginase competes with eNOS for arginine and increased arginase activity leads to decreased NO generation since arginine is converted to ornithine. This reduction in arginase activity is also seen *in vivo* in both rat kidney and human erythrocytes following consumption of cocoa (Schnorr *et al.*, 2008).

All of the above mechanisms explain the direct mechanism by which DC flavonoids increase NO bioavailability. There is, however, an additional indirect mechanism by which DC flavonoids could promote NO bioavailability and which involves angiotensin-II, which is summarised below.

Angiotensin-II is produced through the conversion of angiotensin-I by the enzyme angiotensin-converting enzyme. Procyanidins (dimer to hexamers) and epigallocatechin, but not (+)-catechin, (-)-epicatechin, GA, CGA, caffeic acid, quercetin, kaempferol or resveratrol, have been shown to be effective inhibitors of angiotensin-converting enzyme (Actis-Goretta *et al.*, 2003). In relation to procyanidins, this effect is dependent on the number of flavanol units within the procyanidin (Actis-Goretta *et al.*, 2003). Chocolate extracts have also been shown to inhibit this enzyme in rat kidney membrane, the effect being related to the concentration of flavanols within the chocolate extract (Actis-Goretta *et al.*, 2006). Additionally, cocoa flavonoids have been reported to attenuate angiotensin-II-induced activation of MAPK-dependent pathway (Lee *et al.*, 2006) which is likely to lead to decreased endothelin-1 and ROS production, and subsequently improved NO bioavailability. However, *in vivo* inhibition of angiotensin-converting enzyme by chocolate or cocoa has not yet been reported. This area deserves further investigation since the renin-angiotensin-aldosterone system is known to be an important regulator of blood pressure homeostasis. Moreover, *in vivo* inhibition of angiotensin-converting enzyme in humans following ingestion of polyphenol-rich pomegranate juice has been reported providing further evidence for the need to investigate potential *in vivo* inhibition of this enzyme by polyphenols (Avriam and Dornfeld, 2001).

What is most important about the above mechanisms is that they suggest that cocoa and DC polyphenols have the potential to improve endothelium function as a result of their ability to

modulate NO, as highlighted below, with major consequences to glucose utilisation, blood pressure regulation and FFA oxidation.

Initial reports of improved endothelium function following treatment with cocoa flavonoids came from a study on rabbit aortic rings (Karim *et al.*, 2000). Here, polymeric procyanidins (tetramer through decamer of catechin) induced dose-dependent endothelium-dependent vasodilation. This effect was attributed to the ability of these procyanidins, with the exception of monomers, to improve eNOS activity.

Ever since, several studies have been published which explored both the acute and chronic effects of cocoa and DC on endothelium function *in vivo*. To illustrate, in a short-term study by Heiss *et al.* (2003), consumption of 100ml flavanol-rich cocoa (containing 176mg flavan-3-ols) by patients with at least 1 cardiovascular risk factor (coronary artery disease, hypertension, hyperlipidaemia, diabetes or smoking) for 2 consecutive days increased flow-mediated dilation maximally at 2h, an effect that was correlated with increased nitrosylated and nitrosated species. Brachial artery diameter, endothelium-independent dilation of brachial artery diameter, nitrite or nitrate levels remained unaltered. In another short-term study, consumption of a flavanol-rich beverage for 5 days (containing 821mg flavanol/day) produced vasodilation and improved vasodilator response to ischemia in finger arteries (Fisher *et al.*, 2003). This effect was reversed following an intravenous infusion of the NOS-inhibitor L-NMMA suggesting that flavanols exert their effect via activation of NO system (Fisher *et al.*, 2003). Similar observations were made in smokers following consumption of 100ml cocoa drink containing 176-185mg flavanols (Heiss *et al.*, 2005).

With regard to DC, acute consumption of 100g DC has been shown to increase both resting and hyperaemic brachial artery diameter in healthy subjects (Vlachopoulos *et al.*, 2005), an effect that was not observed in Heiss *et al.* (2003) study. Flow-mediated dilation also increased significantly 60min after DC consumption. This effect occurred irrespective from significant changes in oxidant status (Vlachopoulos *et al.*, 2005). In another study, the increase in flow-mediated dilation reached its maximum at 2h and was then maintained to up to 8h following consumption of 40g DC (Hermann *et al.*, 2006), consistent with the kinetics of (-)-epicatechin. Indeed, Schroeter *et al.* (2006) identified (-)-epicatechin and its metabolite epicatechin-7-O-glucuronide as independent predictors of vascular effects of flavanol-rich cocoa and their effect on NO as the main mechanism by which these compounds improve endothelium function. Improvement in flow-mediated dilation have

also been reported following consumption of 46g of DC containing 213mg procyanidins for 2 weeks in healthy subjects (Engler *et al.*, 2004) and 100g DC containing 500mg polyphenols for 2 weeks in patients with essential hypertension (Grassi *et al.*, 2005b). Interestingly, in both these studies improvements in flow-mediated dilation were of similar magnitude as to the one observed by Vlachopoulos *et al.* (2005) following acute consumption of DC (1.43%, 1.5% vs. 1.3%). This probably suggests that the effect of DC on endothelium function could be sustained with regular consumption of this flavonoid-rich product and that desensitization does not occur. Indeed, consumption of a cocoa beverage containing 446mg flavanols for 6 weeks by hypercholesterolaemic women have been demonstrated to improve hyperaemic brachial artery blood flow, an effect that was correlated with a decrease in vascular adhesion molecule-1 (Wang-polagruto *et al.*, 2006). Similarly Heiss *et al.* (2007) have reported that continual improvement in flow-mediated dilation and sustained augmentation at 2h could be achieved with regular consumption of cocoa. Most importantly, Heiss *et al.* (2007) identified the dose that achieves half-maximal flow-mediated dilation, which was 616mg flavanols.

In the most recent trial, sugar-free cocoa has been shown to ameliorate flow-mediated dilation to a better extent than sugared-cocoa (5.7% vs. 2.0%). This effect was even greater than the one observed with DC containing a similar quantity of cocoa (4.3%) (Faridi *et al.*, 2008). This led Faridi *et al.* (2008) to conclude that while polyphenols improve endothelium function, the sugar fraction attenuates it. This finding is particularly relevant to diabetic patients in whom increased glucose levels promote ROS production leading to decreased NO bioavailability. Reducing the sugar content of the cocoa beverage could thus augment the improvement in endothelium function seen after acute and chronic consumption of cocoa in medicated diabetic patients, particularly that sugared flavanol-rich cocoa, on its own, is able to increase flow-mediated dilation by 30% (Balzer *et al.*, 2008). The use of sugar-free cocoa could also be of relevance to obese patients or those with the metabolic syndrome, since decreasing the sugar content will ultimately allow the delivery of higher quantities of flavanols in a less energy-dense form. Reducing the sugar content is also likely to improve flow-mediated dilation further. To illustrate, Davison *et al.* (2008) observed a 2.4% increase in flow-mediated dilation following acute consumption of sugared-cocoa and a 1.6% increase following 12 weeks of sugared-cocoa. Faridi *et al.* (2008) also observed a similar increase using sugared-cocoa in overweight individuals but when the sugared-cocoa was replaced by a sugar-free version flow-mediated dilation increased strikingly by an additional 2.1%.

All of the above evidence suggests that cocoa and DC could be used to promote vascular health in the overweight and obese population since their beneficial effects on vascular function extends from healthy individuals to those with cardiovascular risk factors, diabetes and the metabolic syndrome.

1.11.4.b Effect of green coffee bean extract on nitric oxide and endothelium function

Improvements in endothelium function following GCBE or CGA consumption have been documented in humans as well as in animals (Ochiai *et al.*, 2004; Chikama *et al.*, 2006; Suzuki *et al.*, 2006). In general, these effects are attributed to both CGA and its metabolites, in particular ferulic acid. Ferulic acid is a recognised NO-donor that has been shown to improve endothelium function (Suzuki *et al.*, 2002; Suzuki *et al.*, 2007). CGA, on the other hand, can improve NO bioavailability by modulating oxidative stress via inhibition of NADPH oxidase activity and subsequent superoxide generation (Suzuki *et al.*, 2006). Consumption of CGA has also been shown to reduce urinary H_2O_2 excretion in spontaneously hypertensive rats (Suzuki *et al.*, 2006). Additionally, CGA has been shown to ameliorate acetylcholine-induced endothelium-dependent vasodilation (Suzuki *et al.*, 2006). Overall, these improvements in endothelium function might have potential implications for the regulation of blood pressure, glucose and lipid metabolism by GCBE.

1.11.5 Polyphenols, Fat metabolism and obesity

1.11.5.a Effect of cocoa and dark chocolate

As discussed in previous sections, NO regulates flow-mediated dilation resulting in improved perfusion of skeletal muscle and substrate delivery and oxidation. Accordingly, Davison *et al.* (2008) hypothesised that cocoa may reduce fat mass and increase fat oxidation in overweight and obese individuals via improvement in NO bioavailability.

Support for this hypothesis stemmed mainly from studies on L-arginine, the precursor of NO. According to these studies L-arginine supplementation decreases fat mass in Zucker-diabetic rat (Fu *et al.*, 2005) while in obese type II diabetes patients consuming 8.3g/day of L-arginine decreases both fat mass and WC while maintaining lean muscle mass (Lucotti *et al.*, 2006). L-arginine supplementation was also shown to improve endothelium function by increasing the levels of NO second messenger, cGMP, while decreasing endothelin-1 levels, an effect that was correlated with improved SBP (Lucotti *et al.*, 2006). According to Lucotti *et al.* (2006), L-arginine supplementation in conjunction with exercise reduces atherogenesis,

as indicated by decreased leptin-to-adiponectin ratio, while producing an additive improvement on glucose metabolism, insulin sensitivity, adiponectin and antioxidant capacity, the latter being indicated by increased superoxide dismutase levels. All of these findings led Davison *et al.* (2008) to investigate the effect of cocoa consumption, on its own or in combination with exercise, on body composition, endothelium function, insulin resistance and blood pressure in obese individuals. Based on the findings of Davison *et al.* (2008), only exercise is capable of raising fat oxidation and reducing abdominal fat mass. High-flavanol cocoa on its own did not improve fat oxidation or decrease abdominal fat mass. However, when comparing the data from the combined low-flavanol and exercise group and the high-flavanol and exercise group, it appears that within the high-flavanol group, fat oxidation was increased and abdominal fat was decreased to a greater extent. Fat oxidation in high-flavanol group was greater by 0.140gmin-1 from baseline versus the 0.064gmin-1 increase from baseline in low-flavanol group. Percentage abdominal fat was lower by 1.31% from baseline in high-flavanol group versus the 0.52% reduction in low-flavanol group. Unfortunately, Davison *et al.* (2008) did not report the p-values or effect size of these results.

Nonetheless, decreased visceral adipose tissue weight has been reported in rats following ingestion of cocoa (Matsui *et al.*, 2005). Ingested cocoa has also been shown to prevent high-fat diet-induced obesity by decreasing the gene expression of the enzymes that regulate fatty acid synthesis in liver and white adipose tissue as well as decreasing the gene expression of fatty acid transport systems in white adipose tissue (Matsui *et al.*, 2005). Enhancement of the gene expression for uncoupling protein-2, which regulates thermogenesis, has also been reported (Matsui *et al.*, 2005).

All of this suggests the need for further research into the effects of different polyphenol compounds on substrate utilisation and thermogenesis, since some polyphenols have been demonstrated to increase fat oxidation (Boschmann and Thielecke, 2007). This also highlights the need to monitor body composition and to obtain anthropometrical data to observe if any changes in these parameters occur in overweight and obese individuals during cocoa or DC consumption. Understanding structure-activity relation is important in elucidating the mechanisms by which diet could prevent obesity, particularly that FFA accumulation in muscle is known to induce insulin resistance. The effect of DC on pancreatic lipase activity should also be investigated since NO modulates its activity. This

could carry important implication to improving lipid profile and post-prandial lipaemia in the overweight and obese population.

1.11.5.b Effect of green coffee bean extract

Blum *et al.* (2007) were the first to report that consumption of decaffeinated GCBE is associated with weight loss in a prospective preliminary study. These findings were subsequently confirmed by Dellalibera *et al.* (2006) in a larger placebo-controlled trial compromising 50 overweight volunteers. In here, consumption of 400mg of decaffeinated GCBE in conjunction with caloric restriction for 60 days was shown to induce a 4.97± 0.32 (mean±SEM) kg reduction in weight. Moreover, a significant elevation of muscle mass-to-fat mass (mean± SEM= 4.1±0.7) following decaffeinated GCBE consumption was observed compared to control which had a minimal effect on body composition (mean± SEM= 1.6± 0.6) (Dellalibera *et al.*, 2006). These findings were postulated to be modulated by GCBE's ability to improve glycaemic control which arguably leads to a rise in fat utilisation and reduced fat storage, a hypothesis based on the close association between glucose and lipid metabolism (Blum *et al.*, 2007). This hypothesis was partially supported by a study on mice wherein consumption of GCBE evoked a reduction in fat absorption and hepatic TG accumulation which was accompanied by a reduction in body weight and visceral fat (Shimoda *et al.*, 2006). However, in contrast to the studies on human volunteers, the GCBE used by Shimoda *et al.* (2006) contained both caffeine (10%) and CGA (27%). Caffeine was identified as the chief component responsible for GCBE's inhibitory effect on fat absorption while CGA was shown to be effective in reducing hepatic TG accumulation (Shimoda *et al.*, 2006). Importantly, though GCBE demonstrated dose-dependent attenuation of hepatic carnitine-palmitoyltransferase activity, this effect was not accounted by CGA. Instead other GCBE components such as neochlorogenic acid and feruloylquinic acid proved to contribute to GCBE-induced inhibition of carnitine-palmitoyltransferase (Shimoda *et al.*, 2006).

1.11.6 Polyphenols, lipid profile and lipid peroxidation

1.11.6.a Effect of cocoa and dark chocolate

DC could improve lipid profile by both decreasing LDL levels and decreasing its susceptibility to oxidation by increasing plasma antioxidant capacity and decreasing ROS formation.

To illustrate, consumption of 22g cocoa and 16g DC, containing 446mg procyanidins, for 4 weeks by healthy subjects have been shown to increase total antioxidant capacity, an effect that was correlated with an 8% increase in LDL oxidation lag time (Wan *et al.*, 2001). HDL cholesterol, but not HDL: LDL ratio, was also found to be increased by 4% (0.05mmol/l) (Wan *et al.*, 2001). Indeed, prolongation of LDL oxidation lag time and improvements in lipid profile have been reported by numerous studies (Table 1.8). In one study, increased LDL oxidation lag time was reported following 1 and 2 weeks of 36g of sugared cocoa powder with a 2610mg polyphenol content, an effect that occurred alongside increased epicatechin excretion in urine (Osakabe *et al.*, 2001). In another study, normocholesterolaemic and mildly hypercholesterolaemic subjects who consumed 26 g cocoa powder and 12 g sugar/d, providing 41.08mg procyanidins and 98.02mg epicatechin, for 12weeks compared to a control of 12 g sugar/d, exhibited a 9% or a 13% prolongation of lag time of LDL oxidation compared to baseline or the control group, respectively, and a 24% increase in plasma HDL cholesterol (Baba *et al.*, 2007a). Improvement in lipid profile have also been reported by Grassi *et al.* (2005b, 2008) who showed that 100g DC with 500mg polyphenols decreases total cholesterol by 7.4% or 6.5% and LDL cholesterol by 11.8% or 7.5% in hypertensive subjects and hypertensive subjects with glucose intolerance, respectively; by Fraga *et al.* (2005) who showed that flavanol-rich milk chocolate reduces total cholesterol by 11% and LDL-cholesterol by 15%, and by Balzer *et al.* (2008) who demonstrated that sugared-cocoa could reduce LDL levels in medicated diabetic patients. Reductions in total cholesterol, TG and LDL-cholesterol following supplementation with cocoa have also been reported in animal models of diabetes (Ruzaidi *et al.*, 2005) and obesity (Jalil *et al.*, 2009). Jalil *et al.*, (2009) attributed these effects to the potential ability of polyphenols to inhibit pancreatic lipase, promote cholesterol excretion in faeces and reduce hepatic lipid accumulation through activation of adenosine monophosphate (AMP)-activated protein kinase, attenuation of hepatic secretion of apolipoprotein-B100 and stimulation of hepatic LDL receptors expressions. Indirect mechanisms involving insulin's regulatory actions on lipid metabolism have also been implicated (Ruzaidi *et al.*, 2005).

However, not all cocoa and DC components affect LDL oxidation and lipid profile positively. For instance, in an *ex vivo* study by Vinson *et al.* (2006), it was demonstrated that while cocoa polyphenols prolong lag time of LDL+VLDL oxidation, cocoa butter decreases it resulting in increased LDL oxidation. This was evident in that defatted cocoa powder and DC increased the lag time of LDL+VLDL oxidation to a greater extent than normal cocoa and DC (Vinson *et al.*, 2006). In relation to LDL+VLDL oxidation, similar observations

were made in humans following consumption of muffins containing 22g DC and 12g cocoa versus muffins with cocoa butter only (Vinson *et al.*, 2006).

It is well acknowledged that the composition of LDL or atherosclerotic plaque, as well as lipid membranes is largely influenced by the composition of dietary fats as evident in that dietary fats are rapidly incorporated into atherosclerotic plaque which then influences plaque composition, hence plaque formation and rupture (Felton *et al.*, 1994). Cocoa butter is rich in monounsaturated oleic acid and polyunsaturated fatty acids (Hannum *et al.*, 2002). The fact that cocoa butter increases LDL oxidation suggest that though oleic acids decreases LDL susceptibility to oxidation (Nicolosi *et al.*, 2004), the polyunsaturated fatty acids in cocoa butter with their large numbers of unsaturated fatty acids bonds appear to override the beneficial effects of oleic acid rendering cocoa butter atherogenic (Vinson *et al.*, 2006).

Nonetheless, such findings may also suggest that the anti-atherosclerotic properties of cocoa or DC could be enhanced by reducing the cocoa butter content of cocoa or DC. It also suggests that the use of cocoa instead of DC may be more appropriate in conditions of increased oxidative stress wherein maximal inhibition of LDL oxidation is required. It is notable that in contrast to Wan *et al.* (2001) and Osakabe *et al.* (2001), Vinson *et al.* (2006) failed to show any correlation between plasma epicatechin and LDL+VLDL oxidation lag time. This led Vinson *et al.* (2006) to suggest that while polyphenols could reduce LDL oxidation through interaction with the LDL particle, this interaction requires time to occur, hence the lack of correlation. Most importantly, this study showed that low-dose cocoa, which corresponded to two 40g DC bars per day, and high dose cocoa prevent the development of early-stage atherosclerosis by 40% and 36%, respectively, in Syrian Golden hamsters (Vinson *et al.*, 2006). The similarity in results between the low and high polyphenols possibly implied that a saturation effect occurs with increasing polyphenols concentration, an effect that was also noted in relation to cholesterol, TG and LDL oxidation (Vinson *et al.*, 2006).

In general, the lag time of LDL oxidation serves as an index of the quantity and quality of antioxidants within LDL particles. Prolongation of LDL oxidation lag time following cocoa or DC consumption suggests that the levels and quality of antioxidant within LDL is increased, which as stated by Vinson *et al.* (2006) is the result of incorporation of cocoa polyphenols into the LDL molecule. Indeed, red wine polyphenols have been shown to bind to human LDL and HDL particles (Ivanov *et al.*, 2001). In relation to quercetin, this effect is

attributed to its ability to form glycosidic ether bonds with the LDL molecule resulting in decreased metal-ion induced LDL oxidation and aggregation, hence decreased lesion size (Aviram and Fuhrman, 2002). Increased paraoxonase activity and flavonoid accumulation in macrophages is another mechanism by which quercetin reduces LDL oxidation and uptake (Aviram and Fuhrman, 2002). It is likely that cocoa polyphenols act via similar mechanisms to inhibit LDL oxidation. This is evident in that consumption of cocoa results in reduced atherosclerotic lesion size in animal models of atherosclerosis (Kurosawa *et al.*, 2005). Cocoa consumption have also been reported to reduce wall thickness and cholesterol content of aorta, although the change in the latter was not significant (Kurosawa *et al.*, 2005).

In addition to the above findings, acute reduction in F-2 isoprostane levels, which according to Wan *et al.* (2001) are a more direct measure of lipid peroxidation, have been reported following ingestion of cocoa (Wiswedel *et al.*, 2004). Such findings were, however, not observed following 6 weeks consumption of a combination of 36.9g DC and 30.95g cocoa by healthy volunteers, despite that lag time of LDL oxidation was prolonged by 9.8% reflecting a reduction in copper-induced LDL oxidisation (Mathur *et al.*, 2002). Mathur *et al.* (2002) explained the lack of significant change in F2-isoprostane to be the result of the use of a single morning urine sample rather than a 24h urine sample. Mathur *et al.* (2002) also failed to report any significant changes in plasma total antioxidant capacity or plasma polyphenols levels. Mathur *et al.* (2002) argued that this effect was due to blood samples having been taken after an overnight fast rather 2h following cocoa ingestion when epicatechin levels are at their highest. However, since reduced LDL oxidation was still observed 12h following DC consumption, this suggests that polyphenols affect LDL oxidation via mechanisms other than their antioxidant activity. For instance, via their sparring effects on α-tocopherol (see studies by Baba *et al.* (2000) and Lotito *et al.* (2000) p. 67 and p. 908).

In Baba *et al.*'s (2007a) study, a negative correlation was observed between plasma concentrations of HDL, but not LDL cholesterol, and oxidized LDL. Baba *et al.* (2007a) suggested that HDL could influence LDL oxidation through inhibition of monocyte chemotaxis, breakdown of lipid peroxide via paraoxonase, reverse cholesterol transporter via lecithin-cholesterol transferase and inhibition of vascular endothelium activation via apolipoproteinA1. Indeed, the ability of HDL to prevent LDL oxidation have been reviewed by Mackness and Durrington (1995) who argued that enzymes like paraoxonase, lecithin: cholesterol acyl transferase, platelet activating factor acetylhydrolase, phospholipase D and

protease, which are present on the surface of HDL may influence LDL oxidation (Mackness and Durrington, 1995). In addition to these enzymes, apolipoprotein AI has been shown to reduce LDL oxidation by increasing HDL's anti-inflammatory action and by enhancing macrophage reverse cholesterol transport (Moore *et al.*, 2005).

It is important to note that some studies failed to report any significant change in lipid profile (Mathur *et al.*, 2002, Engler *et al.*, 2004, Grassi *et al.*, 2005a, Taubert *et al.*, 2007) or in LDL oxidation (Engler *et al.*, 2004) following DC (see Table 1.8). In relation to lipid profile, cocoa polyphenols could prevent atherosclerosis development without significantly altering lipid profile. For instance, consumption of a diet containing 1% cacao liquor polyphenols for 10days by hypercholesterolaemic rabbits have been reported to increase lag time of LDL oxidation and to decrease TBARS without significantly affecting lipid profile (Osakabe *et al.*, 2000). Based on the results of this study, Kurosawa *et al.* (2005) investigated the effect of a similar diet containing 1% cacao liquor polyphenols on atherosclerosis development in rabbits. As with Osakabe *et al.* (2000), Kurosawa *et al.* (2005) demonstrated that cocoa increased resistance against LDL oxidation and decreased TBARS significantly at the end of the first 2 and 3months of the 6 month trial without significantly altering plasma cholesterol, TG or phospholipids (Kurosawa *et al.*, 2005). They argued that the decrement in LDL oxidation will lead to decreased foam cell formation, which was confirmed by the observed reduction in the area of atherosclerotic lesion following cocoa consumption (Kurosawa *et al.*, 2005). The importance of this study lies in that Kusagni rabbits reflect a more advanced and severe model of atherosclerosis (Vinson *et al.*, 2006) which suggests that cocoa and DC polyphenols can act at different stages of atherosclerosis. In rats, consumption of cocoa have also been reported to attenuate the accumulation of lipid peroxides in plasma and to spare plasma α-tocopherol from oxidation by the water-soluble free radical generator 2, 2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) or copper sulphate, with an inverse correlation being observed between plasma epicatechin and lipid peroxides and a positive association between plasma epicatechin and α-tocopherol (Baba *et al.*, 2000). Attenuation of lipid peroxidation as measured by TBARS have also been documented *ex-vivo* (Lotito and Fraga, 2000)

Cocoa is also rich in fiber. In a study by Lecumberri *et al.* (2007), supplementing the diet of rats with 165 g of cocoa fiber per kilogram reduced TG, total cholesterol and LDL-cholesterol, though the latter two did not reach baseline values. Decreased lipid peroxidation in serum and liver was also observed in both hypercholesterolaemic and

normocholesterolaemic rats, although no significant changes in total antioxidant capacity or the activity of antioxidant enzymes and glutathione levels in liver were seen (Lecumberri *et al.*, 2007). In another study, cocoa fiber obtained from cocoa husks was found to counteract the effects of lipid-rich diet by preventing fat-induced decline in HDL-cholesterol and fat-induced rise in total cholesterol (TC), LDL-cholesterol and lipid peroxidation (Ramos *et al.*, 2008). Cocoa fiber also reduced food intake, body weight gain and TG levels to values lower than those in the group fed the cholesterol-free diet (Ramos *et al.*, 2008).

Table 1.8 Summary of studies investigating the effect of polyphenol-rich dark chocolate and cocoa on lipid profile.

Study	Study Design	Population	Number of participants	Dose	Placebo/Control	Assessed parameter	Change significant?
Wan 2001	Randomised, parallel-group, 4 weeks	healthy	23 (10 men, 13 women)	22 g cocoa powder and 16 g DC 466 mg procyanidins	Average American Diet	LDL oxidation, lipid profile	LDL oxidation lag time and HDL increased
Mathur 2002	Non-randomised trial, subjects studied at the end of 6 weeks DC and at the end of 6weeks follow-up period	Healthy	25 (12 females, 13 males)	36.9 g DC and 30.95 g cocoa drink 651mg procyanidins	Habitual diet	LDL oxidation, lipid profile	LDL oxidation lag time
Engler 2004	14days	healthy adults	21	46g DC 213 mg procyanidins, 46 mg epicatechin	Low-flavonoid DC	LDL oxidation, lipid profile	No
Fraga 2005	Crossover, 2 weeks	Soccer players	27 males	105g DC 168mg of flavanols; 39 mg catechin and epicatechin	105g white chocolate	Lipid profile	TC -0.47mmol/L LDL - 0.4mmol/L
Grassi 2005a	Randomised, crossover, 15 days	Healthy	15 (7 males, 8 females	100g DC 500mg polyphenols 88mg flavanols	90g white chocolate	Lipid profile	No
Grassi 2005b	Randomised, crossover, 15 days	never-treated, grade I patients with essential hypertension	20 (10 males, 10 females)	100g DC 500mg polyphenols 88mg flavanols	90g white chocolate	Lipid profile	TC -0.4mmol/L LDL -0.4 mmol/L

Continued...

Table 1.7... *Continued*

Study	Study Design	Population	Number of participants	Dose	Placebo/Control	Assessed parameter	Change significant?
Baba 2007a	Randomised, parallel-group, 12 weeks	normocholesterolaemic and mildly hypercholesterolaemic humans	25	12g sugar and 26g cocoa 41.08mg procyanidins; 98.02mg epicatechin	12 g sugar	LDL oxidation, lipid profile	HDL rose by 24%
Baba 2007b	Parallel-group, acute	normocholesterolaemic and mildly hypercholesterolaemic humans	160	3 polyphenol-rich cocoa drinks 13, 19.5, and 26 g cocoa (65, 97 and 129mg epicatechin)	Low-polyphenol cocoa	Lipid profile and oxidised-LDL	LDL, oxidized LDL, and apolipoprotein B decreased HDL increased in subjects with LDL ≥3.23 mmol/L
Balzer 2008	Randomised, parallel control, 30 days	Type-II diabetics	44 of which 3 dropped-out (29 females and 12 males)	18g cocoa 321 mg flavanols taken thrice daily (Total 963mg flavanols)	18g cocoa 25 mg flavanols taken thrice daily (Total 75mg flavanols)	Lipid profile	LDL decreased by 0.02mmol/L
Grassi 2008	Randomised, crossover, 15 days	hypertensives with impaired glucose tolerance	19 (11 males, 8 females)	100g DC 500mg polyphenols	90g white chocolate	Lipid profile	No
Hamed 2008	Open-label trial, 7 days	Healthy	28	700mg flavonoids	Baseline	Lipid profile	LDL fell by 6% and HDL rose by 9%
Nanetti 2008	Open label, 3 weeks	Healthy	10	50g DC	Baseline	Lipid profile, lipid peroxidation	HDL increased Conjugate formation in HDL and in LDL decreased
Al-Faris 2009	Parallel-group 15days	Healthy	89 females	100g DC 500mg polyphenols	90g white chocolate or habitual diet	Lipid profile	No

1.11.6.b Effect of green coffee bean extract

Coffee prolongs lag time of LDL oxidation (292-948min) to a greater extent than a serving of cocoa (217-444min), green tea (186-338min) or black tea (67-277min) (Richelle *et al.*, 2001) and its individual components particularly CGA possess the ability to inhibit lipid peroxidation (Kono *et al.*, 1997). However certain components present in unfiltered coffee such as diterpenes can increases serum levels of total and LDL cholesterol (Jee *et al.*, 2001) via activation of CETP and inhibition of lecithin-cholesterol acyltransferase (De Roos *et al.*, 2000). Diterpenes are also present in green coffee beans in the following concentrations: 1.3% w: w in *coffea Arabica* and 0.2% w: w in *coffea robusta* (Urgert *et al.*, 1995). Because of the diversity of GCBE components it is important to monitor the effect of CGA-rich GCBE on lipid profile. So far in one study in obese, hyperlipidaemic, and insulin resistant (*fa/fa*) Zucker rats, treatment with CGA was shown to reduce fasting plasma cholesterol and TG by 44% and 58%, respectively (Rodriguez *et al.*, 2002). These results were not replicated in humans wherein ingestion of GCBE supplying 140mg CGA for 4 months failed to produce any significant changes in lipid profile (Ochiai *et al.*, 2004). It is likely that the effect of CGA in rats was exaggerated since CGA was administered via intravenous infusion which does not take into account metabolism by the liver. In humans, the lack of an effect of CGA-rich GCBE on lipid profile could be explained by the method of GCBE administration. Ochiai *et al.* (2004) provided GCBE in the form of a drink (125 ml), and it is well recognised that the food matrix and even fluid volume in which a pharmaceutical compound is administered could influence the bioavailability of the active components (Toothaker and Welling, 1980). In fact some phenolic compounds are more bioavailable in fluid forms (Chen *et al.*, 1997) while others require encapsulation to protect the active components in the digestive track (Parada and Aguilera, 2007). As such the effect of CGA-rich GCBE on TC, TG, HDL and LDL levels remains unclear.

1.11.7 Polyphenols and glucocorticoid metabolism

Polyphenols could influence cortisol and corticosteroid metabolism in several ways (see Table 1.9). For instance, ingestion of liquorice and grapefruit juice polyphenols have been reported to increase plasma cortisol causing severe hypokalaemia and hypertension (Sardi *et al.*, 2002). Similarly, drinking grapefruit juice, rich in the flavonoids naringenin, quercetin and hesperetin, have been shown to decrease urinary cortisone-to-cortisol ratio (Lee *et al.*, 1996). These effects occur through inhibition of 11βHSD2 (Song *et al.*, 1992, Lee *et al.*, 1996, Guo *et al.*, 1998, Sardi *et al.*, 2002).

Table 1.9 Summary of the endocrine actions and the metabolic sequelae of antioxidants and polyphenol-rich products.

Study	Antioxidant or polyphenol-rich food	Model	Endocrine action/ physiological effects
Song 1992	Gossypol glycyrrhetinic acid	Kidney	↓ 11βHSD2
Lee 1996	Grapefruit	Kidney microsomes, Humans	↓11βHSD2 ↓ urinary cortisone/cortisol ratio
Arion 1997; 1998	Chlorogenic acid and Chlorogenic Acid Analogue S 3483	Liver microsomes	↓glucose 6-phosphatase
Hemmerle 1997	Chlorogenic acid	Liver microsomes	↓ glucose-6-phosphate translocase ↓gluconeogenesis and glycogenolysis
Guo 1998	quercetin, tea polyphenols, furosemide, and gossypol	Cortex microsomes	↓11βHSD2
Ohtsuka 1998	Vitamin E	Sprague-Dawley rats	↓lipid peroxidation and skeletal muscle oxidative stress
Sardi 2002	Liquorice Grapefruit	humans	↓11βHSD2 Hypertension, hyperkalaemia
Eid 2003	Dietary polyphenols	Broiler chicken	↓ corticosterone and corticosterone-induced oxidative stress, hyperlipidaemia, abdominal fat content, liver weight, lipid peroxidation
Schweizer 2003	Flavanone	from stably transfected cells	Selective inhibition of 11βHSD1 but not 11βHSD2
Atanasov 2006	Caffeinated/ Decaffeinated coffee	HEK-293 cells	↓11βHSD1
Miguet 2006	Flavonone	*in silico* screening	↓11βHSD1
Ajdžanović 2009	Genistein	Wistar rats	↑DHEA secretion ↓ corticosterone and aldosterone secretion
Gumy 2009	Extract of *Eriobotrya japonica* Roasted coffee	HEK-293 cells	Selective inhibition of 11βHSD1 but not 11βHSD2

By contrast, extracts of freshly ground roasted *Coffea Arabica L* have been shown to inhibit 11βHSD1 activity and to prevent glucocorticoid-receptor translocation, glucocorticoid-induced expression of PEP-CK and gluconeogenesis (Atanasov *et al.*, 2006). These effects were observed even following removal of caffeine suggesting that compounds other than caffeine were responsible for coffee's dose-dependent inhibitory action against 11βHSD1 (Atanasov *et al.*, 2006). Although Atanasov *et al.* (2006) were unable to identify the compound responsible for coffee's inhibitory activity, they characterised the compound as being highly polar and thermo-stable which is consistent with the chemical properties of CGA (see Figure 1.14; note the polar OH groups). CGA has consistently been reported to reduce cortisol concentrations by inhibiting glucose-6-phosphate transport into the lumen of

endoplasmic reticulum (Arion *et al.*, 1997, Hemmerle *et al.*, 1997) (Figure 1.20). In general, glucose-6-phosphate acts as a substrate for hexose-6-phosphate dehydrogenase, an enzyme that generates NADPH (Banhegyi *et al.*, 2004). Because NADPH is necessary for maintaining 11βHSD1's reductase activity (Banhegyi *et al.*, 2004), inhibition of glucose-6-phophate translocase causes a reduction in microsomal glucose-6-phosphate levels leading to diminished NADPH generation, impaired 11βHSD1 reductase activity, hence reduced cortisol generation. Glucose-6-phophatase is also central to the regulation of gluconeogenesis and glycogenolysis (Hemmerle *et al.*, 1997) and inhibition of glucose-6-phosphate translocase have been shown to reduce plasma glucose concentrations in rats and mice (Parker *et al.*, 1998). Treatments with dietary polyphenols and vitamin-E have also been demonstrated to reverse corticosterone-induced oxidative stress, lipid peroxidation, hyperlipidaemia and abdominal fat accumulation in animals (Ohtsuka *et al.*, 1998; Eid *et al.*, 2003). More recently, the composition of dietary macronutrients have been shown to produce both acute and chronic effects on cortisol metabolism (Basu *et al.*, 2006; Wake *et al.*, 2006; Stimson *et al.*, 2007). In fact, consumption of dietary fat or high-fat low-carbohydrate diets have been documented to replicate the changes in cortisol metabolism and 11βHSD1 activity that are seen in obesity and the metabolic syndrome (Morton *et al.*, 2004a; Drake *et al.*, 2005; Stimson *et al.*, 2007).

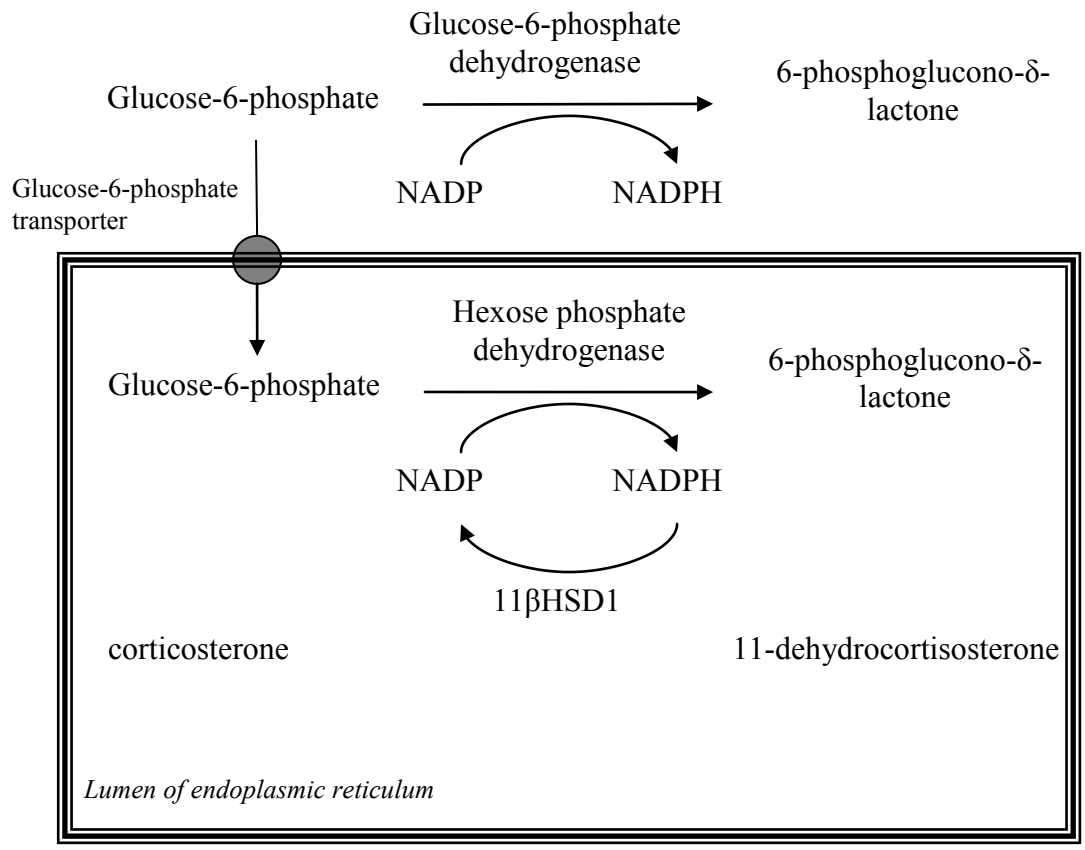


Figure 1.20 Inhibition of glucose-6-phosphate by CGA (Kenyon, see Dickinson, 2007).

However given the limited studies conducted in humans and the growing evidence suggesting a potential role of diet and nutrition in regulating glucocorticoid metabolism and the ability of polyphenols to both adversely and positively affect cortisol metabolism (see Table 1.9 and London and Castonguay, 2009), investigating the effect of phenolic compounds, such as CGA in GCBE and DC polyphenols, on cortisol metabolism in overweight and obese individuals and assessing for a correlation between cortisol, glucose, insulin, and blood pressure and lipid profile might be essential to elucidating the mechanisms by which these phenolic compounds influence the above metabolic parameters. This is particularly true when considering that currently efforts are being made to identify selective 11βHSD1 inhibitors as novel treatments for obesity and the metabolic syndrome (Seckl *et al.*, 2004).

1.12. Overview and rationale for Thesis

The following statements summarise the key themes discussed in Chapter 1:

- Overweight and obesity are complex multi-factorial conditions that are associated with a wide array of metabolic sequelae including insulin resistance, hypertension and dyslipidaemia.
- Insulin resistance lies at the centre of obesity-related complications with this pathophysiological feature being influenced by excess adiposity and defects in glucocorticoid metabolism and oxidative stress.
- Polyphenols are a large and diverse group of antioxidants whose clinical importance arises from their wide distribution in the diet and their ability to regulate various physiological, endocrinological and antioxidant processes.
- Hydroxycinnamic acids and flavonoids are the two main classes of polyphenols that can act as potential therapeutic compounds.
- GCBE and DC are key dietary sources of hydroxycinnamic acids and flavonoids, respectively, and considerable evidence exists to suggest a role for these polyphenols-rich products in modulating glucose metabolism, blood pressure and lipid profile.

Against this background, it could be hypothesised that polyphenol-rich products such as GCBE and DC, with their antioxidant properties and ability to regulate glucose, lipid and blood pressure homeostasis via various biochemical and endocrinological mechanisms (discussed in Section-1.10 to section 1.11.7) may be critical to modulating insulin resistance, hypertension and dyslipidaemia in overweight and obesity. This is particularly evident when considering that these metabolic abnormalities are closely linked to increased risk of type II diabetes, hypertension and CVD. The clinical relevance of polyphenols could also be derived from their potential ability to counteract the endocrinological imbalance and oxidative stress that often underlie overweight and obese-related insulin resistance. Notably, the role of polyphenols in glucocorticoid metabolism emerges as a promising field of research as few studies exit in this area despite the established role of glucocorticoid in the regulation of glucose metabolism, body weight and oxidative stress and the mounting evidence concerning the involvement of HPA hyperactivity in the pathogenesis of obesity-related complications. Moreover, it remains unknown whether polyphenols can selectively and differentially target defects in glucocorticoid metabolism in liver, kidney and adipose tissue, which could have important implication to the treatment of overweight and obesity

and prevention of hepatic FFA accumulation. The diversity and complexity of polyphenols also implies that differences may exist between the mechanisms of action of the various polyphenolic groups. As such improving our understanding of basis of the health-promoting properties of polyphenols is central to identifying the most relevant polyphenolic classes to overweight and obesity and towards designing effective dietary strategy aimed at preventing or reducing the occurrence of chronic conditions. Finally, it remains to be stated that at the time this thesis was first initiated only one randomised-controlled trial had examined the effect of GCBE on weight management in overweight an obese individuals (Dellalibera *et al.*, 2006) and almost none have investigated the potential therapeutic properties of GCBE and DC in counteracting overweight and obesity-related complications.

1.13. Aim of the Thesis

This PhD research aims to investigate the effect of polyphenol-rich GCBE and DC on glucoregulatory biomarkers, blood pressure and lipid profile in overweight and obese individuals, the hypothesis being that polyphenol-rich GCBE and DC may improve glucoregulatory biomarkers, blood pressure and lipid profile in overweight and obese individuals. To test this hypothesis and to achieve this overall aim, a series of *in vitro*, animal and human studies were conducted, the aims of which are described below.

Objective 1: *In vitro* studies

The *in vitro* work consisted of two studies, which aimed:

1. To test the antioxidant properties and free-radical scavenging capacity of green coffee and *Theobroma cacao* bean extracts using three different extraction methods and a panel of *in vitro* antioxidant assays including Folin-Ciocalteu, FRAP, ORAC and DPPH.
2. To explore the potential inhibitory properties of green coffee and *Theobroma cacao* bean extracts on pancreatic lipase with a view of elucidating the mechanisms for the lipid-lowering effects of green coffee and *Theobroma cacao* bean extracts described in the literature.

Objective 2: Animal studies

1. To examine the effect of chlorogenic acid on tissue glucocorticoid levels in mice and to assess for tissue-specific differences in the effect of chlorogenic acid on glucocorticoid metabolism.

Objective 3: Human studies

The human studies consisted of two preliminary studies and one main trial with the following aims:

Green coffee bean extract: Preliminary study

1. To examine the effect of GCBE on fasting glucose, blood pressure, total cholesterol, urinary cortisol, cortisone and cortisone-to-cortisol ratio in overweight and obese subjects

Dark chocolate: Preliminary study

1. To examine the effect of different doses of polyphenol DC on fasting glucose, blood pressure, total cholesterol, urinary cortisol, cortisone and cortisone-to-cortisol ratio in overweight and obese subjects and to identify minimum DC dose capable of inducing a significant reduction in fasting glucose.
2. To determine whether the outcome of the preliminary study necessitates the need to conduct a large-scale, long-term study.

Main dark chocolate study

1. To elaborate further on the effect of polyphenol-rich DC on glucoregulatory biomarkers, blood pressure and lipid profile in overweight and obese females.

Specific rationales for conducting *in vitro*, animal and humans studies and the significance of these studies are also provided below.

1.13.1 Rationale for investigating antioxidant properties of green coffee and *Theobroma cacao* bean extracts

Oxidative stress is one of the underlying and key factors involved in the pathogenesis of chronic diseases (Brownlee, 2001, 2005). Understanding the antioxidant properties of dietary compounds is particularly important in relation to overweight and obesity, since these conditions are characterised by a state of systemic and adipose-tissue oxidative stress (Keaney *et al.*, 2003, Furukawa *et al.*, 2004). Moreover, in recent years multiple forms of insulin resistance have been linked to ROS overproduction (Houstis *et al.*, 2006). Since insulin resistance is central to the pathogenesis of overweight and obesity-related complications (see Section 1.3), examining the potential antioxidant properties of GCBE and *Theobroma cacao* bean extract (TCBE) could be pivotal to elucidating the molecular mechanisms by which these antioxidant-rich products may protect against disturbances in

insulin sensitivity and glucose homeostasis. The rationale behind selecting various extraction methods and antioxidant assays was to give insight into the variability of extraction methods and to provide a detailed antioxidant profile for both GCBE and TCBE (Ou *et al.*, 2001, Huang *et al.*, 2005; Prior *et al.*, 2005). In relation to the latter, the diversity of ROS, the complexity of the antioxidant mixtures present in food products and variations in their mechanisms of action imply that no standard assay is capable of measuring the ability of antioxidants to scavenge all of the different ROS (i.e. total antioxidant activity) in a single reaction medium (Huang *et al.*, 2005, Prior *et al.*, 2005). As a result, both electron transport (ET)-based antioxidant assays such as Folin-Ciocalteu (FC), FRAP and DPPH and hydrogen atom transfer (HAT)-based assays such as ORAC need to be employed if a comprehensive antioxidant profile is to be gained (Huang *et al.*, 2005, Prior *et al.*, 2005).

Data from this experiment was subsequently used to estimate the potential contribution of GCBE, TCBE and *Theobroma cacao*'s products such as polyphenol-rich DC to the total antioxidant intake in the UK adult population. The data obtained from this study also served as the basis for examining GCBE and TCBE's potential inhibitory activities on pancreatic lipase.

1.13.2 Rationale for investigating the effect of green coffee and *Theobroma cacao* bean extracts on pancreatic lipase

It is increasingly recognised that polyphenols can affect the activity of numerous enzymes that regulate glucose, protein and lipid metabolism. Consistent with this several studies have shown that extracts of *Salacia reticulate* (Yoshikawa *et al.*, 2002), grape seed (Moreno *et al.*, 2003), berries (McDougall *et al.*, 2005, McDougall and Stewart, 2005), oolong tea (Nakai *et al.*, 2005) and green tea (Koo and Noh, 2007) inhibit α-amylase, α-glucosidase, trypsin and pancreatic lipase. Inhibition of pancreatic lipase, in particular, by polyphenolic-rich plant extracts offers a natural dietary approach to preventing obesity. This if combined with the ability of polyphenols to improve antioxidant status and reduce risk of diabetes, hypertension and coronary heart disease will result in additional benefits to obesity, the reduction of obesity-related complications

Obesity is characterised by an imbalance between energy intake and energy expenditure. Strategies aimed at preventing or treating obesity often focus on blocking fat absorption and enhancing thermogenesis, amongst others (see review by Bray and Tartaglia, 2000). Both GCBE and cocoa reportably affect various pathways involved in lipid metabolism. In

relation to GCBE, Shimoda *et al.* (2006) has demonstrated that GCBE reduces hepatic TG and visceral fat accumulation while enhancing the activity of carnitine-palmitoyltransferase. Cocoa, on the other hand, has been shown to reduce serum TG, downregulate fatty acid synthesis and transport in liver and adipose in addition to stimulating thermogenesis (Matsui *et al.*, 2005). All of the above evidence highlights the need for investigating the effect of GCBE and TCBE on pancreatic lipase.

1.13.3 Rationale for preliminary green coffee bean extract study

Several studies have linked consumption of GCBE rich in CGA with reduced blood pressure (Suzuki *et al.*, 2002; Kozuma *et al.*, 2005, Suzuki *et al.*, 2006; Watanabe *et al.*, 2006). GCBE have also been shown to regulate glucose metabolism and improve glycaemic control in a range of *in vitro*, animal and human trials (Sirinek *et al.*, 1983; Welsch *et al.*, 1989; Hemmerle *et al.*, 1997; Johnston *et al.*, 2003; Wu *et al.*, 2005; Blum *et al.*, 2007; Bassoli *et al.*, 2008). However, the relevance of these findings to overweight and obese population remains unknown. To date no study has investigated the effect of GCBE consumption on blood pressure regulation in overweight and obesity despite excess body weight being the most common cause of essential hypertension (Fletcher *et al.*, 1999). Moreover data on the blood pressure-lowering effects of GCBE in healthy normotensives subjects are conflicting (Ochiai *et al.*, 2004). In relation to glycaemic control, although Blum *et al.* (2007) has reported improved glucose tolerance in individuals with BMI> 25kg/m^2 following 40 days consumption of 200mg GCBE three times a day, the mechanism concerning this glucose-lowering effect remains speculative. The present open-label prospective non-blinded preliminary trial was based on the hypothesis that GCBE may reduce the above cardiometabolic risk factors in overweight and obese individuals through its potent antioxidant properties (see Section 3.1) and its potential inhibitory activity against pancreatic lipase (Section 3.2) and possibly 11βHSD1 (Section 1.11.7).

1.13.4 Rationale for animal study

Increased 11βHSD1 activity is implicated in the development of the metabolic syndrome (Lindsay *et al.*, 2003, Seckl *et al.*, 2004). Identifying natural compounds that influence 11 βHSD1 activity could lead to novel methods of treating obesity, CVD and diabetes. To elaborate further on the findings of the preliminary GCBE study and to establish the implications of the observed changes in glucocorticoid levels to overweight and obesity, the animal study was conducted to examine the effect of CGA on tissue corticosterone levels in

mice, corticosterone being the analogous of cortisol in humans. This study was part of a larger trial investigating CGA's potential therapeutic effects against the metabolic syndrome, especially in relation to glucose and lipid metabolism (Dickinson, 2007). Examining CGA effects on tissue glucocorticoid metabolism is particularly important considering that the main component of GCBE, CGA, can regulate hepatic FFA accumulation (Dickinson, 2007), an important factor contributing to the development of systemic insulin resistance (see Section 1.7.3). Moreover, emerging findings suggest potential storage of CGA and its metabolite ferulic acid in the liver (Farah, 2009). Consequently, if CGA is proven to influence glucocorticoid metabolism in the liver, this may have important metabolic consequences to overweight and obesity since defects in hepatic 11βHSD1 are closely implicated in the development of obesity-related co-morbidities.

1.13.5 Rationale for preliminary dark chocolate study

Epidemiological studies link high polyphenol intake with reduced risk of oxidative stress-related diseases like diabetes, hypertension and CVD (Buijsse *et al.*, 2006; Pereira *et al.*, 2006; McCullough *et al.*, 2006). In particular consumption of cocoa and DC has been shown to improve endothelium function, insulin sensitivity, blood pressure in healthy individuals, hypertensives with or without glucose intolerance (Grassi *et al.*, 2005a-b, 2008) and obese subjects (Davison *et al.*, 2008). Cocoa and DC are rich sources of polyphenols providing on average more polyphenols per serving than red wine, green tea or black tea (Lee *et al.*, 2003). These polyphenols confer potent antioxidant properties to cocoa and DC (Richelle *et al.*, 2001; Lee *et al.*, 2003) in addition to their ability to regulate NO (Grassi *et al.*, 2005b, 2008).

Obesity is known to be associated with insulin resistance and elevated blood pressure (Section 1.2.3). One of the underlying factors linked to these cardiovascular risk factors is abnormal cortisol metabolism (Section 1.8.3). Cortisol is a counter-regulatory hormone that is essential in the long-term maintenance of blood glucose and which could also unfavourably influence blood pressure (BP) and lipid profile (Section 1.8.1). When present in excess, cortisol induces overproduction of ROS (Iuchi *et al.*, 2003; Bjelaković *et al.*, 2007) leading to reduced endothelial NO synthase expression (Liu *et al.*, 2009). In obesity, particularly abdominal obesity, postprandial hypercortisolism and enhanced peripheral metabolism of cortisol, characterised by increased urinary cortisone-to-cortisol ratio, are observed which are linked to insulin resistance and increased fasting insulin (Vicennati and Pasquali, 2000). Increased expression of subcutaneous adipose tissue 11βHSD1 has also

been reported which is known to impair glucose-stimulated insulin secretion (Alberti *et al.*, 2007). Since improved NO bioavailability is the main mechanism by which DC polyphenols reduce endothelium dysfunction, insulin resistance and hypertension (Grassi *et al.*, 2005a-b, Grassi *et al.*, 2008), this preliminary DC study aimed to assess and compare the effect of DC containing two different doses of polyphenols on fasting capillary whole blood glucose levels, total cholesterol, BP, urinary free cortisol and cortisone excretion in healthy overweight and obese subjects. The other objective was to observe whether improvements in fasting blood glucose, total cholesterol and BP could be correlated with changes in urinary free cortisol or cortisone excretion. A secondary objective was to monitor magnesium intake and excretion since DC is known to contain large quantities of magnesium, which, in turn, could influence BP, insulin action and metabolic syndrome (Meisel, 2005; Song *et al.*, 2005; Song *et al.*, 2007).

1.13.6 Rationale for main dark chocolate study

Given the positive results of the preliminary study and the inheretent weaknesses of using capillary whole blood for measurements of glucose and total cholesterol, the main DC study was designed to elaborate further on the long-term effect of consumption of 500mg polyphenols DC on glucose, BP, lipid and glucocorticoid metabolism using more robust techniques. The objective being to monitor several biomarkers of glucose metabolism including fasting glucose, fasting insulin, HOMA-IR, QUICKI, revised-QUICKI and HOMA-β alongside obtaining a detailed lipid and glucocorticoid profile. In relation to glucocorticoids, this was achieved by quantifying urinary, salivary as well as serum cortisol and cortisone levels. A secondary objective was to assess for differences in response to polyphenol-rich DC between lean females and overweight and obese females and to monitor for any changes in body composition in the overweight and obese group.

Establishing the long-term benefits of consumption of 500mg polyphenols DC is important since it will provide information over the sustainability of the effect of polyphenols on glucose metabolism and BP. Moreover, monitoring body composition is essential in the overweight and obese group given that 20g of DC provided an additional 100kcal and 7.34g of fat per day. If proven to be effective, the advantage of using 500mg polyphenols as opposed to 1000mg could be seen in the potential greater palatability and acceptability of 500mg DC to consumers, as a reduction in the polyphenol content also implies a reduction in the bitterness of chocolate.

2. General Materials and Methods

2.1. *In vitro* studies

2.1.1 Chemicals and validation of antioxidant assays

Ascorbic acid, gallic acid and chlorogenic acid were from Sigma-Aldrich. Folin-Ciocalteu, sodium carbonate, formic acid, ferrous sulphate, sodium acetate, 2,4,6-tris(2-pyridyl)-S-triazine, ferric chloride, fluorescein, 2, 2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), 1, 1 –diphenyl-2-picilhydrazyl, , p-nitrophenyl laurate, Trition X-100 were obtained from Sigma (Poole, Dorset, UK). Lipase was from porcine pancreas Type II (Sigma product L3126). Acetonitrile, ethanol, glacial acetic acid, hydrochloric acid, sodium hydroxide, trolox were from Fisher Scientific (Loughborough, Leicestershire, UK). All other chemicals and reagents were obtained from Sigma-Alrdich (Poole, Dorset, UK) unless otherwise stated.

All antioxidant assays were validated by replicate analyses of known concentrations of standards as summarised in Appendix 1. Accuracy was estimated by calculating the percentage deviation of measured standard from its expected concentration (Medina-Remon *et al.*, 2009). Precision was calculated by dividing the S.D. by the mean measured concentration to obtain the coefficient of variation (CV), which when conveyed as a percentage gives the relative standard deviation (R.S.D.) (Medina-Remon *et al.*, 2009).

2.1.2 Folin-Ciocalteu method

2.1.2.a Principle of method

The Folin-Ciocalteu (FC) method is a colorimetric assay which was adapted by Singleton and Rossi (1965) from an earlier method using Folin-Denis reagent. It measures the ability of a sample to reduce yellow heteropoly phosphomolybdate-tungstate anions in the FC reagent to blue using GA as a standard (Singleton and Rossi, 1965). The extensive use of GA as a standard in FC method is explained by the fact that GA represents the mean reaction between FC reagent and phenol aglycones and conjugates such as quercetin, catechin, procyanidins, caffeic and CGA (Singleton and Rossi, 1965). Albeit non-specific, the simplicity and reproducibility of the FC method has permitted its extensive use in the literature for the quantification of total phenolic compounds in plant extracts and biological samples(Brat *et al.* 2006, McDougall *et al.*, 2005; Roura *et al.*, 2006).

2.1.2.b Preparation of extracts

GCBE tablets were weighed to the nearest 0.0001g, grinded to a fine powder using a mortar and pestle, then dissolved in 20ml ethanol (EtOH) to give ethanol extract, in 20ml of acidified water (0.2% v/v formic acid/ water) to give acidified-water extract or in 20ml acetonitrile-water-formic acid solution (50:50:2 v/v/v) to give acetonitrile extract. The resulting mixtures were then extracted at room temperature at 200rpm for 30min on a Luckham, Model R100/TW rotatest shaker and centrifuged at 4000rpm for 15 min at 4^{o}C to remove any solid material. After centrifugation, the sample was run through porous-free filter using vacuum and the resulting solution collected in a Schlenk flask.

For TCBE, 20g of *Theobroma Cacao* bean was dissolved in 200ml of acetonitrile-water-formic acid solution (50:50:2 v/v/v), homogenised 3 times to remove fat materials using Ultra-Turrax homogenizer (Rose Sci Ltd) and then extracted on a rotatest shaker, as described above. Samples were treated with rotary evaporation to remove acetonitrile then loaded onto a 12-ml bed volume C18-E Giga Tube (Strata, Phenomenex, UK) previously conditioned and equilibrated with 98% methanol (MeOH) and acetone, respectively. Loaded samples were then washed with acidified water to obtain the unbound fraction. The bound fraction, rich in proanthocyanidins, was eluted with 2x 12ml acetonitrile. To extract the polyphenols further, solid-phase extraction was repeated 3 times using the unbound fraction instead of sample.

All extractions were carried out in triplicates and the coefficient of variation for each extraction was calculated (Hammerstone *et al.*, 2000). This was to examine whether any differences in procyanidin content were related to variation in the samples or extraction method (Hammerstone *et al.*, 2000).

2.1.2.c Standards

A 50mg/L GA stock standard was prepared and serially diluted in water to give the following concentrations: 5, 10, 20, 30, 40, 50mg/L GA.

2.1.2.d Measurement of total phenolic content

Total phenol content of extracts was determined using a modification of the method of Singleton and Rossi (1965). Briefly, 250µl of half-strength FC reagent (FC diluted 1:1 in distilled water) was added to a 1.6ml cuvette (Fisher Scientific, Leicestershire, UK)

containing 250µl of standard or diluted sample (1% dilution). Following a 3-min incubation, 500µl sodium carbonate (130g sodium carbonate in 1L water) was added and the samples were left to stand in darkness for 1h. Absorbance was read at 750nm against a water blank using an Ultraspec II spectrophotometer (LKB Biochrom Ltd, UK). A standard curve was constructed by plotting absorbance (nm) against known concentrations of GA standards and results were expressed as mmol gallic acid equivalents (GAE) per litre. All test samples were analysed in triplicates.

2.1.3 Ferric-reducing capacity of plasma

2.1.3.a Principle of the method

The use of the FRAP assay as a measure of total antioxidants was first described by Benzie and Strain (1996). The FRAP assay is a colorimetric assay used to measure the ability of a sample to reduce the straw-coloured ferric-2, 4, 6-tri-2pyridyl-s-triazine (TPTZ-Fe^{3+}) complex to the blue-coloured ferrous-2, 4, 6-tri-2-pyridyl-s-triazine (TPTZ-Fe^{2+}) form under acidic conditions (Benzie and Strain, 1996). Compared to ORAC and DPPH, the FRAP assay has the advantage of directly measuring the concentration of reductants in any given sample (Halvorsen *et al.*, 2002).

2.1.3.b Preparation of extracts

Extracts were prepared as for FC method.

2.1.3.c Standards

Ferrous sulphate (0.278g) was weighed and dissolved in 1 L of distilled water to give a 1mM stock solution. The stock was serially diluted in phosphate buffer saline (PBS) to give the following standard concentrations: 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM Fe^{2+}.

2.1.3.d Acetate buffer

A 300mM acetate buffer solution (pH 3.6) was prepared by dissolving 3.1g sodium acetate and 16ml of glacial acetic acid in 1 L of distilled water.

2.1.3.e Ferric-2, 4, 6-tri-2-pyridyl-s-triazine

A 10mM TPTZ solution was prepared by dissolving 0.031g TPTZ in 10ml of 40mM hydrochloric acid (HCL).

2.1.3.f Ferric chloride

A 20mM ferric chloride solution was prepared by adding 0.054g of ferric chloride in 10ml distilled water.

2.1.3.g Working ferric-reducing capacity of plasma solution

The working FRAP solution was prepared by combining 100ml of 300mM actetate buffer (pH 3.6) with 10ml ferric chloride, 12ml distilled water followed by 10ml of TPTZ. The TPTZ solution was added drop-wise giving a straw coloured working FRAP solution. Any traces of a blue colour were suggestive of contamination with a reducing compound indicating the need to discard the solution. The working FRAP solution was kept in a water bath at 37^{o}C.

2.1.3.h Measurement of ferric-reducing capacity of plasma

To a 96-well microplate, 6µl of standard or diluted sample was added, followed by 200µl of FRAP reagent. The plate was left to incubate in an oven at 37^{o}C for 4min and absorbance read at 600nm using a Dynex Technologies MRX microplate reader (Dynex Technologies Ltd, Worthing, UK). Results were expressed in mmol Fe^{2+} per litre.

2.1.4 Oxygen radical absorbance capacity

2.1.4.a Principle of method

The ORAC method was initially developed by Cao *et al.* (1993, 1995, and 1999), adapted by Ou *et al.* (2001) and subsequently optimised by Girard-Lalancette *et al.* (2009). Its principal is based on the oxidation of a fluorescent probe by peroxyl radicals generated by AAPH (Ou *et al.*, 2001). This oxidation (decay) results in a decline in fluorescence intensity that could be captured over time using a fluorometer (Huang *et al.*, 2005). Since Antioxidants could delay peroxyl-radical induced fluorescence decay, the antioxidant capacity of a compound or biological sample could quantified by comparing the area under the fluorescence decay curve (AUC) of the sample against the AUC of the blank (Ou *et al.*, 2001, Huang *et al.*, 2005).

Unlike most antioxidant assays the ORAC assay has the advantage of measuring the rate and the degree of free-radical inhibition by an antioxidant based on the HAT mechanism (Cao *et al.*, 1998). Most other antioxidant assays like FC and FRAP use a single ET reaction (Prior

et al., 2005). This led a number of authors to suggest that the ORAC assay is physiologically more relevant since it is capable of directly measuring the chain-breaking (free-radical scavenging) activity of a sample as opposed to simply providing a measure of the potential reducing-capacity of a sample (Ou *et al.*, 2001, Huang *et al.*, 2005, Prior *et al.*, 2005). Moreover, ORAC can quantify the ability of an antioxidant to scavenge the peroxyl radical involved in lipid peroxidation (Prior *et al.*, 2005). This could be best illustrated by Equation 1 and Equation 2. Equation 1 shows the underlying principal of HAT reaction wherein the antioxidant inhibits free-radicals by directly donating a hydrogen atom. Conversely, Equation 2 shows the principal of the ET reaction wherein an antioxidant could potentially inhibit free-radicals by initially donating an electron to the free radical and subsequently reacting with H_2O to produce H_3O which then inhibits the free-radical by donating a hydrogen atom (Pior et al, 2005).

Equation 1 $X. + AH \rightarrow XH + A.$

Equation 2
$X. + AH \rightarrow X- + AH.+$ (1)
$AH.+ H_2O \rightarrow A. + H_3O+$ (2)
$X- + H_3O+ \rightarrow XH + H_2O$ (3)
$M(III) + AH \rightarrow AH+ + M(II)$ (4) (Prior *et al.*, 2005).

The use of fluorescein probe also renders the ORAC method more robust and cost-effective because of the photostability of fluorescien, which permits the use of 96-well microplate (Ou et al, 2001). As a result, the ORAC assay is considered the gold standard method for the determination of the antioxidant capacity of biological samples (Cao et al, 1998).

2.1.4.b Preparation of extracts

Extracts were initially prepared in acetonitrile as for FC method and then diluted 1:1000 in (75mM, pH 7.4) phosphate buffer.

2.1.4.c Standards

A 10mM stock solution was prepared by dissolving 0.025g trolox in 10ml (75mM, pH 7.4) phosphate buffer saline (PBS). The stock was serially diluted in PBS to give the following standard concentrations: 0, 6.25, 12.5, 25, 50 and 100 μM trolox equivalents.

2.1.4.d Fluorescein

A 40mM stock solution was prepared by dissolving 0.1505g fluorescein in 10ml (75mM, pH 7.4) PBS and stored at 4^{o}C until required. Prior to use, the stock was diluted in (75mM, pH 7.4) PBS to give a 400nM fluorescein solution.

2.1.4.e 2,2'-azobis-(2-amidinopropane) dihydrochloride

A 375mM solution was prepared daily by dissolving 1.017g AAPH in 10ml (75mM, pH 7.4) phosphate buffer.

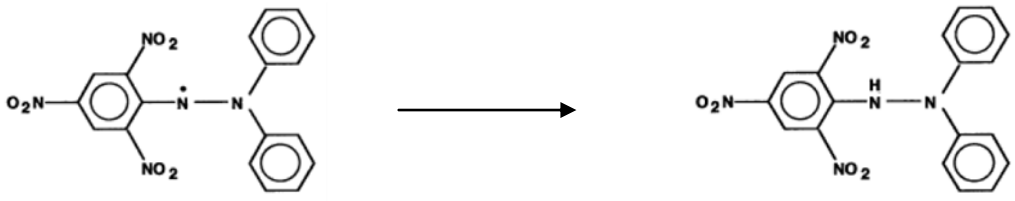
2.1.4.f Measurement of oxygen radical absorbance capacity

To a 96-well fluorescein reading microplate (Wallac, Finland), 25µl of standard or diluted sample was added, followed by 150µl of 400nM fluorescein. The plate was then incubated at 37^{o}C for 15min. Next, 25µl of 375mM AAPH was added and the decay in fluorescein intensity was measured kinetically over 35 min using a Fluoroskan Ascent plate reader (Thermo Scientific, USA) equipped with a 488nm excitation filter and a 515 nm emission filter. Results were expressed in µmol trolox equivalents per litre.

2.1.5 2,2-diphenyl-1-picrylhydrazyl radical

2.1.5.a Principal of method

Unlike the FRAP assay, the DPPH assay serves as an indirect measure of reductants in a sample since it measures the inhibition of ROS in the reaction medium (Halvorsen *et al.*, 2002). The purple DPPH radical has an absorbance wavelength of 515 nm. When an antiradical compound donates H^{+} to the DPPH molecule, DPPH is reduced forming a colourless compound (Brand-Williams *et al.*, 1995; Molyneux, 2004), as illustrated in the following equation:



Purple diphenylpicrylhydrazyl radical Colourless diphenylpicrylhydrazine non-radical

The main limitations of DPPH assay is that colour interferences from samples containing anthocyanidins could lead to underestimation of antioxidant activity (Teow *et al.*, 2007). This could be overcome by preparing extracts in colourless solvents such as EtOH or MeOH (Molyneux, 2004). Measuring the absorbance of samples without DPPH and subtracting from the absorbance of sample with DPPH could also help overcome this limitation. Another limitation of DPPH assay is that DPPH could take up to several hours to react with certain compounds (Teow *et al.*, 2007).

2.1.5.b Preparation of extracts

GCBE and *Theobroma cacao* were ground using a mortar and pestle and dissolved in EtOH to give a 60µM GAE stock solution which was serially diluted to obtain the following concentrations: 3, 6, 15, 30, 60 µM GAE.

2.1.5.c Standards

DPPH standards were prepared in the following concentrations 0, 5, 10, 20, 40 and 60µM. These standards were used to construct a standard curve of DPPH concentrations against absorbance, which permitted the calculation of DPPH concentration in the reaction medium.

2.1.5.d Positive controls

Ascorbic acid, GA and CGA were dissolved in EtOH to give a 60µM stock solution of each antioxidant. These stock solutions were serially diluted to give 3, 6, 15, 30 and 60µM dilutions of each antioxidant.

2.1.5.e Measurement of 2,2-diphenyl-1-picrylhydrazyl radical scavenging

The effect of GCBE and TCBE on DPPH radical was determined using an adaptation of the method described by Blois (1958). Briefly, a 100µl of each GCBE, TCBE and positive control dilutions were incubated with an equal volume of DPPH in a 96-well microplate. A solution of 95% EtOH acted as a blank. The test samples were left to stand in the dark at room temperature and absorbance was read at 500 nm using a Dynex Technologies MRX microplate reader (Dynex Technologies Ltd, Worthing, UK). All solutions were made up in duplicates. Percentage of remaining DPPH was calculated using the following equation.

$$\% \text{ remaining DPPH} = [DPPH]t / [DPPH]t=0$$

Percentage DPPH inhibition was estimated:

%inhibition=((blank-test sample)/blank) x 100 (Ayoola *et al.*, 2008).

The inhibitory concentration 50 (IC50), defined as the concentration of antioxidant required to reduce the initial concentration of DPPH by 50%, was determined by plotting the percentage of remaining DPPH against increasing concentrations of GCBE, TCBE, GA, ascorbic acid and CGA using a non-linear regression model (GraphPad Prism 5, USA). A linear regression model was used for estimating IC50 of CGA since the non-linear regression model did not generate the best-fit curve for the data.

2.1.5.f Statistical analysis

Differences in IC50 between the 5 different extracts and controls were analysed using a one-way analysis of variance (ANOVA) (GraphPad Prism 5, USA).

2.1.6 Pancreatic lipase: Preparation of extracts

GCBE and TCBE were prepared in acetonitrile as described for FC assay (Section 2.1.2.b).

2.1.7 Lipase assay

Pancreatic lipase activity was measured according to the method described by Lin *et al.* (1996) and Gilham *et al.* (2003), with some modification, using p-nitrophenyl laurate (pNP) (10mg/ml) as the substrate. Briefly, 50µl of varying concentration of GCBE or bound TCBE fraction (5, 10, 25 and 50µg total polyphenols (GAE)) were added to a mixture containing 350µl 100mM Tris buffer (pH 8.2) and 150µl of porcine lipase solution (10mg/ml). The reaction was started by adding the substrate solution. The latter was prepared by dissolving 8mg pNP in 10ml of 5 mM sodium acetate (pH 5.0) containing 1% Trition X-100, which was subsequently heated in boiling water for 1 min to aid dissolution, mixed and cooled to room temperature. The samples were then incubated for 2h at 37^{o}C. Following incubation, all samples were centrifuged at 16000rpm for 5 min, transferred to cuvettes and read at 400 nm in a UV spectrophotometer. Samples were assayed in triplicates and appropriate sample controls were prepared for each sample concentration to account for interference from GCBE or TCBE. The percentage inhibitory activity of each sample was calculated using the following equation:

$$\% \text{ inhibitory activity} = \left[1 - \frac{sample(abs) - samplecontrol(abs)}{control(abs)}\right] \times 100$$

2.2. Animals and Metabolic cage study

Seven week old male C57BL6 mice were housed at a constant temperature (22 ^{o}C) and a 12:12 h light/ dark cycle in metabolic cages at the Little France Biomedical Research Facility, Edinburgh, UK. Animals were fed a standard rodent diet with free access to water for 7 days followed by 17 days of a diet containing 0.15% CGA (treatment group) or no CGA (control group). At the end of the study period animals were decapitated, trunk blood samples were collected and organs and adipose tissue removed and weighed. All procedures and experiments were conducted according to UK Animals (Scientific Procedures) Act, 1986. Organs were kindly donated for corticosterone analysis by Dr Chris Kenyon from the Queen Medical Research Institute, Edinburgh, UK.

2.2.1 Steroid Tissue Extraction

Stock solutions of the homogenizing buffer (5mM potassium phosphate, pH 7) and extraction solvent (92.25ml EtOH + 4.75 H20 + 3ml glacial acetic acid) were prepared and stored overnight at 4^{o}C and -80^{o}C, respectively. On the following day, kidney, liver, mesenteric and subcutaneous tissue were weighed (approximately 0.1g) and transferred to a 2ml eppendorf placed on ice. Each sample was subsequently homogenised in 1ml phosphate buffer (brief bursts with cooling), and then added dripwise using a glass Pasteur pipette with continuous stirring to a 20ml scintillation vial chilled on dry ice and acetone and which contained 5ml of the extraction solvent (**Figure 2.1**). Samples were capped and frozen at -80^{o}C for 40h. Next, samples were sonitanked for 2 min (15s bursts with cooling) and transferred to centrifuge tubes using an additional 1ml of extraction solvent in order to wash remnants. Following 20min centrifugation at 4^{o}C x14 000 rpm, the supernatant was collected, placed in Pyrex tubes and evaporated to dryness. Samples were reconstituted in 0.5ml 80% MeOH plus 0.5ml distilled water. A 0.5ml aliquot was taken for corticosterone extraction using Sep-Pak C 18 cartridges (Waters Ltd, Elstree, Hertfordshire, UK) previously preconditioned and equilibrated with 5ml 100% MeOH and 2ml water. The samples were first eluted with 1ml 40% MeOH, and then with 2ml 100% MeOH. This produced two fractions of corticosterone: sulphate and glucoronide-conjugated corticosterone and free corticosterone. Both fraction were analysed for corticosterone using enzyme-linked immuno-sorbent assay (ELISA) according to the method described by Al-Dujaili *et al.* (2005, 2006) (see p. 146).

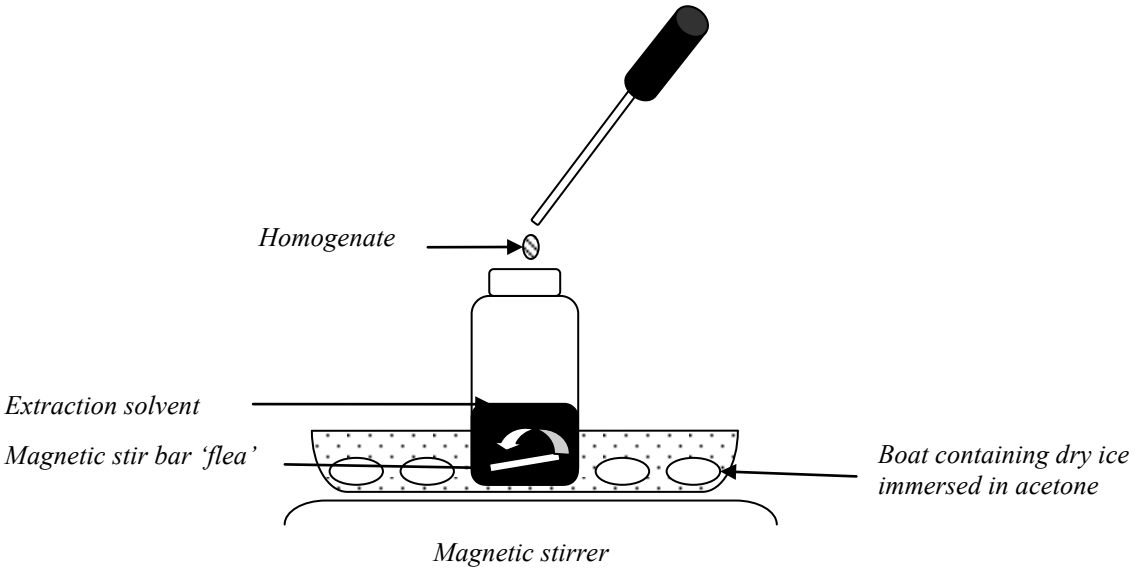


Figure 2.1 schematic showing drip-wise addition of the homogenate using a Pasteur pipette with continuous stirring into a 20ml scintillation vial containing extraction solvent, placed in a bath of dry ice and acetone.

2.3. Human studies

2.3.1 Rational for study design: Preliminary green coffee bean extract study

A self-controlled open-label single-subject experimental AB design was selected for the preliminary GCBE trial. Following an initial 7-day run-in phase, eligible volunteers were given 200mg GCBE twice daily for 14 days (Figure 2.2). Capillary fasting glucose, BP and anthropometrical measurements were assessed at the end of the run-in phase (baseline) and after 1 week and 2 weeks of GCBE administration. Capillary fasting total cholesterol was assessed at baseline at at the end of 2 weeks of each dietary intervention. Likewise, 24h urine was collected at baseline and at the end of Week 2 to assess 24 urinary free cortisol, urinary free cortisone, cortisone-to-cortisol ratio and in order to monitor any changes in sodium, potassium and magnesium excretion. Salivary cortisol and cortisone concentrations were also quantified in a sub-sample of the study population at baseline and at the end of the intervention (n=9).

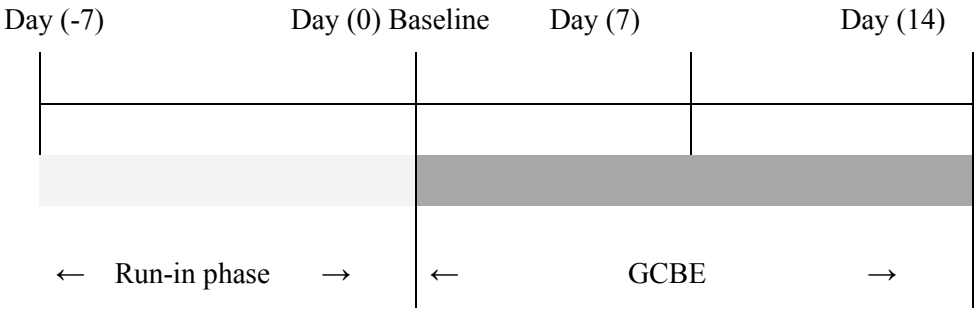


Figure 2.2 Summary of the study design

The self-controlled open-label single-subject experimental AB study design was selected primarily due to the lack of appropriate placebo tablets matched for size, shape, colour, taste and smell of GCBE tablets. Open-label trials are generally used for Phase I studies and serve as an initial investigation for new treatments (Mellis *et al.*, 2002). They are the opposite of double-blind studies in that both the investigator and the participant are aware of the treatment offered (Mellis *et al.*, 2002). As a result, they are likely to overestimate outcomes due to expectation bias (Mellis *et al.*, 2002). The single-subject AB design is also the most basic type of study designs (Satake *et al.*, 2008). It implies that the study consists of a pre-treatment period and a post-treatment period. Its main advantage lies in that subjects act as their own control which is particularly important when investigating a condition that shows

large inter-subject variations such as blood pressure (Cleophas, 1990). However, the AB design has several limitations since it does not control for the effect of time. To overcome this limitation, several baseline measurements need to be taken prior to intervention (Satake *et al.*, 2008).

In general, open-label single-subject trials are unlikely to prove the efficacy of treatment due to the inherent limitations associated with their design (Mellis *et al.*, 2002). Nonetheless, a number of studies have used this design to investigate the effect of new treatments on blood pressure and other parameters of the cardiovascular system (Shand *et al.*, 2003, O'Brien *et al.*, 2007). Investigators have also used this design to explore the potential *in vivo* activities of novel polyphenol-rich products (Blum *et al.*, 2007). An open-label single-subject experimental AB design could thus be deemed ideal for feasibility studies that could then direct future research and justify the need for more rigorous research designs.

2.3.2 Rational for study design: Preliminary dark chocolate study

To assess the feasibility of the main study, the preliminary DC study used a randomised single-blind two-period two-treatment crossover design where each subject acted as his or her own control. Following a 1-week run-in phase, eligible subjects were randomly assigned to one of the two polyphenol doses: 500 mg polyphenols DC or 1000 mg polyphenols DC. Participants followed each intervention for 2 weeks, after which they were crossed-over to the next intervention separated by a 1-week washout period (Figure 2.3). As with preliminary GCBE study, capillary fasting glucose, BP and anthropometrical measurements were assessed at the end of the run-in phase (baseline) and after 1 week and 2 weeks of each of the dietary interventions. Capillary fasting total cholesterol was assessed at baseline at at the end of 2 weeks of each dietary intervention. Likewise, 24h urine was collected at baseline and at the end of Week2 to assess 24 urinary free cortisol, urinary free cortisone, cortisone-to-cortisol ratio and in order to monitor any changes in sodium, potassium and magnesium excretion.

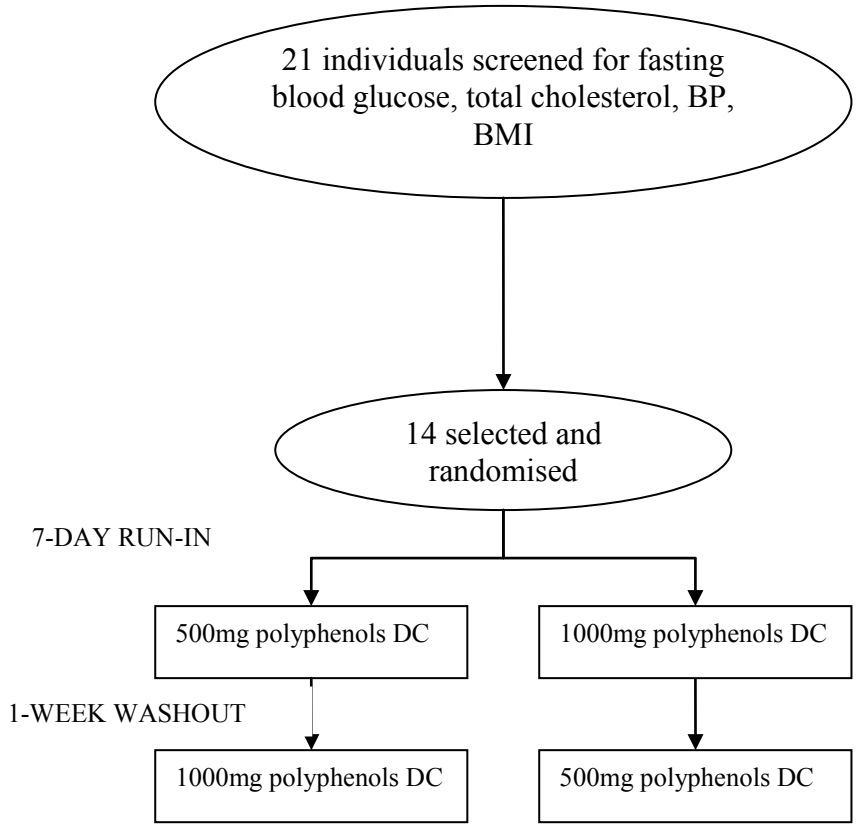


Figure 2.3 Diagram showing random allocation of subjects into the different dietary interventions

In general, the decision to use a parallel group or a cross over design depends on the balance between the merits and the weaknesses of each design given a specific condition or an outcome. In crossover studies, participants are randomly allocated to two or more sequences of treatment, each treatment period being separated by a washout period (Senn, 2002). Because each subject act as their own control, cross-over studies have the advantage of reducing systemic variations between groups and using a smaller number of participants to achieve a similar statistical power as parallel group designs (Prescott *et al.*, 1999, Richens, 2001, Grahame-Smith and Aronson, 2002). As a result, several authors argue in favour of using crossover trials in cardiovascular and hypertension research, conditions characterised by large inter-subject variations (Sever *et al.*, 1989, Cleophas, 1990). There is also some evidence to suggest that cross-over trials are statistically more sensitive than parallel-group studies when comparing treatments with a similar biological action or chemical composition (Cleophas and De Vogel, 1998, Cleophas and Zwinderman, 2002), although this view is not

held by some authors (Jackson and Yeo, 1997). The main limitation with crossover trials lies in their duration, which could be inconvenient to subjects leading to high dropout rates (Sever *et al.*, 1989). Lack of knowledge of the pharmacokinetic properties of the compound under investigation might also lead to inappropriately designed crossover trials in which carry-over and order effects are not controlled for (Senn, 2002).

Conversely, in parallel group studies participants are randomly allocated to receive treatment or control (Senn, 2002). Parallel-group studies are consequently shorter, more convenient to participants and allow for multiple comparisons of treatments of varying chemical compositions (Cleophas, 2000). They are also not susceptible to carry-over and order effects and are ideal for examining both short-term and long-term effects of a treatment (Sever *et al.*, 1989). However, the time needed to recruit large numbers of participants is a disadvantage (Richens, 2001).

Both cross-over and parallel group studies have the advantage of reducing selection bias as a result of random allocation of subjects to treatment groups (Edwards *et al.*, 1998). However this also implies that randomly allocated groups can differ in a number of baseline characteristics which according to Cleophas (1997) may or may not influence outcome. Parallel groups designs appear to be particularly susceptible to such an imbalance (Cleophas, 1995) resulting in the need to use specialised randomisation softwares to ensure groups are matched for age, gender and other baseline variables. This could be particularly difficult when recruitment rates are poor and when few eligible subjects are available. As a result, the need to adopt a multi-centre approach is often inevitable with parallel group design (Richens, 2001). Moreover, difficulties with obtaining consent might arise when patients have strong preferences for a specific treatment or when they refuse randomisation (Prescott *et al.*, 1999, Cleophas *et al.*, 1997). Excluding participants, in this case, might result in a smaller sample, which might hinder the study's external validity (Cleophas *et al.*, 1997) or render generation of matched-groups difficult. There are thus several issues relating to randomisation, patient recruitment, informed consent and ethics that need to be considered before adopting a parallel-group design. It is also important to note that in many cases, the decision to use parallel or crossover design largely depends on the number and duration of treatment periods (Prescott *et al.*, 1999). The risk of carry-over effects is also a determinant factor (Cleophas, 1990, Prescott *et al.*, 1999; Senn, 2002). In the present study the use of a cross-over design was deemed appropriate for both the preliminary and the main study since both consisted of two treatment periods each lasting for 2-4 weeks. Risk of a carry-over

effect was minimal since epicatechin has a short half-life and is metabolised and excreted rapidly. In fact, if using the criteria described by Graham-Smith and Aronson (2002) which suggests that a washout period should be equivalent to four to five half-lives of a compound, and assuming that the half-life of epicatechin is 2h (Richelle *et al.*, 1999), then a minimum washout period of 8-10h should be sufficient for this study. Nonetheless, because the effect of any chemical could persist beyond what is anticipated by its half-life (Grahame-Smith and Aronson, 2002), this study used a washout period of 1-2 weeks which is similar to the washout periods used by Taubert *et al.* (2003) and Grassi *et al.* (2005a-b).

However, a major criticism of the crossover design, particularly the one used in the preliminary study, is its inability to control for the effect of time compared to a parallel-group design (Richens, 2001). This occurs when the severity of a disease or a parameter changes over time regardless of the intervention (i.e. BP) (Cleophas and Tavenier, 1995). Efforts were made to overcome this limitation, by randomising participants using a Latin square design such that one group received 500mg polyphenols DC followed by 1000mg polyphenols DC while the other group received 1000mg polyphenols DC followed by 500mg polyphenols DC. This randomisation reduces both order (Grahame-Smith and Aronson, 2002) and time effects (Senn, 2002). Additional statistical analyses were also conducted to detect any carry-over effects in the preliminary DC study (see Section 2.18.2, p. 153)

2.3.3 Rational for study design: Main study

The main study adopted a placebo-controlled single-blind crossover design. Following a 1-week run-in phase, eligible subjects were randomly assigned to receive 500mg polyphenols DC or placebo. Participants followed each intervention for 4 weeks, after which they were crossed-over to the next intervention separated by a 2-week washout period (Figure 2.4).

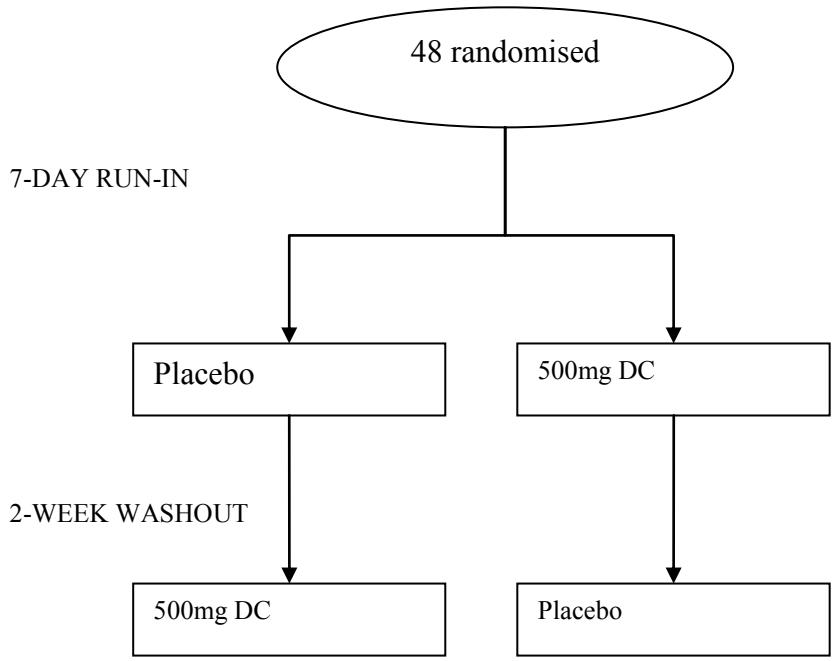


Figure 2.4 Diagram showing random allocation of subjects into the different dietary interventions

This design is generally used to measure the efficacy of a treatment under controlled conditions while controlling for placebo effects. To overcome the limitations observed in the pilot study, baseline data were collected prior to each intervention period. Given an adequate washout period, incorporating baseline measurements as covariates in statistical analysis has been suggested to improve data precision by reducing standard error (Senn, 2002). Additionally, the main study used a placebo to control for time effects and to asses for a causal relation. Notably, the main study has the advantage of using a DC placebo as compared to a white chocolate which has been generally used in previous studies (see Table 1.5, p. 77). The lack of adequate blinding in previous research might have acted as a confounding factor increasing expectation bias since both the investigator and the participant were aware of the treatment received (Desch *et al.*, 2009).

Crossover studies are generally designed to measure the efficacy of a treatment while a parallel-group design measures a treatment's effectiveness. Efficacy implies whether a treatment works under controlled conditions and effectiveness under less astringent conditions. The efficacy of a drug does not necessarily determine its effectiveness and broader long-term studies are needed to confirm the effectiveness of an intervention (Pittler and White, 1999). This suggests that regardless of the efficiency of cross-over trials, the use

of parallel-design studies remains indispensable for confirming the findings of cross-over trials and ensuring their generalisation (Sever *et al.*, 1989, Cleophas and Tavenier, 1995).

2.4. Ethical approval

Ethical approval was gained from Queen Margaret University ethics committee and all interventions involving human volunteers were conducted according to the guidelines laid down in the Declaration of Helsinki. For studies involving human subjects, information sheets were provided to all potential volunteers and written informed consent was obtained prior to participation (Appendix 1). All collected data was stored according to the Data Protection Act (1998).

2.5. Subjects sources and selections

For the pilot studies, volunteers were recruited via an email sent to all staff and students at Queen Margaret University and using fliers or word-of-mouth.

For the main DC study, a detailed recruitment strategy was developed to ensure that the obtained study sample provided a good representation of the general population. As a result, in addition to recruiting volunteers from various universities, colleagues and business parks across Edinburgh, advertisement using fliers and posters in newspapers, community centres and websites such as clinicaltrials.gov were used. Websites yielded the least response but were nonetheless central to the success of newspaper and community centre advertisements as they permitted the general population to access detailed information on the study's objectives and design without necessitating the need for contacting the investigator. This helped facilitate recruitment by concentrating the sample which permitted the focus on the target population.

2.6. Inclusion and exclusion criteria

2.6.1 Preliminary studies

These studies included healthy volunteers aged 19-50years with a BMI $\geq$25kg/m^2 and no history of diabetes, hypotension, hypertension or CVD (**Table 2.1**). They, therefore,

included both overweight (BMI 25-29.99kg/m^2) and obese subjects (BMI ≥30kg/m^2) as defined by the criteria set by WHO (2000).

Table 2.1 Definition of diabetes, hypotension and hypertension

Disease	Definition	Reference
Diabetes	▪ Diabetes symptoms (i.e. polyuria, polydipsia and unexplained weight loss) plus ▪ fasting plasma glucose≥7.0mmol/l (whole blood ≥6.1mmol/l) or ▪ 2-hour oral glucose tolerance test plasma glucose concentration ≥11.1mmol.l 2h after 75g anhydrous glucose.	Diabetes UK, 2006
Hypotension	BP<90/60mmHg	National Institute of Health, 2010
Hypertension	BP≥140/90mmHg on two or more seated BP readings on two or more visits	Khatib and El-Guindy, 2005

Exclusion criteria consisted of smokers, people taking dietary supplements, BP or cholesterol-lowering drugs, and those with soy, nut and coffee allergies. Smokers were excluded to minimise confounding factors since nicotine consumption is known to enhance HPA activity, hence resulting in elevated cortisol levels (Rohleder and Kirschbaum , 2006). Subjects who consume more than one portion of cocoa or DC a week (Taubert *et al.*, 2007), one or more cup of green tea a week (Iso *et al.*, 2006) and one or more cup of coffee a day were also excluded (Salazar-Martinez *et al.*, 2004). Exclusion criteria was based on that multivariate odds ratio for type II diabetes is highest for people who consume less than 1 cup of green tea per week compared to people who consume green tea 1 or more times per week (Iso et al, 2006). Likewise, relative risk for type II diabetes is higher for coffee abstainers as compared to individuals who consume one or more cup of coffee per day (Salazar-Martinez, 2004). All potential participants were screened for fasting glucose, total cholesterol, BP, BMI, WHR prior to the intervention and completed a screening questionnaire to determine their eligibility (Appendix 3).

2.6.2 Main study

The afore-mentioned inclusion and exclusion criteria was applicable for the main study. Additionally, the main study included females with BMI between 18.5 and 24.99kg/m^2 (WHO, 2000), as to allow for comparison of the baseline characteristics between the two BMI categories. This also permitted detection of differences in glucoregulatory biomarkers, BP, lipid profile, mineral, 24-h urinary cortisol or cortisol-to-cortisone ratio across the two BMI groups in response to polyphenol-rich DC or placebo. The choice of female volunteers

as opposed to both men and women was based on the wide variations in urinary cortisol and cortisone seen in the preliminary studies as well as available evidence in the literature suggesting differences in 11βHSD1 regulation and cortisol secretion between men and women (Andrew *et al*., 1998; Rask *et al*., 2002). Consequently, only females were recruited for the main study as to minimise the confounding effect of gender.

2.7. Sample size

For DC pilot study, sample size was calculated to detect a 0.3mmol/l reduction in fasting glucose with a power of (1-β) =0.8, α=0.05 and an effect size of 0.6546537 (G-power software version 3.0.8, Dusseldorf, Germany). This reduction was similar to the change in fasting glucose reported by Grassi *et al.* (2005b) in their study on polyphenols-rich DC [baseline mean±SD glucose =4.8±0.5mmol/l, post-DC mean±SD glucose=4.5±0.4]. Accordingly, a sample of 21 volunteers was identified as the minimum required to detect a significant reduction in fasting capillary glucose levels from baseline.

For GCBE, the initial sample was selected to match the sample size of the DC pilot study. Once preliminary data was obtained, power analysis was conducted using the data from urinary glucocorticoid metabolism and sample size was adjusted accordingly in order to detect an effect on glucocorticoid metabolism. Based on G-power, a sample size of 23 volunteers was required to detect a significant change in urinary cortisone with a power (1-β) =0.80, alpha=0.05 and an effect size of effect size dz=0.7172830 [baseline mean±SD urinary cortisone =56.58±36.20 nmol/d, post-GCBE mean±SD urinary cortisone=37.26±15.79 nmol/d].

For the main DC trial, a sample size of 22 subjects per group was required to detect a reduction in fasting glucose of 0.5mmol/L with a power of (1-β) =0.8, alpha=0.05 and an effect size of effect size dz=0.6546537 (G-power software version 3.0.8, Dusseldorf, Germany). These Power calculations were based on the results of the pilot study, which showed that DC containing 500mg polyphenols reduces fasting glucose from 4.42±0.7 to 3.92±0.86 mmol/l.

2.8. Diet and nutritional composition of green coffee bean extract

Table 2.2 shows the nutritional composition of GCBE. Following an initial 7-day run-in phase, participants were given 200mg GCBE containing 90mg CGA (Quest Vitamins Ltd, Birmingham, UK) twice daily for 14 days. They were instructed to consume one tablet at breakfast between 8-10am with food or white bread and then another tablet 2 hours after the evening meal between 8-10pm. This was to ensure plasma concentrations of CGA were maintained throughout the study period.

Most previous studies have incorporated GCBE or CGA supplements into drinks and foods like strawberry jam and soup bases, all of which contain little or no CGA (Olthof *et al.*, 2001b, Ochiai *et al.*, 2004, Kozuma *et al.*, 2005). White bread is also a low source of polyphenols and CGA and it is therefore unlikely to interfere with the absorption of GCBE (Clifford, 1999). In fact, two studies have indicated that carbohydrate-rich foods like bread could actually increase the bioavailability of polyphenols (Wang *et al.*, 2000, Holt *et al.*, 2002).

Table 2.2 The composition of green coffee bean extract

Component	content
Green coffee bean extract (mg)	200
Chlorogenic acid (mg)	90
Caffeine (%)	<2

With regard to the dosages, although the manufacturer recommends up to 3 tablets of GCBE per day, only 2 tablets per day were used in the preliminary GCBE study. This is because, consumption of 3 tablets of GCBE per day have been reported to produce side-effects including headaches, nausea and discomfort (Blum *et al.*, 2007). By contrast, the dose selected for use in the preliminary GCBE is in the range of doses used in previous studies on GCBE and CGA supplements, which showed no adverse effects (Olthof *et al.*, 2001a-b, 2003; Kozuma *et al.*, 2005; Watanabe *et al.*, 2006) but still reduced BP (Kozuma *et al.*, 2005; Watanabe *et al.*, 2006). It is noteworthy that one study did fail to show any significant reduction in BP following consumption of 140mg CGA for 4 months (Ochiai *et al.*, 2004). However, the insignificant result seen in this study is likely to be attributed to the small sample size used (n=10). This is confirmed by the fact that a later study with a larger sample size did demonstrate that 140mg CGA significantly reduces both SBP and DBP (Watanabe

et al., 2006). Differences in the GCBE doses used in this preliminary study and previous studies are shown in Table 2.3.

Table 2.3 Comparison of the dosages and compositions of green coffee bean extracts between the present study and previous studies

Content per dose(g)	GCBE[1] (mg)	Chlorogenic acid (mg)	Caffeine%
Kozuma *et al.* (2005)	46	25	12
	93	50	
	185	100	
Ochiai *et al.*, 2004	500	140	6
Watanabe *et al.*, 2006	-	140	-
Present study 2 tablets	400	180	<2

GCBE[1], green coffee bean extract

2.9. Diet and nutritional composition of dark chocolate

Table 2.4 and Table 2.5provide a summary of the nutrient composition of the DC used as part of the preliminary DC (500mg and 1000mg polyphenols DC) and main study (placebo and 500mg polyphenols DC) alongside a comparison of the composition of these DC with DC from previous trials.

Table 2.4 **Nutritional composition of 20 g of 500/1000 mg polyphenol dark chocolate and placebo.**

Component	Placebo	500 mg DC[1]	1000 mg DC[1]
Polyphenols (mg)	NG	500	1000
Epicatechin and catechin (mg)	NG	18.99	37.98
Theobromine (mg)	160	160	160
Energy (kJ)	425.8	425.8	425.8
Fat (g)	7.34	7.34	7.34
Protein (g)	1.34	1.34	1.34
Carbohydrate (g)	7.44	7.44	7.44
Magnesium (mg)	33.42	33.42	33.42
Sodium (mg)	1.4	1.4	1.4
Potassium (mg)	168.42	168.42	168.42

NG, negligible.

Table 2.5 Comparison between the polyphenol composition of dark chocolate in the present study and previous studies

Content per dose(g)	Polyphenols (mg)	Epicatechin (mg)	Catechin (mg)	Sum of epicatechin and catechin (mg)	epicatechin-to-catechin ratio
Grassi *et al.* (2005b) 100g DC	500	65.97	21.91	87.88	1:3
Taubert *et al.* (2007) 6.3g DC	30	5.1	1.7	6.8	1:3
Engler *et al.* (2004) 46g DC	-	46	-	-	-
Present study	500-1000	-	-	18.99-37.98	-

On average, DC was provided in the form of 20g containing negligible amount of polyphenols (placebo) or 500mg polyphenols supplying 18.99mg of catechin and epicatechin or 1000mg polyphenols with 37.98mg of catechin and epicatechin (Barry Callebaut, Lebbeke, Belgium). The 500mg polyphenols dose was chosen to correspond to the total polyphenol content of DC used by Grassi *et al.* (2005a-b) and Taubert *et al.* (2003). However due to the great variation in the levels of epicatechin and catechin between the chocolate used in this study and the one used by Grassi *et al.* (2005a-b), and the importance of these monomers given their large bioavailability compared to other DC polyphenols (Manach *et al.*, 2004), a higher DC dose was also chosen. This 1000mg polyphenols dose was selected to provide similar quantities of polyphenol to what is consumed by the Kuna population (Bayard *et al.*, 2007) and about 43.2% of the epicatechin and catechin dose used by Grassi *et al.* (2005a-b). Overall, subjects were instructed to distribute DC doses throughout the day in order to achieve a high steady state concentration. Additional data on the nutritional composition of DC is provided in Appendix 4. In general, DC contained 65.5% cocoa solids, and was found to be a rich source of magnesium and iron providing on average 11.14% recommended dietary allowance of magnesium and 27.52% recommended dietary allowance of iron per 20g.

For the main DC study, the 500mg polyphenols DC was selected. This choice was based on the results of the pilot study.

It is noteworthy that the current trial possesses several advantages over previous studies. This is because most previous studies used DC doses of 100g providing 500mg polyphenols.

In a recent meta-analysis, Desch *et al.* (2009), has risen concern over the long-term use of 100g polyphenol-rich DC. This is because high doses of DC provide a substantial quantity of fat and sugar which in the long-term can counteract any beneficial effect of polyphenols by promoting weight gain (Desch *et al.*, 2009). By contrast, the current study uses 20g DC with 500-1000mg polyphenols, a product that delivers large quantities of polyphenols in a less energy-dense and more palatable form than common chocolate. Moreover, in this study, the placebo was especially manufactured in the form of DC to ensure no differences in macronutrient or micronutrient composition exits. This helped control for the confounding effects of varying fat, theobromine and magnesium content, all of which are likely to influence glucose, BP and lipid profile. Earlier studies investigating the effect of polyphenols-rich DC on insulin sensitivity, BP and lipid profile used white chocolate as placebo (Table 1.5, p. 77 and Table 1.8, p. 96). Apart from introducing bias due to differences in the nutritional composition, the use of white chocolate placebo would not have permitted effective blinding of volunteers to treatment which could have resulted in expectation bias. As such, the present DC studies are likely to yield more reliable results because of the use of a more robust and a stronger study design.

2.10. Compliance and validation of diet diaries

Overall, all participants were instructed to maintain their usual diet throughout the study but to refrain from polyphenol-rich foods and beverages that supply 15 mg/kg epicatechin or CGA and 4 mg/l epicatechin or CGA, respectively (Olthof et al., 2001a-b, 2003) (see Appendix 1: Table 7.6, p. 305 and Table 7.7, p. 308). Subjects completed a 3-d (two weekdays and one weekend) diet and physical activity (Bouchard *et al.*, 1983) diary during the run-in phase and at the end of each dietary intervention. The Photographic Atlas of Food Portion Sizes was used to assist subjects in describing their portion sizes (Nelson *et al.*, 2002). Diet diaries were analysed for energy, fat, protein, carbohydrate and mineral intake using Windiet software (Windiet Research, Univation Ltd, Robert Gordon University, Aberdeen, UK). Compliance with the study's protocol was assessed by direct interviewing, assessment of diet diaries and returning of empty chocolate foils or counting of GCBE tablets and measurement of total polyphenols excreted in urine using FC, FRAP and ORAC, as described below (Khan *et al.*, 2003, Taubert *et al.*, 2007). The validity of diet diaries was established by interviewing 13 volunteers using a validated questionnaire devised by Lindroos *et al.* (1999). Accordingly, energy (r=0.705; P=0.007), protein (r=607; P=0.028), carbohydrate (r=0.936; P=0.000), magnesium (r=0.731; P=0.004), sodium (r=0.735; P=0.004) and potassium intake (r=0.808; P=0.001) as estimated by the diet diaries were

found to be strongly and positively correlated with the corresponding reported intake from dietary questionnaire (Pearson's product-moment correlation). A weak correlation was also observed for fat intake (r=0.103; P=0.737).

2.11. Anthropometry

For the preliminary studies, anthropometrical data was collected at baseline, Week 1 and at the end of each intervention period. For the main DC, anthropometrical data was collected at baseline and at the end of each intervention period.

2.11.1 Height

Height was measured using a stadiometer. Participants were asked to stand barefoot with their knees straightened, their heels placed together against the backdrop and the head positioned such that the plane that passes between the upper margin of the ear canal and the lower border of the eye (Frankfurt Plane) was parallel to the floor (**Figure 2.5**). Height was recorded as the last completed millimetre.

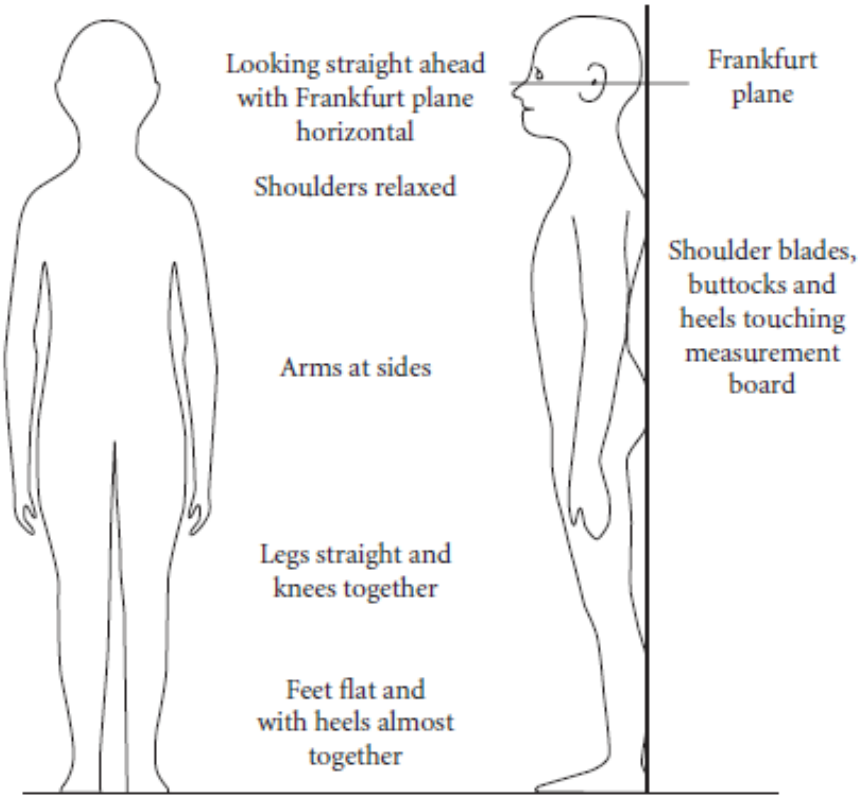


Figure 2.5 Positing of participant when measuring height. Note the of the placement of the head in the Frankfurt plane (Gibson, 2005).

2.11.2 Weight and body composition

Body weight [recorded to the nearest 0.1kg] and body composition (water percentage and fat percentage) were quantified using BF681W Tanita scales with body fat monitor (Tanita, UK). The scales were placed on a firm, flat surface and participants were asked to remove their shoes and any heavy clothing, to stand still with their arms on the side and to look forward (WHO, 2008).

Tanita scales use the Bioelectrical Impedance Analysis technique to measure body composition. This involves sending a low-grade electrical current through the body and measuring the speed at which this current travels from one foot electrode to the other foot electrode. Because fat does not conduct electricity, the higher the percentage body fat, the lower is the speed and intensity of the electrical signal, hence the greater the impedance (Tanita, 2009a).

The advantage of using Tanita scales to monitor weight and body composition is its relative low-cost and convenience to subjects compared to the conventional bioelectrical impedance analysis which require the subject to be placed in a supine position while several electrodes are being attached to specific anatomical sites on their skin (Jebb *et al.*, 2000). Moreover, according to Jebb *et al.* (2000) the estimates for body fat do not differ significantly between those obtained using the Tanita scales or the traditional bioelectrical impedance analysis. Tanita scales also permit the categorisation of body into underfat, healthy, overfat and obese on the basis of the National Institute of Health and WHO guidelines (**Figure 2.6**).

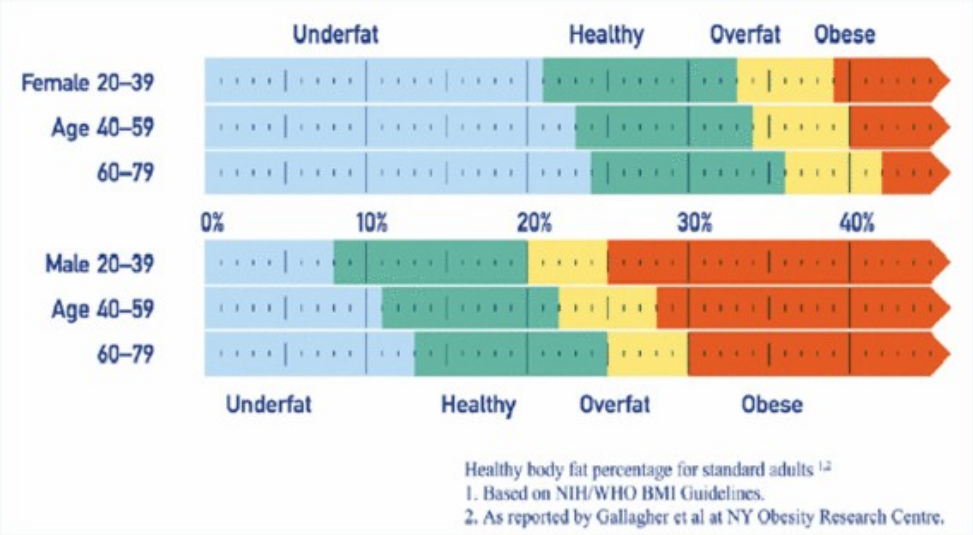


Figure 2.6 Classification of body fat according to Tanita body fat monitors (Tanita, 2009b)

2.11.3 Body mass index

BMI was calculated as weight in kilograms divided by the square of height in meters. The WHO classification was subsequently adopted to categorise participants as lean (BMI<18.5 kg/m^2), overweight (BMI 18.5-24.99 kg/m^2) or obese (BMI≥25 kg/m^2). This BMI classification is based on the morbidity risk conveyed by each BMI category with the BMI categories ≥25 kg/m^2 being associated with a progressive rise in risk of chronic diseases (see Table 2.6) (WHO, 2000; James *et al.*, 2004).

Table 2.6 Risk of morbidity associated with different BMI categories (WHO, 2000)

BMI category	Value (kg/m^2)	Risk of associated morbidity
Underweight	<18.5	Low
Normal weight	18.5-24.99	Moderate
Overweight	25-29.99	increased
Obesity Class I	30-34.99	High
Obesity Class II	35-39.99	Very high
Obesity Class III (morbidly obese)	≥ 40	Extremely high

It is important to acknowledge that the WHO classification of BMI is based on the assumption that the association between BMI, morbidity and mortality follows a linear relation. However, on reviewing the literature it appears that although BMI is a strong predictor of metabolic risk factors, inconsistencies still remain with regard to the shape of the relation curve between this index of adiposity, morbidity and mortality. This is because while some authors have reported a J- or U-shape relation between BMI, morbidity and mortality, with the lower and the highest BMI categories being associated with increased risk of morbidity and mortality (Manson *et al.*, 1995; Allison *et al.*, 1997; Calle *et al.*, 1999; Jee *et al.*, 2006), others have documented a linear association between BMI and mortality (Lindsted *et al.*, 1991; Dorn *et al.*, 1997). This linear relation is particularly apparent after adjustment for smoking status (Manson *et al.*, 1995) or fat free mass (Allison *et al.*, 2002) and is clearly expressed in WHO BMI categorisation (Table 2.6).

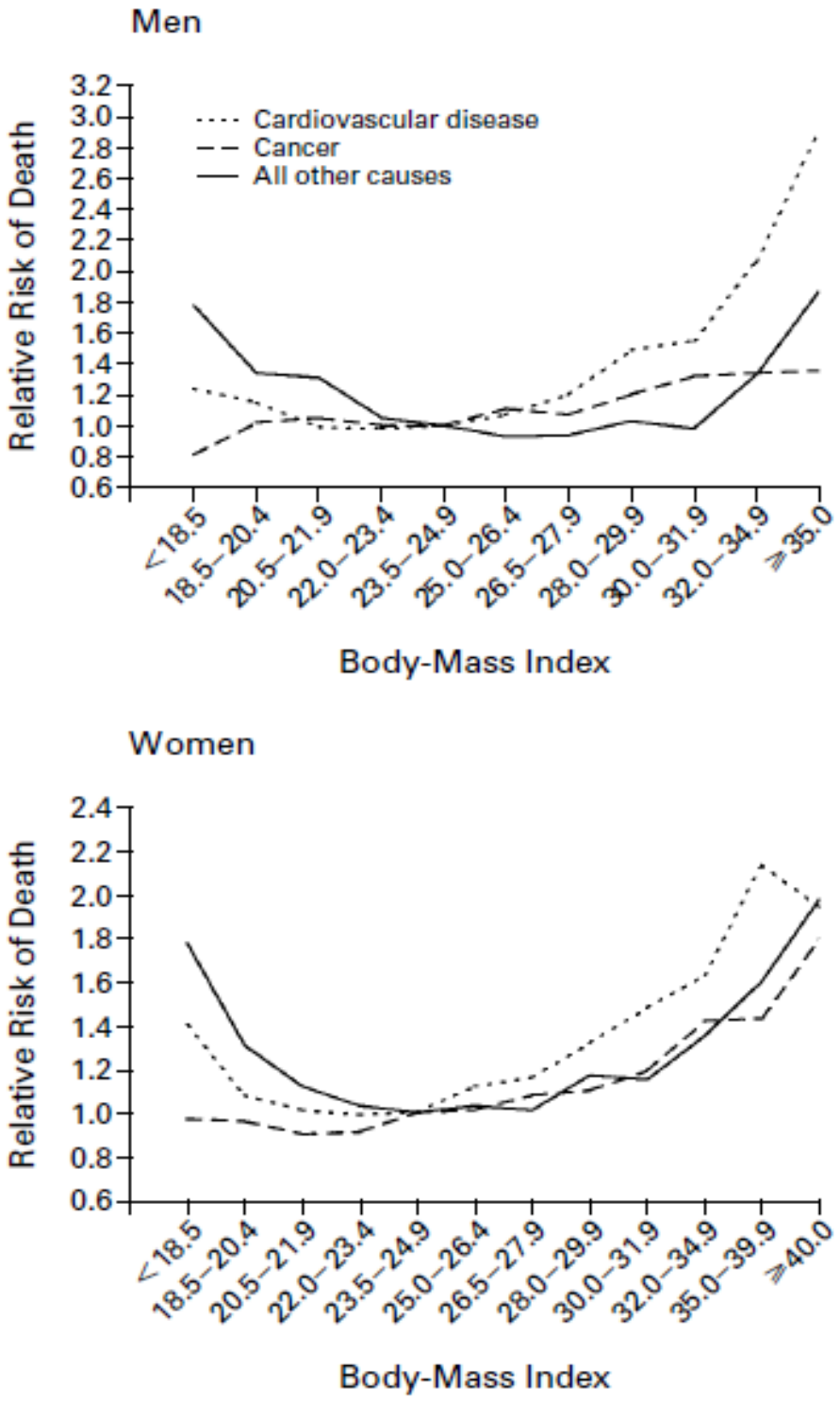


Figure 2.7 Relative risk of death from cardiovascular disease, cancer and other diseases among men and women stratified according to Body-Mass Index. Note, the J-shaped relation between BMI and relative risk of cardiovascular in both men and women. Also note, the U-shaped relation between relative risk of death from other causes and BMI in both men and women (Calle *et al.*, 1995).

Overall, these inconsistencies could arise from the fact that despite its common use and simplicity, BMI remains a crude index of adiposity (WHO, 2000). Consequently, the use of BMI as a measure of adiposity can pose limitations since it does not distinguish between fat mass and fat-free mass. To exemplify, consider **Equation 3**.

Equation 3 $$BMI = \frac{weight(kg)}{height(m)^2} = \frac{fatmass}{height^2} + \frac{fatfreemass}{height^2}$$ (Allison *et al.*, 1997)

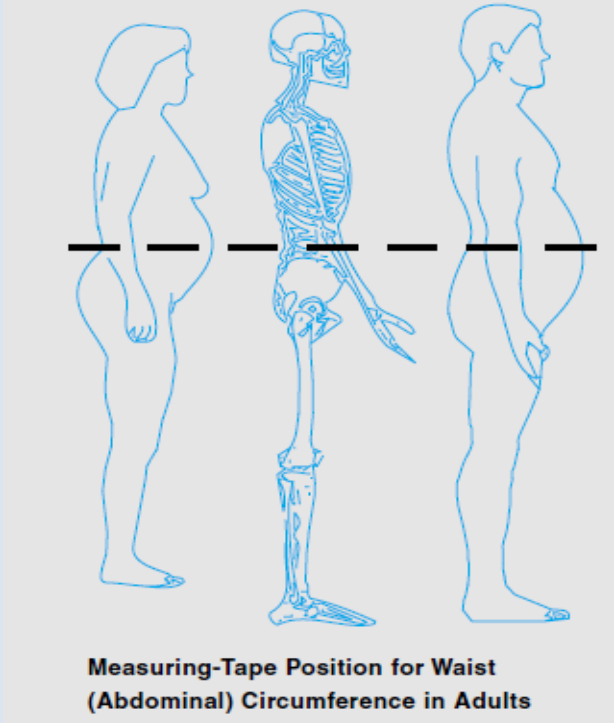
This equation demonstrates that BMI consists of two main components: fat mass/height^2 and fat free mass/height^2 (Allison *et al.*, 1997). This entails that under certain conditions when fat-free mass (i.e muscle mass) is high, such as in atheletes, individuals could mistakenly be classified as overweight (WHO, 2000). This also implies that individuals with an identical BMI do not necessarily have a similar quantity of body fat (WHO, 2000). All the more, it suggests that individuals with low or very low fat free mass (such as those with BMI< 25 kg/m^2 or <18 kg/m^2) could be mistakenly classed at a low morbidity risk despite having a high fat mass: fat free mass ratio (Deurenberg-Yap *et al.*, 2000; 2002), a phenomenon recently named by Professor Bell (2009) as TOFI (Thin on the Outside Fat on the Inside).

Consequently, it could be argued that BMI could only provide a rough estimate of the relative risk of developing chronic diseases. Moreover, it appears that the current cut-offs for lean, overweight and obese are not optimal for defining metabolic risk factors in all populations (Deurenberg *et al.*, 1999; 2000; WHO, 2000; Deurenberg-Yap *et al.*, 2002; Lin *et al.*, 2002; WHO, 2004). The latter is particularly evident in the Asia-Pacific Cohort Studies Collaboration study wherein increments in BMI above 21 kg/m^2 were found to be associated with a raised risk of CVD in the Asian population (James *et al.*, 2004). Regardless of these inconsistencies, a BMI> 30 kg/m^2 remains a definite cut-off for excess fat (WHO, 2000).

Another main limitation of BMI is that it does not take in account the distribution of body fat and the subsequent risk conveyed to health. As such BMI should be used in combination with other indices of adiposity such as WC and WHR in order to provide a clear picture of metabolic risk of developing overweight and obesity-related co-morbidities. Moreover, the earlier evidence described above, argues for the need for recruiting individuals with normal physical activity and to exclude atheletes and for controlling for ethnicity when recruiting lean individuals.

2.11.4 Waist circumference, hip circumference and waist-to-hip ratio

For waist and hip measurement, participants were asked to stand with their feet together and both arms relaxed at the sides. Waist was measured in centimetres (cm) at the end of normal expiration half way between the lowest rib and the iliac crest with the investigator standing at the side to ensure that the measuring tape is horizontal across the back and the front of the participant (National Institute of Health, 2000; WHO 2008) (**Figure 2.8**).



The National Institute of Health recommends that the tape measure should be placed in horizontal plane round the abdomen, parallel to the floor and should be snug without compressing the skin

Figure 2.8 Waist circumference measurement (National Institute of Health 2000)

Hip circumference (HC) was measured at the maximum circumference over the buttocks (WHO 2008). Data on waist and HC were then used to calculate WHR using the following equation:

Equation 4 $$WHR = \frac{waistcircumference(cm)}{hipcircumference(cm)}$$

As with BMI, WC serves as a good indicator of the degree of insulin resistance in overweight and obese individuals (Farin *et al.*, 2006) and as shown in Table 2.7, WC ≥94cm in men and ≥80cm in women is generally associated with increased risk of metabolic complication (Table **2.7**). By contrast, WHR is a strong predictor of the presence of

hypertension (Fuchs *et al.* 2005) and of HPA hyperactivity, characterised by high baseline plasma cortisol levels and low 24-h urinary cortisol excretion in obese women (Vicennati and Pasquali, 2000). WHR also permits the classification of obesity as peripheral or abdominal obesity with WHR >1.0 in men and >0.85 in women being indicative of abdominal fat distribution (WHO, 2000).

Table 2.7 Risk of metabolic complications as predicted by waist circumference (WHO, 2000)

Risk of metabolic complications	Waist circumference (cm)	
	Men	Women
Increased	≥94	≥80
Markedly increased	≥102	≥88

The importance of differentiating between abdominal and peripheral fat distribution lies in that abdominal obesity is closely related to obesity-related co-morbidities and the metabolic syndrome than peripheral obesity (WHO, 2000; Grundy, 2004, 2005). Moreover, abdominal obesity appears to be associated with a greater risk of hypertension, diabetes and CVD compared to peripheral obesity (Rosmond *et al.*, 1998; WHO, 2000; Vicennati and Pasquali, 2000; Grundy, 2004, 2005; Duclos *et al.*, 2005; Wang *et al.*, 2005). This is particularly evident when considering that subjects with high WC are at a relatively higher risk of developing hypertension, diabetes, dyslipidaemia, and the metabolic syndrome than individuals with normal WC in the same BMI category. In fact according to Janssen *et al.* (2002a), even within the lean BMI category, subjects with high WC show a greater risk of developing hypertension, diabetes, dyslipidaemia and the metabolic syndrome than individuals with normal WC. This effect remains apparent even after adjustment for age, race, poverty-income ratio, physical activity, smoking, and alcohol intake and is comparable to the health risk of overweight and obese individuals with an identical WC (Janssen *et al.*, 2004). By contrast, in the overweight category, individuals with high WC are twice more likely to exhibit elevated TG, insulin levels, and the metabolic syndrome, compared to normal WC group (Janssen *et al.*, 2005). Accordingly, several authors have concluded that WC provides an estimation of disease risk similar to or above that explained by BMI (Janssen *et al.*, 2004; Wildman *et al.*, 2005; Farin *et al.*, 2006), especially in relation to diabetes (Janiszweski *et al.*, 2007). As a result, the US National Institute of Health (2000) recommends that abdominally-obese individuals should be placed at one health risk category above peripherally obese individuals with similar BMI (Table 2.8). Moreover, Janssen *et al.* (2002b) have concluded that the current cut-off values for WC are pivotal to identifying

individuals at increased disease risk within each of the lean, overweight, and class I obese BMI categories.

Table 2.8

Classification of overweight and obesity by BMI, waist circumference and associated health risk (adapted from National Institute of Health, 2000)

	BMI (kg/m2)	Obesity Class	Disease Risk* (Relative to Normal Weight and Waist Circumference)	
			Men ≤40 in (≤ 102 cm) Women ≤ 35 in (≤ 88 cm)	> 40 in (> 102 cm) > 35 in (> 88 cm)
Underweight	< 18.5		-	-
Normal†	18.5–24.9		-	-
Overweight	25.0–29.9		Increased	High
Obesity	30.0–34.9	I	High	Very High
	35.0–39.9	II	Very High	Very High
Extreme Obesity	≥ 40	III	Extremely High	Extremely High

* Disease risk for type 2 diabetes, hypertension, and CVD.

† Increased waist circumference can also be a marker for increased risk even in persons of normal weight.

Nonetheless, it is essential to recognise that BMI and WC may serve as independent and additive predictors of total and regional fat distribution. This could be illustrated in the work of Janssen *et al.* (2002b) who observed that BMI explains the greatest proportion of variance in nonabdominal and abdominal subcutaneous fat while WC is a stronger predictor of visceral fat accumulation than BMI. Accordingly, within each of the 3 BMI categories, individuals with higher WC were shown to have more visceral fat than individuals from the same BMI category but with a lower WC. Importantly, Janssen *et al.* (2002b) demonstrated that addition of WC to BMI increases the percentage variance accounted for in abdominal subcutaneous fat. This, according to Janssen *et al.* (2002b) could have important implications since abdominal subcutaneous fat is closely related to insulin resistance (Goodpaster *et al.*, 1997). Combining WC with BMI has also been shown to help explain a greater proportion of the variance in SBP (Janssen *et al.*, 2005), which has led Janssen and colleagues (2002a, 2004, 2005) to emphasise the need for monitoring both BMI and WC. The National Institute of Health (2000) also suggests that monitoring WC may help detect changes in abdominal fat distribution in the absence of changes in weight or BMI. It is important to acknowledge that computed tomography (Jensen *et al.*, 1995; Yoshizumi *et al.*, 1999), dual-energy X-ray absorptiometry (Glickman *et al.*, 2004; Lee *et al.*, 2005) and magnetic resonance imaging (Thomas *et al.*, 2000; Thomas and Bell, 2003) act as more accurate measurements of abdominal fat but that WC and WHR provide a more feasible approach to monitoring changes in abdominal fat accumulation (National Institute of Health, 2000).

2.12. Measurements (Preliminary studies)

2.12.1 Capillary fasting glucose and total cholesterol

To assess fasting glucose and total cholesterol in the preliminary GCBE and DC studies, a 12-hour fasting finger-prick blood sample was taken using a calibrated AccutrendGC system (Roche diagnostics, Mannheim, Germany). Quality controls were also run on the day of analyses and for each new batch of glucose or cholesterol strips. The precision and accuracy of AccutrendGC system are shown in Table 2.9. Overall, normal fasting glucose was defined by values <6.1 mmol/l but ≥3.9 mmol/l (Roche Diagnostics, 2007a) while fasting plasma glucose 6.1-7.0 mmol/l were considered as impaired fasting glucose (Diabetes UK, 2006)). Total cholesterol values <5.2 mmol/l were deemed optimum (Roche Diagnostics, 2007b). Appropriate Health & Safety Risk assessment were undertaken for all sample collections and all samples were disposed off in accordance with the Health & Safety regulations.

Table 2.9 Precision and accuracy of AccutrendGC system (Roche Diagnostics, 2007c)

	Glucose	Total cholesterol
Measuring ranges	20 - 600 mg/dl (1.1 - 33.3 mmol/l)	150 - 300 mg/dl (3.88 - 7.76 mmol/l)
Accuracy (Mean systemic deviation)	±5 % compared with a hexokinase protein free precipitate method	±5% compared with the standard CHOD-PAP method
Repeatability, within-run imprecision (Coefficient of variation)	<5%	≤3.7%
Reproducibility, between-run imprecision: (Coefficient of variation)	<5%	≤3.8% for concentration of 191mg/dl (5mmol/l) ≤5% for concentration between 270mg/dl (7mmol/l)

All participants were instructed to consume the last dose of DC or GCBE 12hr prior to analysis (Taubert *et al.*, 2007) and to avoid heavy physical activity and alcohol intake 24hr prior to testing. They were also instructed to consume the same diet the day before each test for each of the interventions as to avoid additional confounding factors (Olthof *et al.*, 2003).

2.12.2 Blood pressure

Blood pressure (BP) was examined using an automated A&D Medical UA-767 Plus BP monitor (A&D Medical, San Jose, USA) according to the procedure described by Grassi *et al.* (2005b). Accordingly, BP was measured following a 10 minutes rest in warm room with the cuff placed at the left upper arm of each subject, 2-3cm above the antecubital vein. The investigator ensured that the arm rested on a flat surface so that BP-cuff was positioned in line with the heart and that the cuff size was appropriate for the volunteer, as shown below:

- Small Cuff 6.3 - 9.4" (16-24cm)
- Medium Cuff 9.4 - 14.2" (24 - 36cm)
- Large Cuff 14.2 - 17.7" (26 - 45cm)
- Extra Large Cuff 16.5-23.6" (42 - 60cm)

To increase the reliability of the results three values were taken with 2 minutes intervals. According to Grassi *et al.* (2005b), such protocol increases the reliability of results and is likely to reduce white coat hypertension. The accuracy of the A&D Medical UA-767 BP monitor against the cuff/stethoscope auscultation method has been previously validated and has been shown to achieve grade A for both SBP and DBP according to the British Hypertension Society standard (Rogoza *et al.*, 2000; Kobalava *et al.*, 2003; Verdecchia *et al.*, 2004). Mean difference in SBP and DBP reading between mercury sphygmomanometer and UA-767 BP monitor are in the range of –0.4±5.4 to -0.93±5.1 and -0.04±4.8 to –0,41±4,73 mmHg, respectively (Rogoza *et al.*, 2000; Kobalava *et al.*, 2003). Normal BP was defined as levels <120/80 mmHg (Khatib and El-Guindy, 2005) and hypotension and hypertension as in Table 2.1 (see, p. 127).

Repeatability of A&D Medical UA-767 BP monitor was further assessed in a sample of 10 volunteers. Three readings were taken 1 week apart according to the protocol described above and the average of the three readings obtained. Bland-Altman plots were constructed using MedCalc software version 11.1 (Mariakerke, Belgium) to show inter-day variability in SBP and DBP and in order to obtain information on the limits of agreement and mean inter-day differences in SBP and DBP readings (see Appendix 5). The mean difference in SBP and DBP reading between Day 1 and Day2 were 0.17±2.17 and 0.33±2.19mmHg, respectively. Both differences and standard deviation fell within the American Association for the Advancement of Medical Instrumentation (AAMI) criteria for accuracy (mean(SD) = 5(8)). Repeatability was further confirmed by a paired sample t-test which demonstrated that inter-day differences in SBP (P=0.813) and DBP (P= 0.642) were not significant.

2.13. Urinary mineral excretion

24-h urine samples were obtained for measurement of urinary magnesium, sodium and potassium levels using an automated platform (Olympus, UK) at the Clinical Biochemistry Laboratory, Royal Infirmary of Edinburgh, Scotland, UK. Magnesium, sodium and potassium are primarily excreted through the kidneys (Eunsook and Willis, 2000; Tasevska *et al.*, 2006) which implies that changes in their dietary intake could be directly reflected in their urinary levels. This is particularly important when considering that changes in dietary intake of magnesium, sodium and potassium may influence BP levels. Urinary magnesium, sodium and potassium could also be viewed as biomarkers of micronutrient intake that can be used to validate the accuracy of the reported dietary intake (Eunsook and Willis, 2000). Additional reasons for monitoring the urinary excretion of these minerals in the present thesis are described below.

2.13.1 Potassium and sodium

Potassium is an important mineral that regulates insulin secretion by enhancing proinsulin's conversion to insulin (Dale and Haylett, 2004). Its normal serum concentration range from 3.5 to 5mmol/L (Kumar and Clark, 2005). However, according to Zillich *et al.* (2006) maintaining serum potassium >3.9mmol/L is essential to prevent glucose intolerance. This is because decreased dietary potassium intake is associated with increased fasting glucose levels (Gordon, see Zillich *et al.* 2006) while supplementation is linked to improved insulin sensitivity in obese individuals (Norbiato *et al.*, 1984).

Overweight and obese individuals exhibit several abnormalities in potassium metabolism. For instance, overweight and obese individuals excrete significantly more potassium than lean individuals, an effect thought to be caused by increased aldosterone secretion (Bentley-Lewis *et al.*, 2007). Generally, aldosterone secretion is reduced in response to increased potassium secretion. However, in obesity, increased angiotensin-II secretion counteracts the body's normal homeostatic mechanisms leading to enhanced aldosterone secretion, hence increased potassium losses (Bentley-Lewis *et al.*, 2007). Although such increased losses are not necessarily reflected in serum potassium levels, a reduction in intracellular potassium could still occur, as observed in obesity (Colt *et al.*, 1981) which may then affect insulin secretion. Obesity is also associated with decreased natriuretic peptide levels and increased sodium retention (Wang *et al.*, 2004), which when combined with increased potassium losses, as reflected by high urinary sodium-to-potassium ratio, is known to be associated with

hypertension (Langford, 1983). Consequently, it is essential to monitor urinary sodium and potassium excretion in overweight and obese individuals, to compare their levels to the levels excreted by lean individuals, and to observe whether changes in insulin sensitivity or BP could be correlated with an improvement in urinary sodium-to-potassium ratio particularly that DC is rich in potassium.

2.13.2 Magnesium

Magnesium acts as a co-factor in the regulation of various ATP-requiring enzymes, many of which are implicated in glucose metabolism (Palanivel *et al.*, 2005) and insulin's transduction pathways (Takaya *et al.*, 2004). It also regulates cholesterol synthesis and lipid homeostasis by controlling the activity of lecithin cholesterol acyltransferase and LPL both of which are required to increase HDL (Inoue, 2005) and TG's clearance (Rayssiguier, 1984). Magnesium is also essential for maintaining vascular tone, BP (Barbagallo and Dominguez, 2007), calcium, sodium and potassium metabolism (Hordyjewska and Pasternak, 2004). This implies that magnesium regulates virtually all intracellular reactions and that many of its metabolic effects are mediated through other ions' dependent pathways. It is therefore unsurprising why minor changes in magnesium, whether found on their own or accompanied by increased intracellular calcium, have been related to numerous metabolic abnormalities from insulin resistance, unfavourable lipid profile to hypertension (Barbagallo and Dominguez, 2007, Haglin *et al.*, 2007, Schulze *et al.*, 2007, Sharma *et al.*, 2007).

To illustrate, it has been shown repeatedly that hypomagnesaemia is associated with increased insulin resistance (Nadler *et al.*, 1993), reduced insulin secretion (Paolisso *et al.*, 1989) and action (Paolisso *et al.*, 1990) and impaired insulin binding with its receptor (Tonvai *et al.*, see Paolisso *et al.*, 1994). Conversely, correction of these deficiencies leads to reduced plasma glucose and increased insulin response (Paolisso *et al.*, 1989). Increased insulin response, particularly, is explained in terms of magnesium's ability to regulate potassium permeability, thereby increasing β-cell response to glucose (Paolisso *et al.*, 1989). Similarly, magnesium supplementation has been shown to enhance insulin activity by increasing peripheral glucose uptake and glucose oxidation (Paolisso *et al.*, 1992),

With regard to lipid metabolism, animal models suggest that magnesium deficiency is associated with increased plasma TG, altered lipoprotein composition, increased susceptibility of lipoproteins and tissues to oxidative damage and raised apolipoprotein-B,

the latter being indicative of increased plasma TG-rich lipoproteins (Gueux *et al.*, 1995). Conversely, high serum magnesium concentrations have been related to higher HDL (Guerrero-Romero and Rodríguez-Morán, 2000), while magnesium supplementation has been shown to lower LDL-cholesterol (Olatunji and Soladoye, 2007). Magnesium deficiency is also linked to increased oxidative stress and free radical generation (Bussière *et al.*, 2002). This, as discussed earlier, diminishes NO bioavailability causing endothelium dysfunction and hypertension.

Maintaining adequate magnesium intake in overweight and obese individuals could therefore be essential in reducing risk of insulin resistance, diabetes, dyslipidaemia and hypertension, especially that a direct association between low magnesium intake and increased prevalence of the metabolic syndrome exists (Ford *et al.*, 2007). A strong correlation between low magnesium intake and BMI in women also exists, suggesting that magnesium may help regulate women's body weight (Song *et al.*, 2007).

Both coffee and DC are rich in magnesium (Salmeron *et al.*, 1997) providing on average 311 and 100mg magnesium per 100g decaffeinated instant coffee or DC, respectively (Bruinsma and Taren, 1999; USDA, 2007b). In fact, according to Bruinsma and Taren (1999), DC could be used to correct magnesium deficiencies. Magnesium in DC is also thought to account for the reduction in BP and insulin resistance seen in Grassi *et al.*'s studies (2005a-b) (Meisel, 2005). This indicates that foods like coffee and DC could contribute towards improving magnesium intake in overweight and obese subjects, thereby protecting them against insulin resistance, hypertension and dyslipidaemia. Since, in the present thesis DC provided a daily intake of 33.42mg of magnesium, monitoring magnesium excretion could have been essential to explaining some of the mechanisms by which DC may affect glucose levels, BP, and lipid profile in overweight and obese subjects.

2.14. Urinary phenolic content, ferric-reducing capacity of plasma and oxygen radical absorbance capacity

Urine samples were thawed at 4^{o}C, centrifuged and subsequently diluted 1:10 for measurement of total phenolics and FRAP. For ORAC, urine samples were diluted 1:100. Total polyphenol content, FRAP and ORAC were measured according to the methods described in section 2.1.2, section 2.1.3 and section 2.1.4.

2.15. Validation of urine collection

All 24-h urine collections were validated by measuring creatinine excretion (Eunsook and Willis, 2000, Rios *et al.*, 2003, Roura *et al.*, 2006) at the Clinical Biochemistry Laboratory at the Royal Infirmary, Edinburgh. According to Orth (1995), day-to-day variations in urinary creatinine concentration should not exceed 10%.

2.16. Cortisol and Cortisone ELISA

2.16.1 Principle of method

Analyses of urinary free and salivary cortisol and cortisone levels were undertaken using an indirect competitive double-sandwich ELISA according to the protocol devised by Al-Dujaili *et al.* (2005, 2006) (see below). In this assay, the microplate is coated with a constant amount of cortisol/ cortisone conjugate. This conjugate competes with the cortisol/cortisone in standards or samples for binding to a selective anti-sheep or anti-rabbit antibody. Because the levels of the conjugate are held constant, the varying concentration of cortisol/cortisone in standard or sample can indirectly determine the amount of antibodies that bind to the conjugate. An enzyme-linked antibody tracer is then added to the microplate which binds the conjugate-antibody complex. This double antibody sandwich produces a distinct yellow colour upon addition of the substrate and stop solution. The intensity of the colour, which is inversely proportionate to the concentration of cortisol/ cortisone in sample, is measured using an ELISA reader at 450nm and then compared to the absorbance of the cortisol/ cortisone standards.

In general, measurements of 24-h urinary free cortisol and urinary free cortisone offers the advantage of obtaining a complete 24h glucocorticoid profile (Orth, 1995). Although previously researchers used a single measurement of 24-h urinary free cortisol in diagnosing

imbalances in glucocorticoid metabolism, Remer and Maser-Gluth (2007) argue that the use of a combination of 24-h urinary free cortisol and urinary free cortisone "provides more meaningful assessment of functional glucocorticoid activity" mainly because the concentrations of 24-urinary free cortisol could strongly be influenced by the activity of renal 11βHSD2 (Westerbacka *et al.*, 2003). Accordingly, several researchers have used both 24-h urinary free cortisol and urinary free cortisone in assessing glucocorticoid metabolism in Cushing syndrome (Lin *et al.*, 1997), apparent mineralcorticoid excess (Palermo *et al.*, 1996) and type-I diabetes (Remer *et al.*, 2006).

In the present thesis, urinary cortisone-to-cortisol ratio was also calculated because of the importance of this ratio as a marker of 11βHSD2 (Duclos *et al.*, 2005). Moreover, monitoring the activity of this enzyme helps detect changes in peripheral metabolism of cortisol (Palermo *et al.*, 1996). Under normal physiological conditions, 24-h urinary free cortisone is present in concentrations two-fold higher than 24- urinary free cortisol (Lin *et al.*, 1997; Taylor *et al.*, 2002; Remer *et al.*, 2006; Remer and Maser-Gluth, 2007) with the urinary free cortisol-to-cortisone ratio being equivalent to (mean± SE) 0.54±0.05 (Palermo *et al.*, 1996). However, in obesity, this ratio is elevated possibly due to increased cortisol metabolism, which according to Duclos *et al.* (2005) causes a reduction in plasma cortisol levels, and a subsequent increase in stress-induced cortisol secretion. The 24h urinary free cortisol, on the other hand, serves as a measure of 24h free plasma cortisol (Vicennati and Pasquali, 2000). In obesity decreased 24-h urinary free cortisol has been attributed to increased α-reductase activity, which catalyses the conversion of active cortisol to its metabolite 5α-THF (Andrew *et al.*, 1998), as well as reduced cortisol generation from cortisone caused by 11βHSD1 inhibition (Stewart *et al.*, 1999).

The use of a combination of urinary, serum and salivary cortisol/ cortisone was deemed more appropriate for the main study given the wide variation observed in urinary free cortisol and urinary free cortisone excretion in the preliminary GCBE and DC studies. Indeed, measurements of urinary cortisol and cortisone could be confounded by urine volume (Shi *et al.*, 2008) and fluid intake (Fenske, 2006). As with urine, salivary cortisol or cortisone permits the measurements of the biologically active free form of cortisol and cortisone. In fact according to Viardot *et al.* (2005), salivary cortisol reflects 3-6% of the total serum cortisol concentrations. Moreover, unlike urine, the concentration of cortisol and cortisone in saliva is not influenced by the rate of saliva production (Guechot *et al.*, 1982; Riad-Fahmy *et al.*, 1982). Moreover, collection of saliva is generally non-invasive and more convenient

since saliva is one of the most readily available body fluids and studies suggest that salivary glucocorticoids can remain stable at room temperature for up to 7d (Vining *et al.*, 1987; Aardal *et al.*, 1995, Aardal-Eriksson *et al.*, 1998). This suggests that saliva could be collected at different time points during that day which could be important in monitoring changes in diurnal glucocorticoids production (Riad-Fahmy *et al.*, 1982). Finally, measurement of cortisol/ cortisone in saliva avoids interferences from renal 11βHSD2.

It is important to note that the use of high-performance liquid chromatography (HPLC) (Weykamp *et al.*, 1989; Lindsay *et al.*, 2003), gas chromatography-mass spectrometry (GC-MS) (Wudy and Hartmann, 2004), liquid chromatography- tandem mass spectrometry (LC-MS/MS) (Taylor *et al.*, 2002; Janzen *et al.*, 2008; Soldin and Soldin, 2009) permits specific identification and quantification of urinary cortisol and cortisone metabolites which, in turn, enables more accurate determination of the activity of the different enzyme regulating glucocorticoid metabolism (see Figure 1.10; p. 46). However, due to the expensive and time-consuming nature of such analyses, quantification of 24-h urinary free cortisol and urinary free cortisone was deemed more appropriate, particularly that most previous studies investigating the effect of polyphenols on glucocorticoid metabolism measured 24-h urinary free cortisol and urinary free cortisone rather than cortisol or cortisone metabolites (see Section1.11.7).

2.16.2 Reagents and standards

Dichloromethane, HCL, sodium hydroxide (NaOH), diethylether, disodium phosphate, dipotassium phosphate, sodium chloride, potassium chloride, bovine serum albumin (BSA), Tween 20, sodium acetate anhydrous, citric acid, 3,3,5,5 Tetramethyl-benzidine, dimethyl sulfoxide, H_2O_2 were obtained from Sigma-Aldrich (Poole, Dorset, UK). Ultrapure water (Milli-Q) was obtained from Millipore System (Belford, MA).

For the standards, a series of dilutions were prepared by diluting the stock cortisol standard in 0.05M PBS to give the following concentrations: 0, 2.5, 10, 50, 250 and 1000ng/ml. For cortisone, the following dilutions were prepared: 0, 0.5, 1.0, 2.5, 10 and 50ng/ml.

2.16.2.a Phosphate buffer saline (0.05M, pH 7.4)

PBS was prepared by adding 14.2g disodium phosphate, 3.4g dipotassium phosphate to a flask containing 1L distilled water. The solution was mixed on the bench and 10g of sodium

chloride and 1g of potassium chloride were added and the flask filled with distilled water to the 2.5L mark. An iron flee was placed in the flask and the solution mixed on low heat on a magnetic stirrer and hotplate until the salts were completely dissolved. PBS was stored at 4^0C until required

2.16.2.b Assay buffer (0.025M)

Assay buffer was prepared fresh on the day by diluting PBS 1:1 in distilled water. BSA was then added, without stirring, to give a 0.1% BSA solution.

2.16.2.c Coating buffer (0.02M)

Coating buffer was prepared by diluting PBS1:1.5 in distilled water. The buffer was stored at 4^0C until required.

2.16.2.d Block Buffer (0.25M)

Block buffer was prepared fresh on the day by adding 0.2g BSA to 20ml PBS to give a solution with a final concentration of 0.5% BSA.

2.16.2.e Wash buffer

Wash buffer was prepared by adding 200ml PBS to 800ml distilled water (1:5 dilution) followed by 5ml Tween 20 (0.05% Tween 20 solution).

2.16.2.f Substrate solution (pH 4-4.5)

The substrate buffer was made by adding 8.2g sodium acetate anhydrous and 13g of citric acid to 0.5L distilled water and stored at 4^oC until required. The stock substrate consisted of 200mg 3,3,5,5 Tetramethyl-benzidine (TMB) dissolved in 20ml of dimethyl sulfoxide (DMSO). This stock substrate was stored in an amber glass bottle and 1ml distilled water was added to prevent it from freezing upon storage at 4^oC. Overall, the substrate solution was prepared directly prior to use by combining 11ml of the prepared substrate buffer, 300µl stock substrate and 50µl H_2O_2.

2.16.2.g Preparation of plates

For a 96-well plate, 20 ml of coating buffer was prepared by adding 50µl (0.5mg/ml) cortisol-conjugated to BSA or 30µl (0.5mg/ml) cortisone-conjugated to BSA. The solution was vortexed and a 200µl aliquot of the diluted cortisol-conjugate or 180µl of the diluted cortisone-conjugate was added to the plate using a multidispenser. The plate was covered with parafilm and a lid and left at 4^{o}C overnight.

2.16.2.h Preparation of antibodies

A 60µl aliquot of cortisol/cortisone antibody was added to 11ml of assay buffer to give a 1:1000 dilution prior to use.

2.16.2.i Preparation of enzymes

To prepare the cortisol enzyme, a 10µl cortisol anti-sheep enzyme was added to 10ml assay buffer (1:1000 dilutions). For the cortisone enzyme, a 50µl cortisone anti-rabbit enzyme was added to 10ml assay buffer (1:200 dilution). All enzymes were prepared directly prior to use.

2.16.3 Preparation of urine samples

Urine samples were initially defrosted and centrifuged at 6000rpm for 5minutes to remove any impurities. Glucocorticoids were subsequently extracted from urine samples according to the method described by Al-Dujaili *et al.* (2005, 2006). Briefly, 1ml urine was added to an extraction tube containing 8ml dichloromethane. The samples were vortexed for 10min and the resulting steroid-free urine was discarded using a Pasteur-pipette. Next, NaOH (0.05M) was added and the samples inverted gently 3 times. Then, using a Pasteur pipette, NaOH was discarded and HCL (0.05M) was added and the inverting and discarding procedures were repeated again. The same discarding and inverting procedure was repeated with water. The obtained steroid-rich fraction was transferred to disposable Pyrex glass tubes and dichloromethane was evaporated in a water bath at 37^{o}C. The samples were then reconstituted in assay buffer and vortexed vigorously on V400 multi-tube vortexer (Alpha Laboratoiries, the Netherlands) before being stored overnight at 4^{o}C. The reconstituted samples were vortexed again prior to sampling.

2.16.4 Preparation of saliva samples

As with urine, saliva samples were thawed and centrifuged at 6000rpm for 5 minutes to remove any impurities. Next, a 500µl aliquot was pippetted to Fischer extraction tubes containing 4ml diethlyether, vortexed for 10 minutes on muti-tube vortexer, stored at -80°C for 10minutes. Once, the aqueous phase was frozen, the remaining unfrozen steroid-rich fraction was decanted into disposable Pyrex glass tubes and diethylether was evaporated in a water bath at 37^{o}C. The samples were then reconstituted in assay buffer and vortexed vigorously on V400 multi-tube vortexer (Alpha Laboratoiries, the Netherlands) before being stored overnight at 4^{o}C. The reconstituted samples were vortexed again prior to sampling.

2.16.5 Preparation of serum samples

Serum samples were extracted according to the method described for urine.

2.16.6 Assay

A 96-well microplate was coated with cortisol/cortisone conjugate and incubated at 4^{o}C overnight. On the following morning, the fluid was discarded and the microplate was washed 3 times with 250µl wash buffer. Next, 200µl blocking buffer was added and the plate was covered with parafilm and left to incubate for 1h at 37^{o}C. Following the incubation period, the fluid was discarded, and 50µl of standards and urine, saliva or serum samples were aliquoted in duplicates. A 100µl of cortisol or cortisone antibody was added to all wells using a multi-channel pipette and the microplate mixed on the bench for 1 minute. After a 2h incubation in the dark at room temperature, the fluid was discarded and the plate washed 3 times with 250µl of wash buffer. A 100µl of enzyme was added and the plate was left to incubate for 1h. Following incubation, the discarding and washing procedures were repeated again and 100µl of freshly prepared substrate buffer was added to all wells and the plate was left in the dark for 15min to allow colour development. Finally, 50µl of stop solution was dispensed into all wells using a multi-channel pipette and the absorbance read at 450nm using a Dynex Technologies MRX microplate reader (Dynex Technologies Ltd, Worthing, UK).

Intra-assay precision for cortisol and cortisone ELISA ranged from 3.65% to 6.12% and 5.5% to 11.7%, respectively while inter-assay precision ranged from 4.74% to 8.66% and from 8.7% to 12.8%, respectively (Al-Dujaili *et al.*, 2007; 2009; Baghdadi *et al.,* 2010). Additional validation data are provided in Appendix 1.

2.17. Measurements (Main study)

2.17.1 Haematological, biochemical assessment and blood pressure

Fasting glucose was measured using a commercial hexokinase assay (Sentinel, UK) while fasting insulin was measured using a commercial insulin ELISA kit (Mercodia, Sweden). Data on the precision and accuracy of the methods as determined by the manufacturers is provided in Appendix 1. Assessment of the lipid profile was undertaken at the Clinical Biochemistry Laboratory, Royal Infirmary of Edinburgh, Scotland, UK, using an automated platform (Olympus, UK). Serum NEFA were quantified using an automated enzymatic colorimetric method at the Rowett Institute of Nutrition and Health, Dundee, Scotland, UK (Wako, Germany). BP, 24-h urinary magnesium, sodium and potassium, urinary, salivary and serum cortisol and cortisone were measured as described for preliminary studies. The following equations were used to calculate HOMA-IR, HOMA-β, QUICKI and revised-QUICKI.

Equation 5 HOMA-IR= [fasting glucose (mmol/L) * fasting insulin (uLU/mL)]/22.5
(Matthews *et al*., 1985)

Equation 6 HOMA-β= 20* fasting insulin (mU/L)/ (fasting glucose (mmol/L) - 3.5)
(Matthews *et al*., 1985)

Equation 7 QUICKI= 1/[log (I0)+log (G0)],
where I0= fasting insulin in (uLU/mL) and G0= fasting glucose in (mmol/L) (Wallace *et al*., 2004)

Equation 8 Revised-QUICKI= 1/[log (I0)+log (G0)+log (N0)],
where I0= fasting insulin in (uLU/mL), G0= fasting glucose in (mmol/L) and N0= fasting NEFA in (mmol/L) (Perseghin *et al*., 2001)

2.17.2 Serum phenolic content, ferric-reducing capacity of plasma and oxygen radical absorbance capacity

To avoid interferences from proteins, serum samples were deproteinised according to the method of Cao *et al.* (1998). Briefly, 500µl of 0.5M pechloric acid was added to a similar quantity of serum and the samples were left to cool for 15 min at 4^{o}C. Samples were then centrifuged for 30min at 13 000rpm and the resulting supernatant collected for estimation of total phenolics and FRAP. For ORAC, deproteinised serum samples were further diluted to give a final dilution factor of 1:100.

2.18.Statistical analyses

2.18.1 Preliminary GCBE study

All data are expressed as mean ± SDs unless otherwise stated. Changes in fasting capillary glucose, BP and anthropometrical measurements from baseline across Week1 and Week 2 of the intervention were compared using repeated measures ANOVA with Bonferroni post-hoc tests. Differences in total cholesterol, urinary and salivary cortisone, cortisol and cortisone-to-cortisol ratio, dietary intake and energy expenditure were analysed using a Paired sample t-test. Significance was set at a p-value ≤ 0.05. The relationship between capillary fasting glucose levels, total cholesterol, SBP, DBP, BMI, weight, WC, HC, WHR, urinary magnesium, sodium and potassium levels, salivary and 24-h urinary free cortisol, cortisone, cortisol-to-cortisone ratio were assessed using Pearson product-moment correlation coefficient, r. The coefficient of determination was estimated by obtaining r^2. All statistical analyses were performed using SPSS for Windows version 16.0.0 (SPSS Inc, Chicago, IL, USA).

2.18.2 Preliminary DC study

All data are expressed as mean ± SDs unless otherwise stated. Mixed between-within subjects analysis of variance (SPANOVA) was performed for multiple comparison, where time (baseline, week 1, week 2) was the within-group variable and intervention group (500 mg, 1000 mg DC) was the between-group variable and the continuous variable were glucose, SBP, DBP BMI, weight, WC, HC, WHR. A p-value ≤ 0.05 was deemed statistically significant. Within each intervention group (500 mg, 1000 mg DC) changes in fasting blood glucose levels, SBP and DBP were analysed using repeated measures ANOVA with Bonferroni post-hoc tests. A separate SPANOVA was also performed to detect any carry-over effects between the two interventions and to ensure changes in fasting glucose, SBP and DBP following each treatment were not affected by the sequence of DC administration (1000mg followed by 500mg vs. 500mg followed by 1000mg). Two-tailed paired sample t-tests were used to assess changes between baseline and post-intervention total cholesterol, urinary free cortisol or cortisone, urinary cortisol-to-cortisone ratio, mineral excretion, dietary intake and energy expenditure. Similarly, differences in response to both DC doses amongst the various ethnic groups were assessed using one-way between-groups ANOVA with fasting glucose, SBP and DBP as the dependent variables and ethnicity as the Factor. An independent sample t-test was also used to compare the response to DC polyphenols between the abdominally obese individual and the peripherally obese individuals. The

relationship between fasting blood glucose levels, total cholesterol, SBP, DBP, BMI, weight, WC, HC, waist-to-hip circumference, urinary magnesium, sodium and potassium levels, 24-h urinary free cortisol, cortisone, cortisol-to-cortisone ratio were assessed using Pearson product-moment correlation coefficient, r. The coefficient of determination was estimated by obtaining r^2. All statistical analysis was performed using SPSS for Windows version 16.0.0 (SPSS Inc, Chicago, IL, USA).

2.18.3 Main study

Continuous normally distributed data are expressed as mean ± SD unless otherwise stated. Data distribution was assessed using the Explore function of the Descriptive Statistics in SPSS. Differences in baseline characteristics between the two BMI categories (BMI< $25kg/m^2$ and BMI≥ $25kg/m^2$) were examined using an independent sample t-test with BMI category placed as the grouping variable and age, BMI, WC, HC, WHR, fasting insulin, glucose, total cholesterol (TC), TG, HDL, LDL, TC: HDL ratio, SBP, DBP, urinary free cortisol, urinary free cortisone, urinary cortisone-to-cortisol ratio, urinary and serum FC, Fe^{2+}, ORAC as the dependent variables. For multiple comparisons, data were analysed with a three-factor repeated measures ANOVA with time (pre- and post-) and treatment (DC and placebo) as the 2 within-subject factors and BMI category as the between-subject factor. Post hoc comparisons of the main effects of time, treatment and BMI category were conducted using Bonferroni correction with significance level set at a $P \leq 0.05$. For statistically significant treatment-by-time interactions, differences from baseline for each of treatment and placebo group were obtained and compared using a paired-sample t-test. Analyses were also repeated using the split-file function in order to compare differences in response within each of the BMI categories. If statistically significant treatment-by-time-by-BMI category interactions were also observed, then differences from baseline were also used to compare the response between the two BMI categories using an independent sample t-test. Significance was set at a $P \leq 0.05$, unless otherwise stated. Changes in physical activity, energy, macronutrient and micronutrient intake were assessed using SPANOVA with time as the within-subject factor and BMI as the between-subject factor. Significant differences, if any, were tested by applying a Bonferroni correction.

The relationship between all assessed variables was assessed using Pearson product-moment correlation coefficient, r. The coefficient of determination was estimated by obtaining r^2. All statistical analyses were performed using SPSS for Windows version 16.0.0 (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Antioxidant capacity of green coffee and Theobroma cacao bean extracts

Table 3.1 shows mean polyphenol content of GCBE described as GAE per g extract. As observed, the polyphenol content in the acidified water extract varied greatly amongst the triplicates as opposed to other extracts with a large variation being present between the polyphenol content of ethanol, acidified-water and acetonitrile extracts. Similar findings were observed with the FRAP assay, wherein the acetonitrile extract exhibited a stronger ferric-reducing ability than the ethanolic extract and acidified-water extracts (Figure 3.1). With regard to TCBE, the bound fraction was found to contain the majority of phenolic compounds (2952.2±135.4µg GAE per ml bound fraction vs. 296.2±70.4µg GAE per ml unbound fraction).

The ORAC values of GCBE and TCBE are shown in Figure 3.2.

Figure 3.5 shows dose-dependent inhibition of DPPH-radical by each of the samples tested. One-way ANOVA revealed no significant difference in IC50 between the tested samples (P=0.097) although CGA was found to have the lowest DPPH-radical scavenging activity. The IC50 of different antioxidants and GCBE and TCBE are shown in Figure 3.4. As observed, a high concentration of CGA was required to inhibit the DPPH-radical by 50%. Compared to CGA alone, GCBE rich in CGA had a lower IC50 value.

Table 3.1 Mean polyphenol content and coefficient of variation between triplicate analyses of the same sample and between different samples of green coffee bean extract

Extraction solvent	Mean (SD) of triplicates (mg/g extract)	CV	Mean (SD) of three different samples (mg/g extract)	CV
Acidified-water	130.91 (0.66)	0.50	116.47 (14.55)	12.49
Ethanol	122.77 (0.23)	0.19	128.40 (5.76)	4.49
acetonitrile	211.05 (0.51)	0.24	221.82 (8.63)	3.89

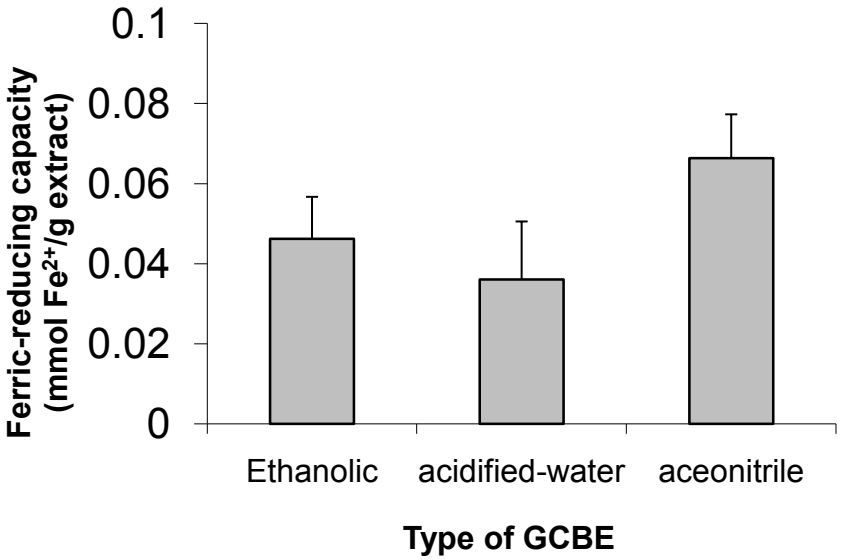


Figure 3.1 Ferric-reducing capacity of various extracts of green coffee bean. Data are expressed as mmol Fe^{2+} per g extract. Data are expressed as mean of three different samples ±SEM.

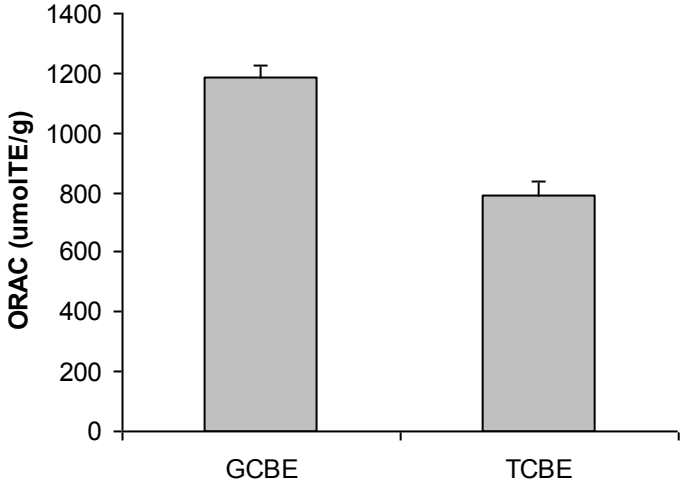


Figure 3.2 Antioxidant capacity of green coffee bean extract (GCBE) and Theobroma cacao bean extract (TCBE) as measured by the Oxygen Radical Absorbance Capacity (ORAC) assay. Data are represented as mean ±SEM (n=4). Data are expressed in µmol trolox equivalents per g extract.

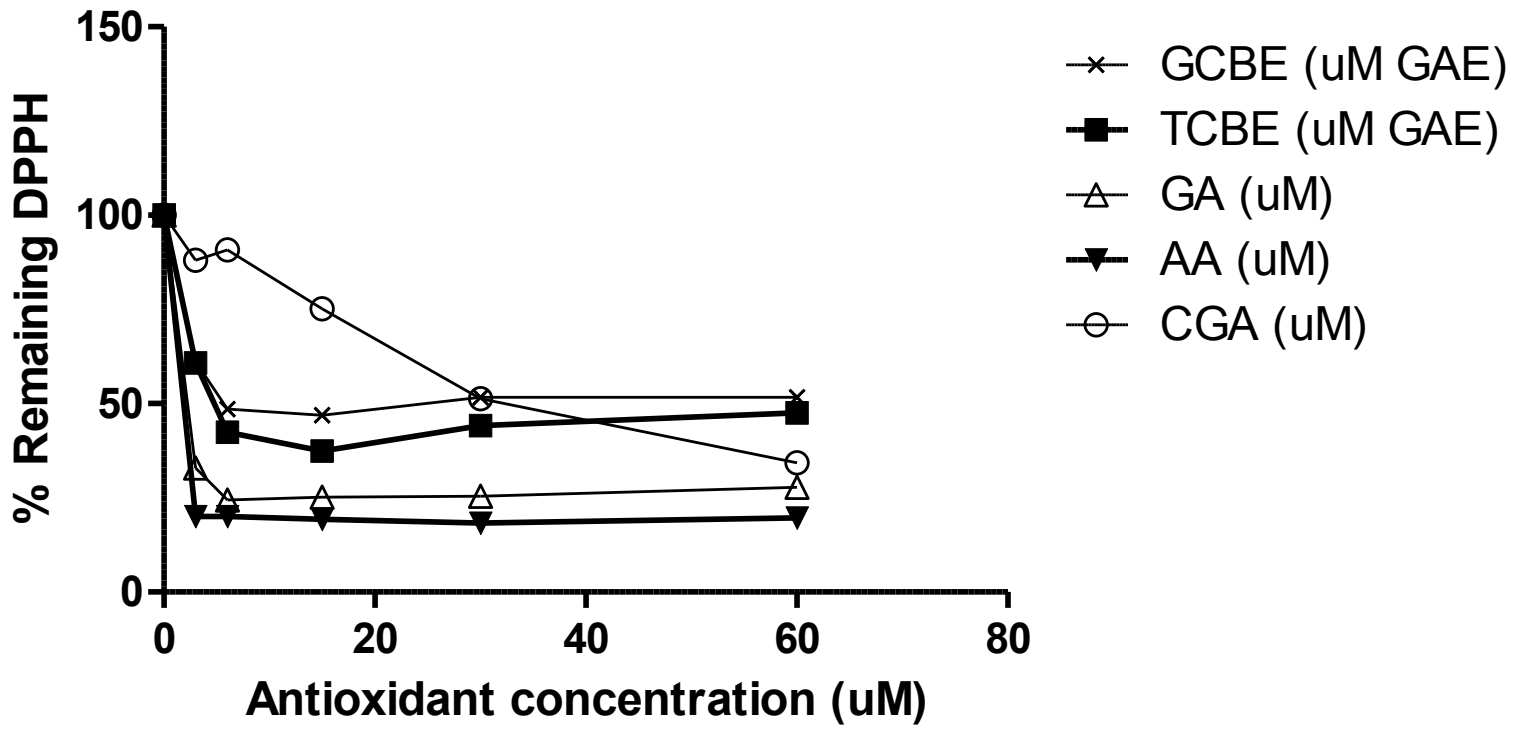


Figure 3.3The percentage remaining 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) against increasing concentrations of green coffee bean extract (GCBE), Theobroma cacao bean extract (TCBE), gallic acid (GA), ascorbic acid (AA) and chlorogenic acid (CGA). Data are represented as mean of duplicates with standard deviations.

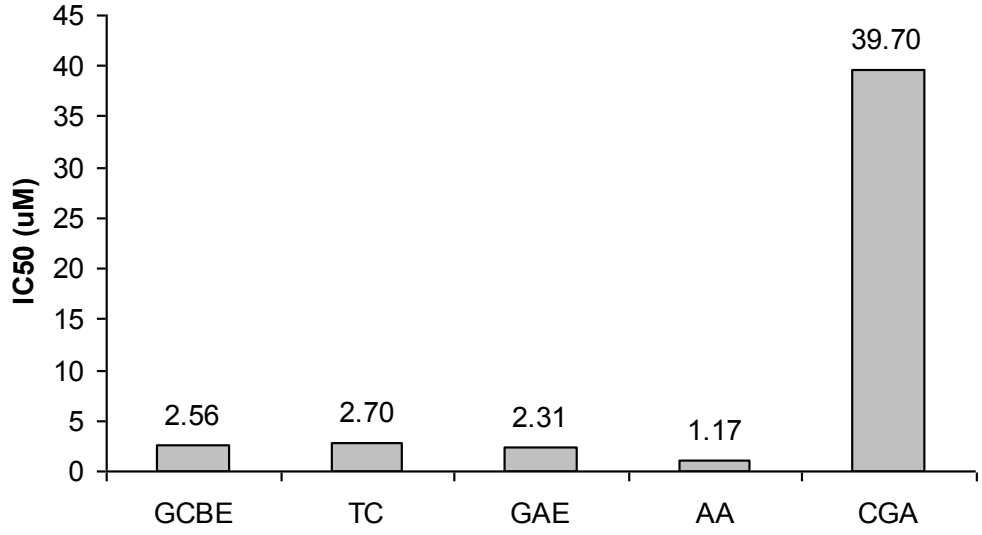


Figure 3.4 **The inhibitory concentration (IC)50 of different phenolic compounds as measured by DPPH. Data are represented as mean of duplicates.**

3.2. Lipase assay

Figure 3.5 shows the effect of GCBE and TCBE on pancreatic lipase activity. GCBE showed a J-shaped dose-dependent inhibition of pancreatic lipase with the percentage inhibition ranging from 11.8 to 61.5%. Similar concentrations of TCBE, on the other hand, had a negligible effect on lipase activity. Non-linear regression analysis revealed that the concentration of GCBE required to elicit a 50% inhibition of pancreatic lipase activity (IC50) was 43.37µg.

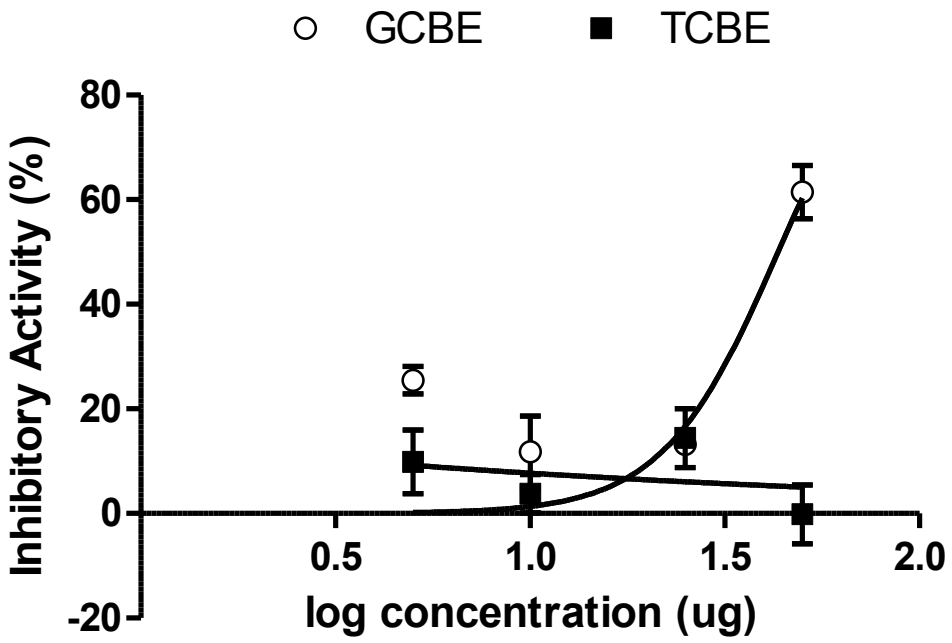


Figure 3.5 percentage inhibition of pancreatic lipase by each of the green coffee bean extract (GCBE) and *Theobroma cacao* bean extract (TCBE). Data are represented as mean of triplicates.

3.3. Preliminary green coffee bean extract study

Table 3.2 shows baseline characteristics of the study population. Overall, Twenty three healthy overweight or obese volunteers were recruited (9 males, 14 females). Of these participants, twenty were peripherally obese and three were abdominally obese. All subjects completed the trial with the exception of one volunteer who failed to complete the second urine (Week 2) collection. Data from this subject for the remaining non-urinary tests was still included in the analyses. Based on volunteers' reports and capsule counting, all GCBE tablets were ingested as directed in the subject information sheets.

Table 3.2 Baseline characteristics of the study population (n=23)

Parameter	Values (mean ± SEM)
Age (yr)	34.09 ± 11.27
BMI (kg/m^2)	27.79± 2.15
WC (cm)	89.02± 9.91
Waist-to-hip ratio	0.84 ± 0.08

Repeated measures ANOVA revealed a significant reduction in SBP following consumption of GCBE (P=0.043). This reduction as demonstrated by post-hoc Bonferroni test was present after 2 weeks of GCBE consumption (P=0.036) but not after 1 week (P=0.276) (Figure 3.6). No significant changes in fasting glucose (Baseline 4.46±0.79, Week 1 4.20±0.74, Post-GCBE 4.22±0.65; P=0.258), TC (Baseline 4.87±0.94, Post-GCBE 4.90±0.81; P=0.694) or DBP (P=0.156; Figure 3.6) were seen.

Paired sample t-test showed that 24h urinary free cortisone excretion significantly declined from 64.95± 34.47 to 42.61±24.47 nmol/d (P=0.0015) (Figure 3.7). A rise in urinary free cortisol from 21.47 ±50.55 to 42.61±24.47 nmol/d (p=0.279) and a fall in urinary cortisone-to-cortisol ratio from 3.32 ±5.83 to 1.26±1.04 (p=0.085) were also observed but neither reached statistical significance (Figure 3.7). Reductions in urinary free cortisone remained significant even after adjustment for weight (Table 3.3). In a subset sample of nine volunteers, salivary free cortisone was found to be significantly raised following 2-week consumption of GCBE while no significant changes in salivary cortisol or salivary cortisone-to-cortisol ratio were detected (Figure 3.8).

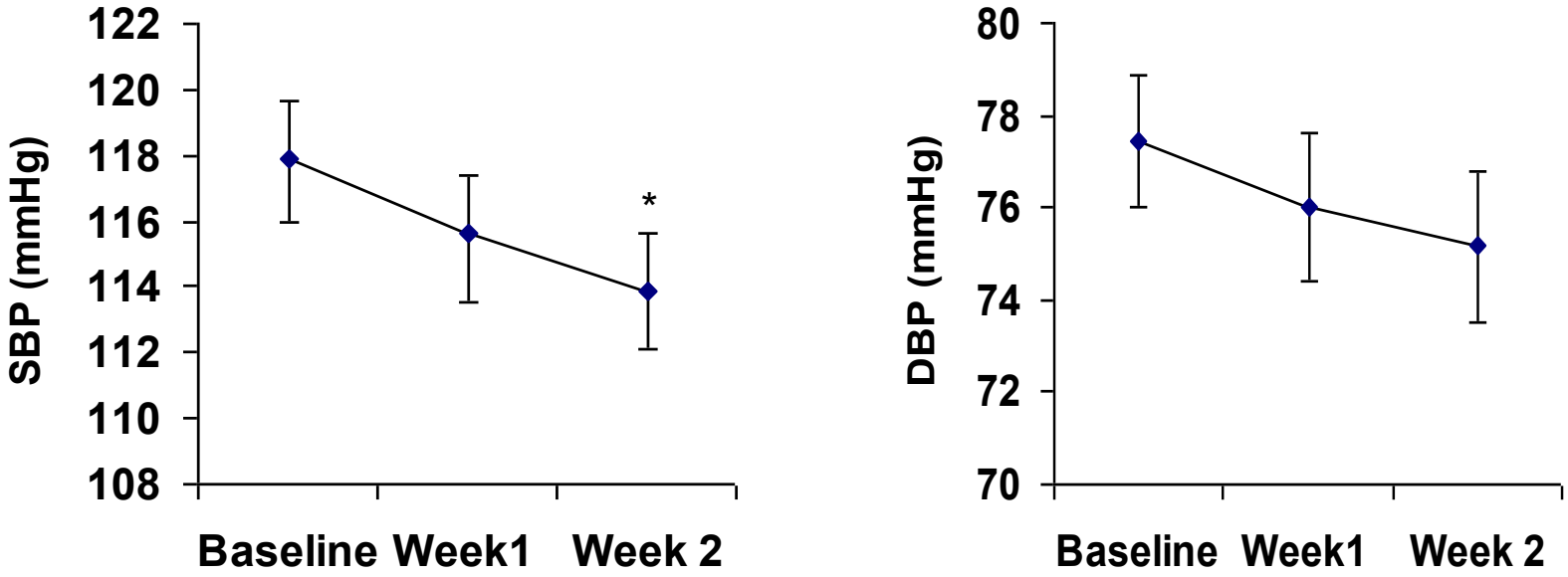


Figure 3.6 Effect of green coffee bean extract consumption for 14 days on systolic (SBP) and diastolic blood pressure (DBP) in healthy overweight subjects (n=13). The asterisk indicates that there was a significant reduction in blood pressure following consumption of green coffee bean extract. Data expressed as mean values ± SEM

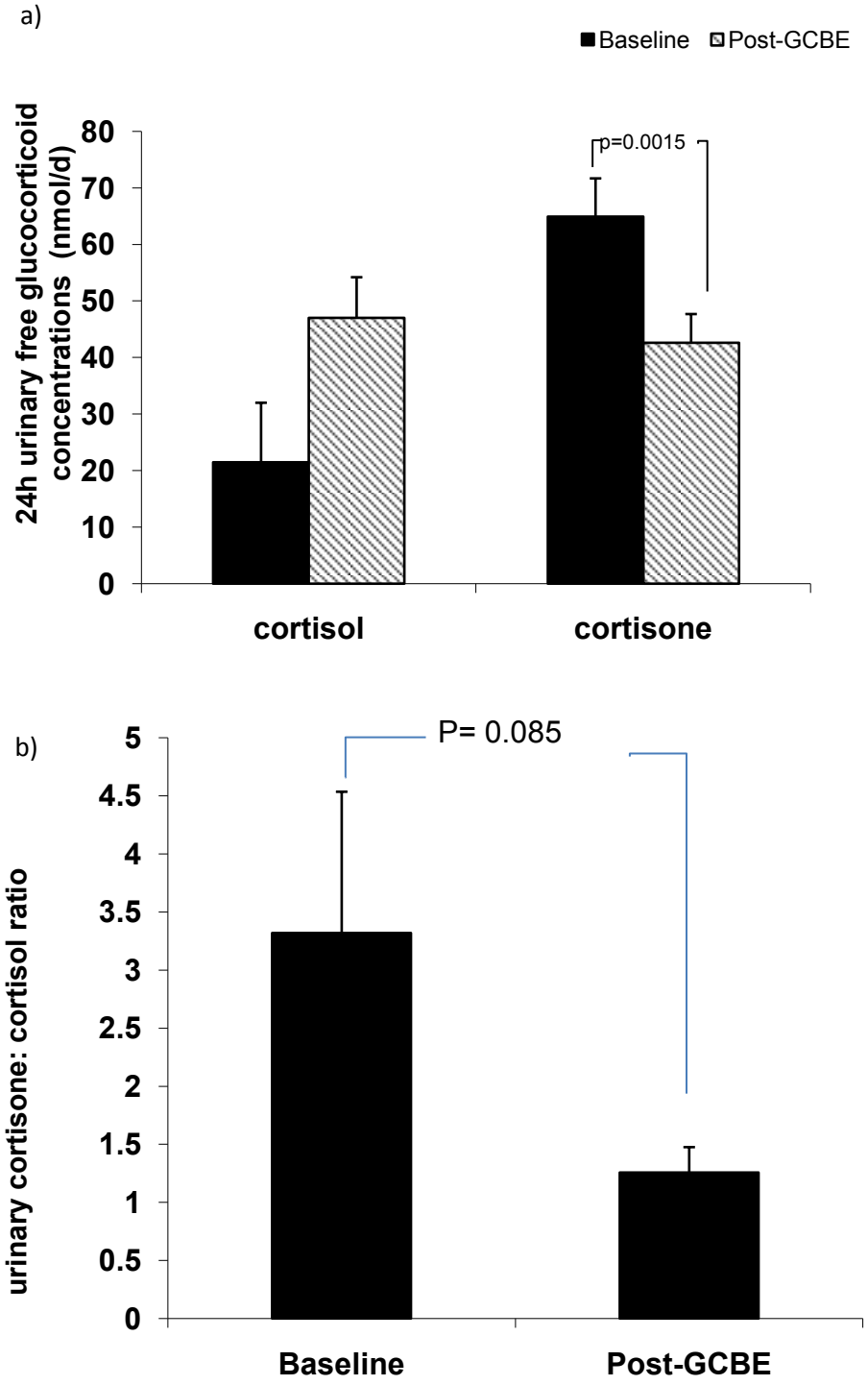


Figure 3.7 Effect of green coffee bean extract consumption for 14 days on urinary free cortisol, urinary free cortisone (a) and urinary cortisone-to-cortisol ratio (b) in healthy overweight subjects (n=22). Data expressed as mean ± SEM.

Table 3.3 Changes in urinary glucocorticoids after adjustment for weight (n=22)

variable	Baseline		Week 2		Paired Sample t-test
	mean	SD	mean	SD	P-value
Urinary free cortisol (nmol/kg/d/)	0.75	0.75	0.60	0.46	0.339
Urinary free cortisone (nmol/kg/d)	0.86	0.46	0.55	0.32	0.0025
Cortisone-to-cortisol ratio	3.46	5.94	1.29	1.06	0.083
Magnesium (mmol/d)	3.27	2.23	3.23	2.13	0.934
Sodium (mmol/d)	124.09	76.57	105.64	58.95	0.299
Potassium (mmol/d)	94.14	83.28	77.29	79.42	0.227

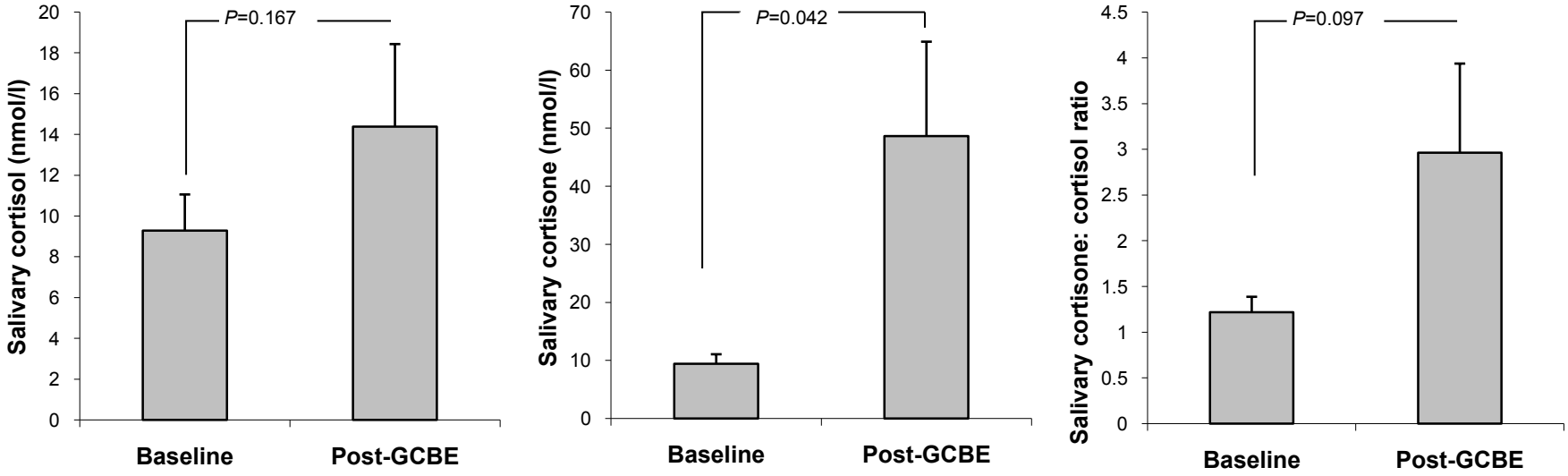


Figure 3.8 Effect of green coffee bean extract consumption for 14 days on salivary free cortisone, salivary free cortisol and salivary cortisone-to-cortisol ratio in healthy overweight subjects (n=9). Data expressed as mean ± SEM.

3.3.1 Urinary total phenolics and ferric-reducing capacity of plasma

In vivo antioxidant activity was determined using FC and FRAP. There was a significant correlation between urinary polyphenols excretion as determined by FC and FRAP (0.637, $p<0.0001$). However no significant increase in urinary antioxidant activity was observed (total phenolics: Pre-GCBE 126.89 (SD 100.76) mg/g GAE equivalents/creatinine vs. Post-GCBE 135.81 (SD 130.77) mg/g GAE equivalents/creatinine, $p>0.05$; FRAP: Pre-GCBE 1.77 (SD 1.29) mmol/g Fe^{2+}/creatinine vs. Post-GCBE 1.49 (SD 0.78) mmol/g Fe^{2+}/creatinine, $p>0.05$).

3.3.2 Weight and anthropometry

No significant changes in weight, BMI, HC, percentage body water or fat were noted after consumption of GCBE (repeated measures ANOVA > 0.05).

Table 3.4 Changes in anthropometrical measurements(n=23)

variable	Baseline		Week 1		Week 2		Repeated-ANOVA
	mean	SD	mean	SD	mean	SD	Wilk's Lambda
Weight(kg)	79.70	11.14	79.57	10.83	79.43	10.95	0.651
BMI[1] (kg/m^2)	27.79	2.15	27.76	2.13	27.71	2.20	0.707
HC[2] (cm)	105.76	5.60	105.37	5.77	105.46	5.75	0.355
WHR[3]	0.842	0.0797	0.840	0.083	0.837	0.084	0.086

BMI[1]bosy mass index; HC[2]hip circumference; WHR[3]waist-to-hip ratio

However, a significant reduction in WC was observed (P=0.013) (Figure 3.9). Post-hoc tests using Bonferroni further revealed that the reduction in WC was gradual and persisted from baseline (89.02±9.91) across Week 1 (88.50±9.69, P=0.055) and into Week 2 of GCBE ingestion (88.24±9.64; P=0.008). WHR was also decreased following GCBE by 0.004. However, this reduction did not reach statistical significance (P=0.086).

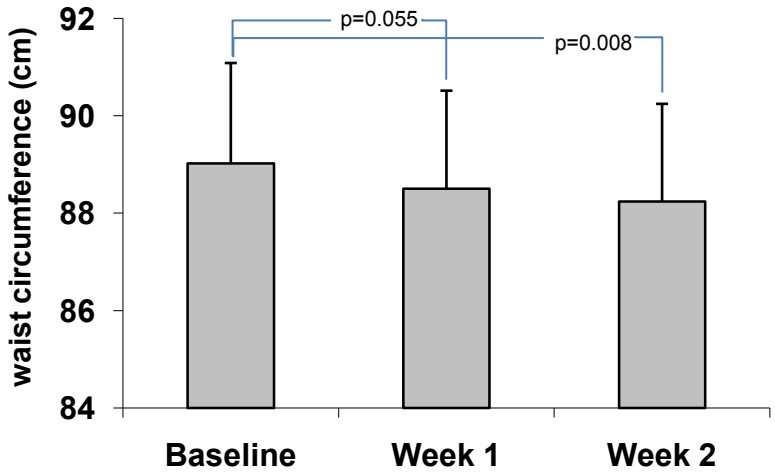


Figure 3.9 Effect of green coffee bean extract consumption for 14 days on waist circumference (n=23). The asterisk indicates a significant reduction in waist circumference following consumption of green coffee bean extract. Data are expressed as mean ± SEM

3.3.3 Energy intake and expenditure

No significant changes in energy intake, energy expenditure or macronutrient, micronutrient intake were observed during the study (Paired sample t-test, P >0.05) (Figure 3.10).

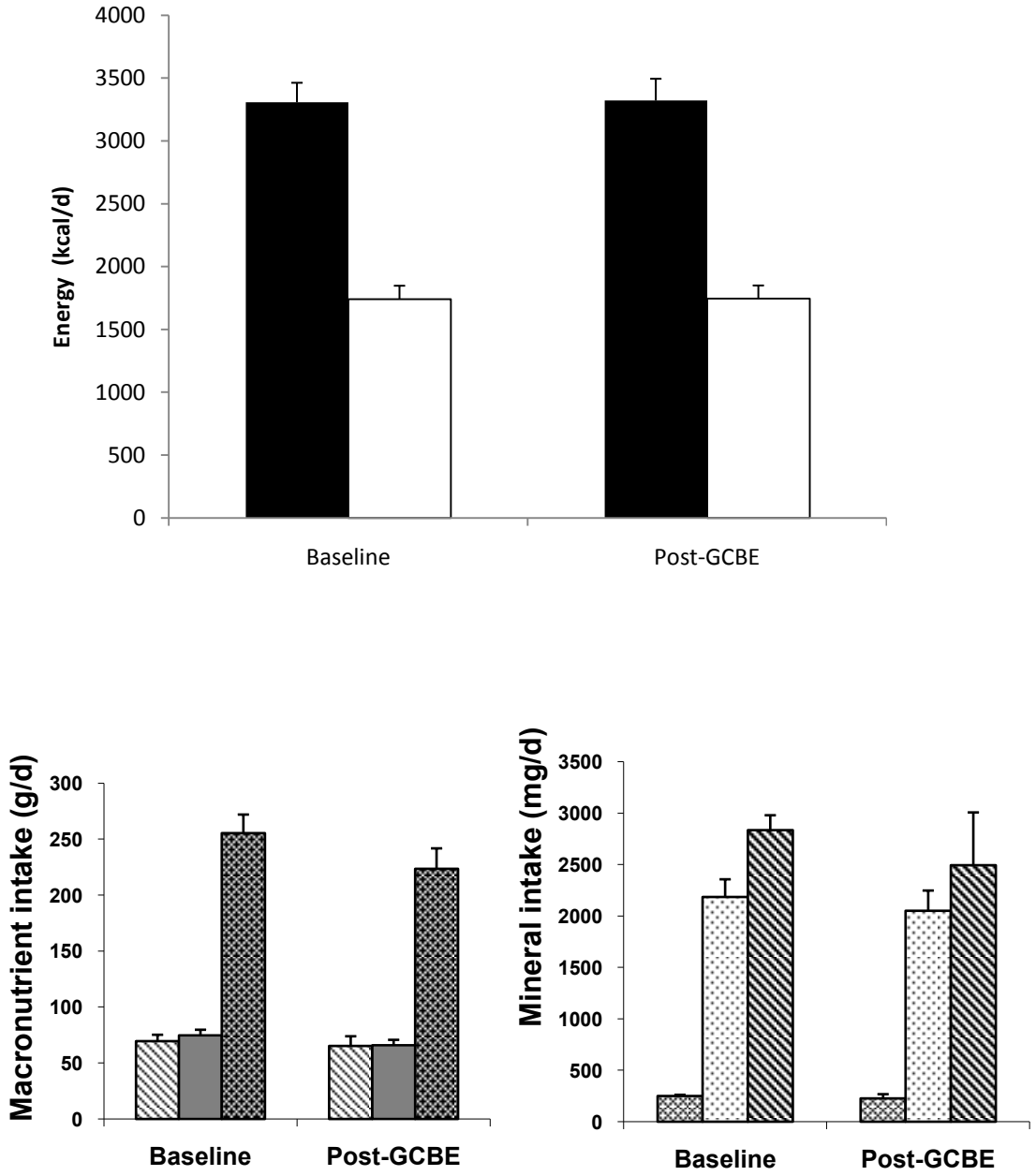


Figure 3.10 Energy, Macronutrient and mineral intake at baseline and at the end of 2-week intervention (n=22). Values are mean, with standard errors represented by vertical bars. (■) energy expenditure; (□) energy intake; (▨) fat intake; (▩) protein intake; (▩) carbohydrate intake; (▩) magnesium intake; (▩) sodium intake; (▨) potassium intake).

3.4. Animal study

Figure 3.11 shows free corticosterone levels in different tissues in both the control group and the treatment group. Compared to control, mice receiving CGA showed a trend towards an increase in the concentration of free corticosterone in kidney. Conversely in the liver, there was a trend towards a reduction in free corticosterone. None of these values reached statistical significance (P= 0.108 and 0.219). However, calculation of Eta^2 revealed a large effect of treatment on free corticosterone levels in kidney (Eta^2 = 0.26) and liver (Eta^2 =0.14). No differences in free corticosterone in mesenteric (P=0.521, Eta^2 =0.05) or subcutaneous tissues (P=0.950 Eta^2 < 0.001) were observed.

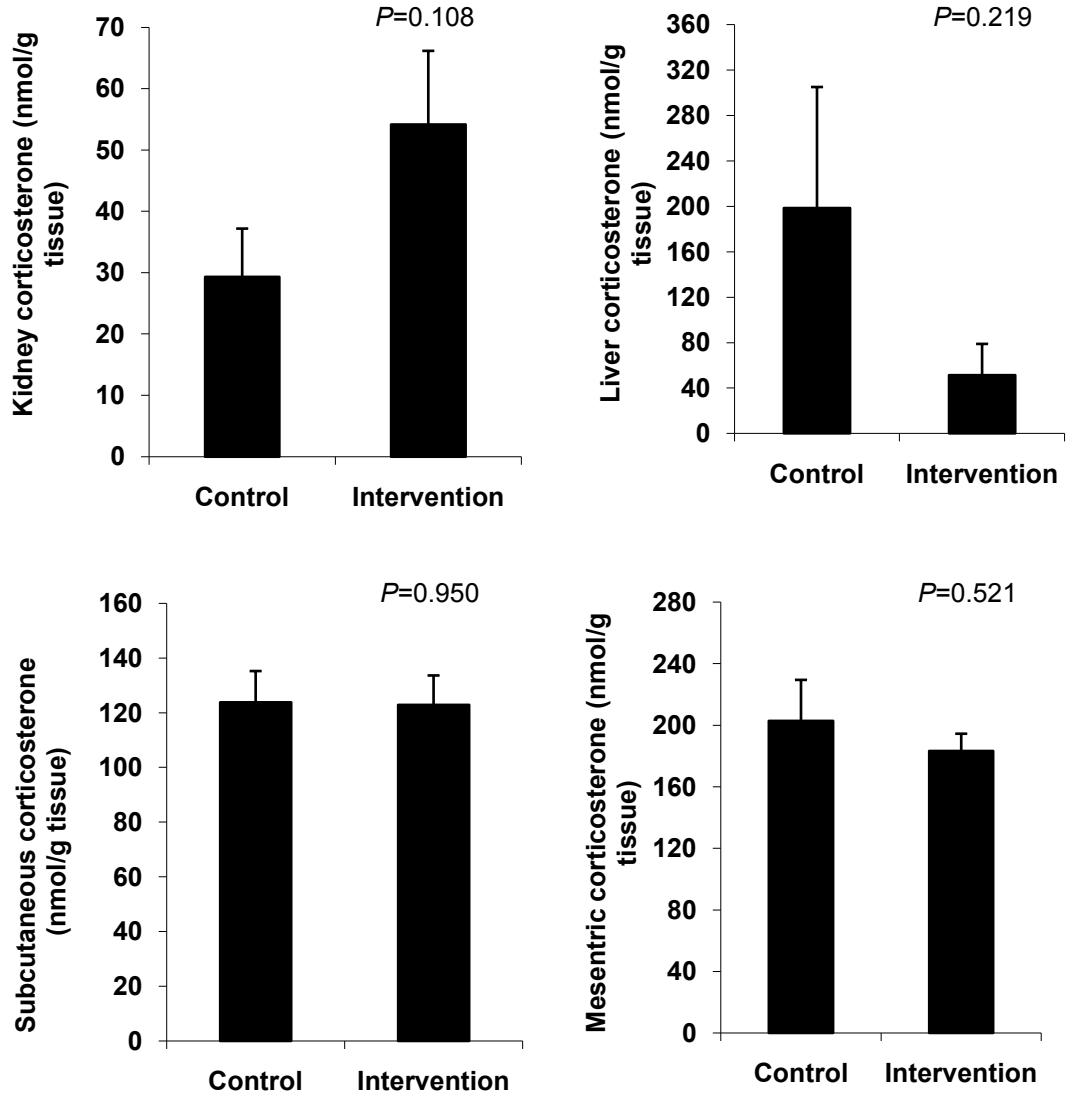


Figure 3.11 Tissue free corticosterone in control mice (n= 5) and in mice fed a diet containing 0.15% chlorogenic acid (n= 6). Data are expressed as mean ± SEM.

Figure 3.12 shows glucoronidated- and sulphated-corticosterone levels in different tissues in both the control group and the treatment group. No significant changes in glucoronidated- or sulphated- corticosterone concentrations were observed in kidney, liver or mesenteric tissue. In subcutaneous tissue, mice receiving CGA showed a trend towards a reduction in glucoronidated- and sulphated-corticosterone as compared to mice receiving a control diet. However, this difference did not reach statistical significance.

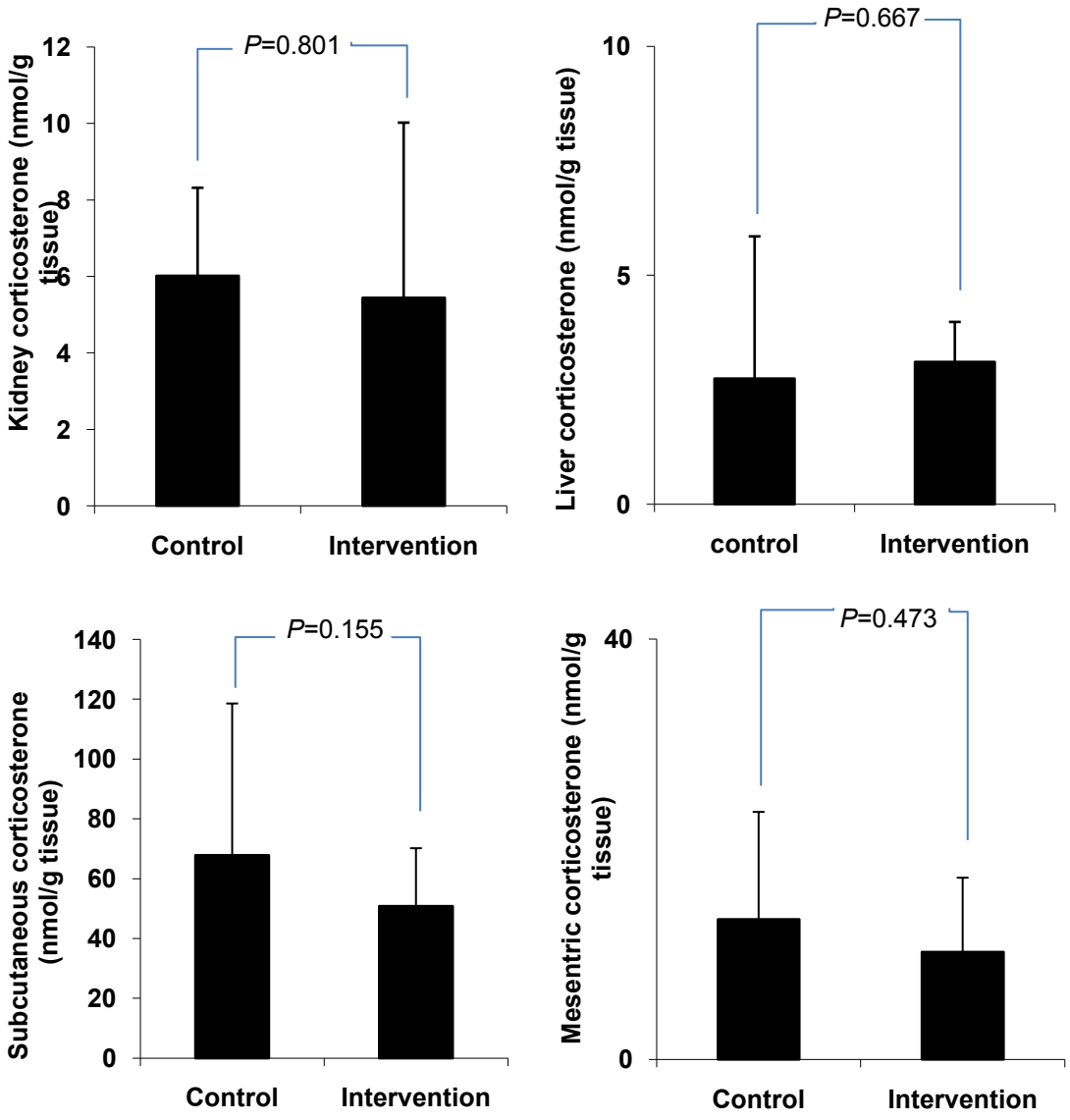


Figure 3.12 Tissue glucoronidated- and sulphated-corticosterone in control mice (n= 5) and in mice fed a diet containing 0.15% chlorogenic acid (n= 6). Data are expressed as mean ± SEM.

3.5. Preliminary dark chocolate study

Figure 3.13 shows flow of volunteers through the preliminary DC study. This study included fourteen healthy volunteers [eight males (five Caucasians, two Asians, one African) and six females (five Caucasians and one Hispanic], 21-50 years old, mean age 26.4± 11.5 years) with a BMI of 27.7± 2.5 kg/ m^2. Of these participants, thirteen were peripherally obese and one was abdominally obese (African). Based on volunteers' self-reports and returned empty bags, all volunteers were found to have ingested the chocolate products provided.

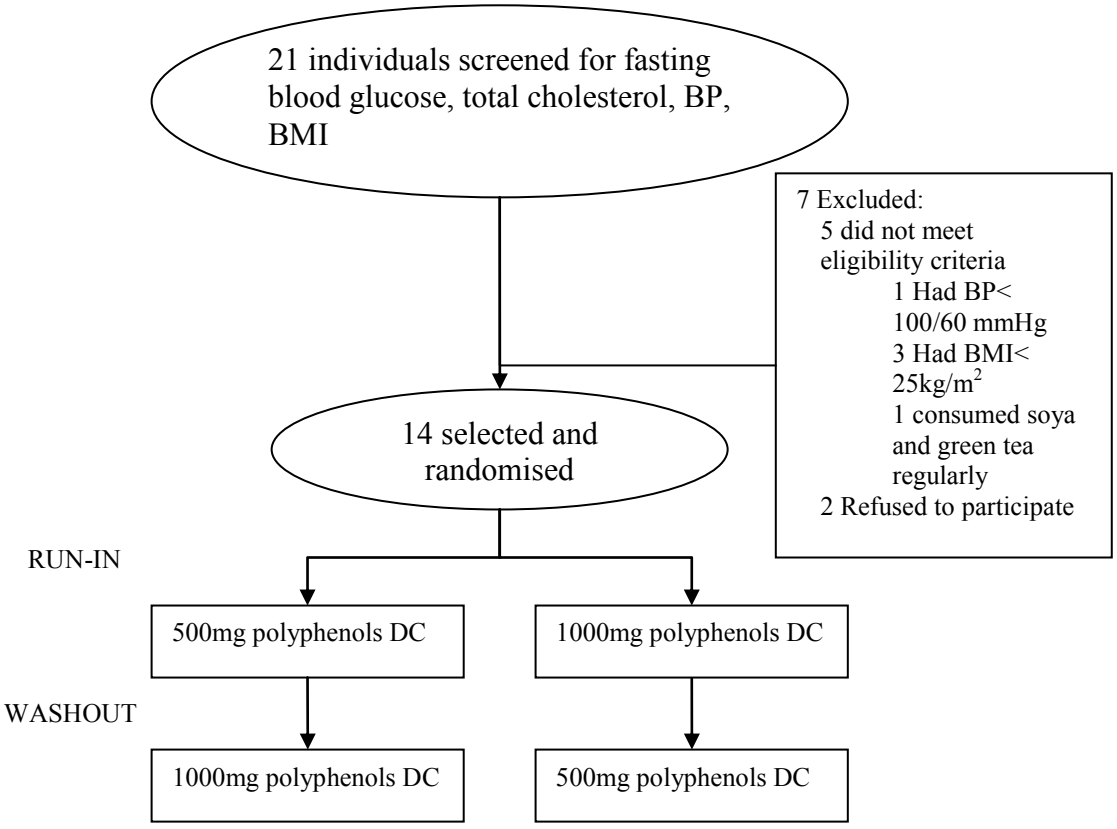


Figure 3.13 Flow of participants through the preliminary dark chocolate trial.

Mixed between-within subjects analysis of variance revealed a significant reduction in fasting capillary blood glucose concentrations (P=0.002), SBP (P<0.0001) and DBP (P<0.0001) following DC consumption. These effects were independent of the sequence of DC administration and no significant interaction between time, intervention group and

sequence of DC administration was observed (fasting glucose F(2, 11)= 1.057, P =0.380; SBP F (2, 11)=0.431, P=0.660; DBP F (2, 11)=0.653, P=0.539) (Figure 3.14 and Figure 3.15). No significant differences between the effect of 1000mg and 500mg polyphenols DC on fasting capillary blood glucose (P > 0.05) and BP (P > 0.05) were observed indicating that both doses have a similar efficacy.

To explore the results further, a one-way repeated measures ANOVA was conducted to compare fasting blood glucose levels, SBP and DBP at baseline, week 1 and week 2 for each of the two dietary interventions. A significant effect of DC on fasting blood glucose levels (F(2, 12)=4.305, P=0.039), SBP (F(2,12)=12.330, P =0.001) and DBP (F(2,12)=13.937, P =0.001) was observed after consumption of 1000 mg DC. Post-hoc comparisons using Bonferroni test indicated that mean fasting blood glucose levels and SBP at week 2 were significantly decreased after 1000mg DC ingestion (fasting glucose 3.97 (SD 0.54) vs. baseline 4.42 (SD 0.70)mmol/l; SBP 112.12 (SD 9.68) vs. baseline 119.38 (SD 10.51) mmHg). Mean DBP levels were significantly lower at week 1 (74.45 (SD 7.17) mmHg) and week 2 (74.57 (SD 7.39)mmHg) compared to baseline (78.62 (SD 7.74)mmHg).

A significant effect of 500mg DC on fasting glucose levels (F(2,12)=5.026, P=0.026), SBP (F(2,12)=11.971, P=0.001) and DBP (F(2,12)=7.709, P =0.007) was also observed. Post-hoc comparisons indicated that the mean fasting glucose levels at week 2 were significantly different from baseline (3.92 (SD 0.86) vs. baseline 4.42 (SD 0.30) mmol/l). Mean SBP was also reduced at the end of week 1 (114.24 (SD 9.53) mmHg) and week 2 (112.40 (SD 9.51)mmHg) as compared to baseline (119.38 (SD 10.51)mmHg). Similar findings were observed with DBP (Week 1= 74.62 (SD 4.27) and Week 2= 73.00 (SD 5.06) vs. baseline 78.62 (SD 7.74) mmHg).

Total cholesterol did not change significantly after 1000mg (4.98 (SD 0.90) mmol/l vs. baseline 5.21 (SD 1.01) mmol/l; P=0.191) or 500mg polyphenols DC (5.03 (SD 0.77) mmol/l vs. baseline 5.21 (SD 0.77) mmol/l; P=0.246). There was a trend towards a reduction in 24h urinary free cortisone levels in both the 1000 mg and 500 mg DC groups, although this reduction did not reach statistical significance even after adjustment for weight. No changes in anthropometrical data (Table 3.5), 24h urinary free cortisol, cortisol-to-cortisone ratio, 24h urinary magnesium, sodium, potassium were observed (Table 3.6). Likewise, one-way ANOVA revealed no significant effect of ethnicity on changes in fasting glucose, SBP, DBP in both DC groups, with the exception of one female subject (Hispanic)

who experienced a greater reduction in SBP following both polyphenol doses as compared to Caucasians (1000mg P =0.009; 500mg P <0.0001), Asians (1000mg P =0.014; 500mg P =0.003) and African (1000mg P =0.0001; 500mg P <0.009). The subject with abdominal obesity showed an increase in urinary free cortisol following 1000mg polyphenols (+52.44 nmol/d) compared to peripherally obese individuals who demonstrated a reduction (-11.05 nmol/d; P=0.037). This subject also had a greater reduction in DBP following 500mg DC compared to other individuals (-15.7 mmHg versus -3.10 mmHg; P=0.017).

No significant changes in urinary total polyphenols were observed following consumption of either 500mg or 1000mg polyphenol-rich DC (total phenolics: Baseline 173.21 (SD 137.77) mg/g GAE equivalents/creatinine vs. Post-500mg polyphenols DC 160.81 (SD 74.37) mg/g GAE equivalents/creatinine and Post-1000mg polyphenols DC 207.61(SD 115.20) mg/g GAE equivalents/creatinine p>0.05; FRAP: Baseline 2.07 (SD 0.91) mmol/g Fe^{2+}/creatinine vs. Post-500mg polyphenols 2.15 (SD 1.05) mmol/g Fe^{2+}/creatinine and Post-1000mg polyphenols 3.01 (SD 1.57) mmol/g Fe^{2+}/creatinine , p>0.05). However, three subjects (Subject 5, 10 and 14) were found to have higher urinary FC and Fe^{2+} values at baseline as compared to post-polyphenol-rich DC (Figure 3.16 and Figure 3.17). These extreme values acted as outliers and following their exclusion a significant dose-dependent increase in urinary antioxidant capacity was observed (total phenolics: Baseline 115.54 (SD 54.59) mg/g GAE equivalents/creatinine vs. Post-500mg polyphenols DC 166.21 (SD 82.22) mg/g GAE equivalents/creatinine, p=0.024 and Post-1000mg polyphenols DC 227.46(SD 122.28) mg/g GAE equivalents/creatinine p=0.007; FRAP: Baseline 1.76 (SD 0.67) mmol/g Fe^{2+}/creatinine vs. Post-500mg polyphenols 2.36 (SD 1.09) mmol/g Fe^{2+}/creatinine, p=0.033 and Post-1000mg polyphenols 3.45 (SD 1.48) mmol/g Fe^{2+}/creatinine , p=0.001).

Pearson's product moment correlations revealed a significant correlation between changes in 24h urinary free cortisol, cortisone and changes in 24h sodium excretion (Table 3.7). There were no significant correlations between age and changes in fasting glucose, SBP, and DBP following both DC doses (P > 0.05). Addition of DC to the diet did not affect magnesium intake or excretion significantly. Moreover, no significant correlations were found between changes in magnesium intake or excretion and the reductions in fasting blood glucose and BP seen following DC consumption. Energy, macronutrient and mineral intake did not change significantly through the study period (Figure 3.18). Physical activity at the end of 500mg DC (3572.40±933.90; Paired sample t-test, P=0.935) and 1000mg DC intervention (3589.39±949.69kcal/d; Paired sample t-test P=0.857) did not differ from baseline (Baseline: 3561.17±813.25kcal/d).

Table 3.5

Effect of either 500mg or 1000mg polyphenol dark chocolate on anthropometrical measurements (n= 14).

(Mean and standard deviations)

	1000 mg DC[1]							500 mg DC[1]							
	baseline		Week-1		Week-2			baseline		Week-1		Week-2			
	Mean	SD	Mean	SD	Mean	SD	P	Mean	SD	Mean	SD	Mean	SD	n	P
Weight (kg)	81.64	13.40	81.66	12.84	81.36	12.80	0.447	81.29	13.99	80.85	13.69	81.13	13.35	14	0.789
BMI (kg/m^2)	27.73	2.53	27.75	2.38	27.66	2.44	0.517	27.58	2.70	27.46	2.75	27.56	2.56	14	0.796
WC[2] (cm)	90.82	11.85	89.86	11.16	89.93	11.412	0.175	90.82	11.85	90.04	11.29	90.00	11.31	14	0.326
HC[3] (cm)	105.61	5.99	105.64	6.21	105.57	6.30	0.089	105.61	5.99	105.11	6.19	105.11	6.96	14	0.810
WHR[4]	.86	.09357	.85	.09	.85	.10	0.165	.86	.09	.86	.11	.86	.11	14	0.992

DC[1], dark chocolate; WC[2], waist circumference; HC[3], hip circumference; WHR[4], waist-to-hip ratio.

Table 3.6

Results for 24h urine collections.

(Data are expressed as mean and standard deviations)

	1000 mg DC[1]							500 mg DC[1]						
	baseline		Week-2					baseline		Week-2				
	Mean	SD	Mean	SD	n	df	P	Mean	SD	Mean	SD	n	df	P
Free cortisol (nmol/d)	77.33	27.09	71.16	38.90	13	12	0.477	86.83	44.05	78.58	47.28	14	13	0.620
Free cortisone (nmol/d)	54.34	26.90	45.82	17.33	13	12	0.159	59.64	32.56	45.80	20.34	14	13	0.139
Cortisol-to-cortisone ratio	1.6812	0.75	1.60	.63	13	12	0.664	1.68	0.72	1.77	0.62	14	13	0.682
Cortisone-to-cortisol ratio	0.71	0.32	0.75	0.37	13	12	0.744	0.71	0.31	0.63	0.21	14	13	0.455
Free cortisol (nmol/kg/d)	0.98	0.37	0.89	0.44	13	12	0.400	1.0529	0.43	0. 98	0.64	14	13	0.731
Free cortisone (nmol/kg/d)	0.69	0.36	0.57	0.17	13	12	0.162	0.73	0.38	0.55	0.23	14	13	0.180
Creatinine (mmol/d)	12.74	5.35	11.78	4.40	13	12	0.219	12.83	5.15	19.29	27.37	14	13	0.387
Magnesium (mmol/d)	3.26	1.23	3.00	1.55	13	12	0.497	3.29	1.19	3.26	1.34	14	13	0.928
Sodium (mmol/d)	122.07	57.90	114.63	48.71	13	12	0.802	124.35	56.28	132.69	54.44	14	13	0.644
Potassium (mmol/d)	58.20	22.12	56.14	24.08	13	12	0.770	61.19	24.03	67.58	22.42	14	13	0.377

DC[1], dark chocolate.

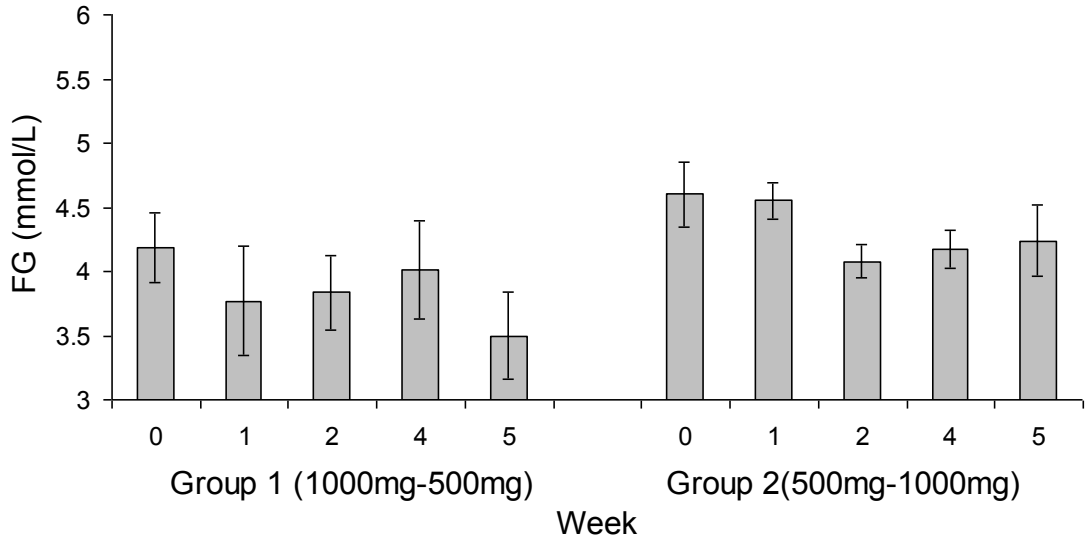
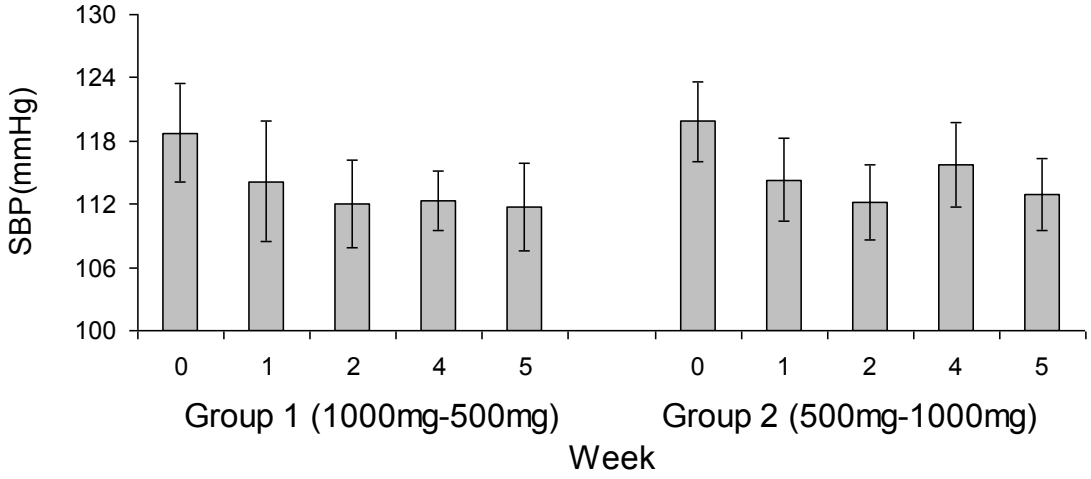


Figure 3.14

Capillary fasting glucose levels (FG) at baseline (Week 0), and at the end of 1 week and 2 weeks of each of the polyphenol doses. Group 1 received 1000mg polyphenols dark chocolate (Week 1-2) followed by 500mg polyphenols dark chocolate (Week 4-5). Group 2 received 500mg polyphenols dark chocolate (Week 1-2) followed by 1000mg polyphenols dark chocolate (Week 4-5). Changes in fasting glucose were independent of the sequences of chocolate administration ($p>0.05$). Values are mean, with standard errors represented by vertical bars.



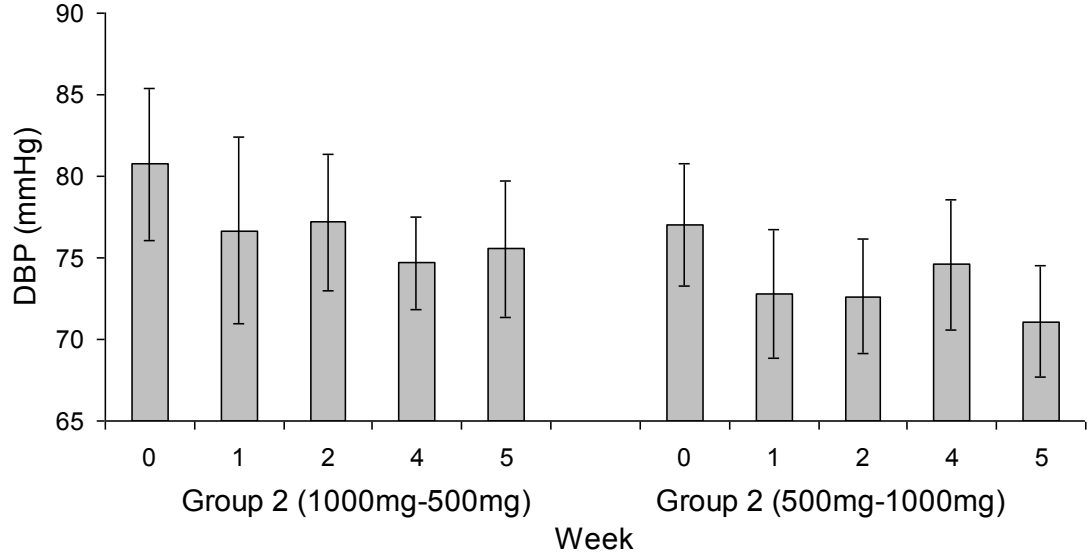


Figure 3.15

Systolic (SBP) and diastolic blood pressure (DBP) at baseline (Week 0), and at the end of 1 week and 2 weeks of each of the polyphenols doses. Group 1 received 1000mg polyphenols dark chocolate (Week 1-2) followed by 500mg polyphenols dark chocolate (Week 4-5). Group 2 received 500mg polyphenols dark chocolate (Week 1-2) followed by 1000mg polyphenols dark chocolate (Week 4-5). Changes in SBP and DBP were independent of the sequences of chocolate administration ($p>0.05$). Values are mean, with standard errors represented by vertical bars.

Figure 3.16
Total polyphenol concentration in urine at baseline and at the end of 2 weeks of 500mg and 1000mg polyphenols DC (n=14). Lines represent individual responses. Data are expressed in mmol GAE per gram creatinine. Note the Outliers (Subjects 5, 10 and 14)

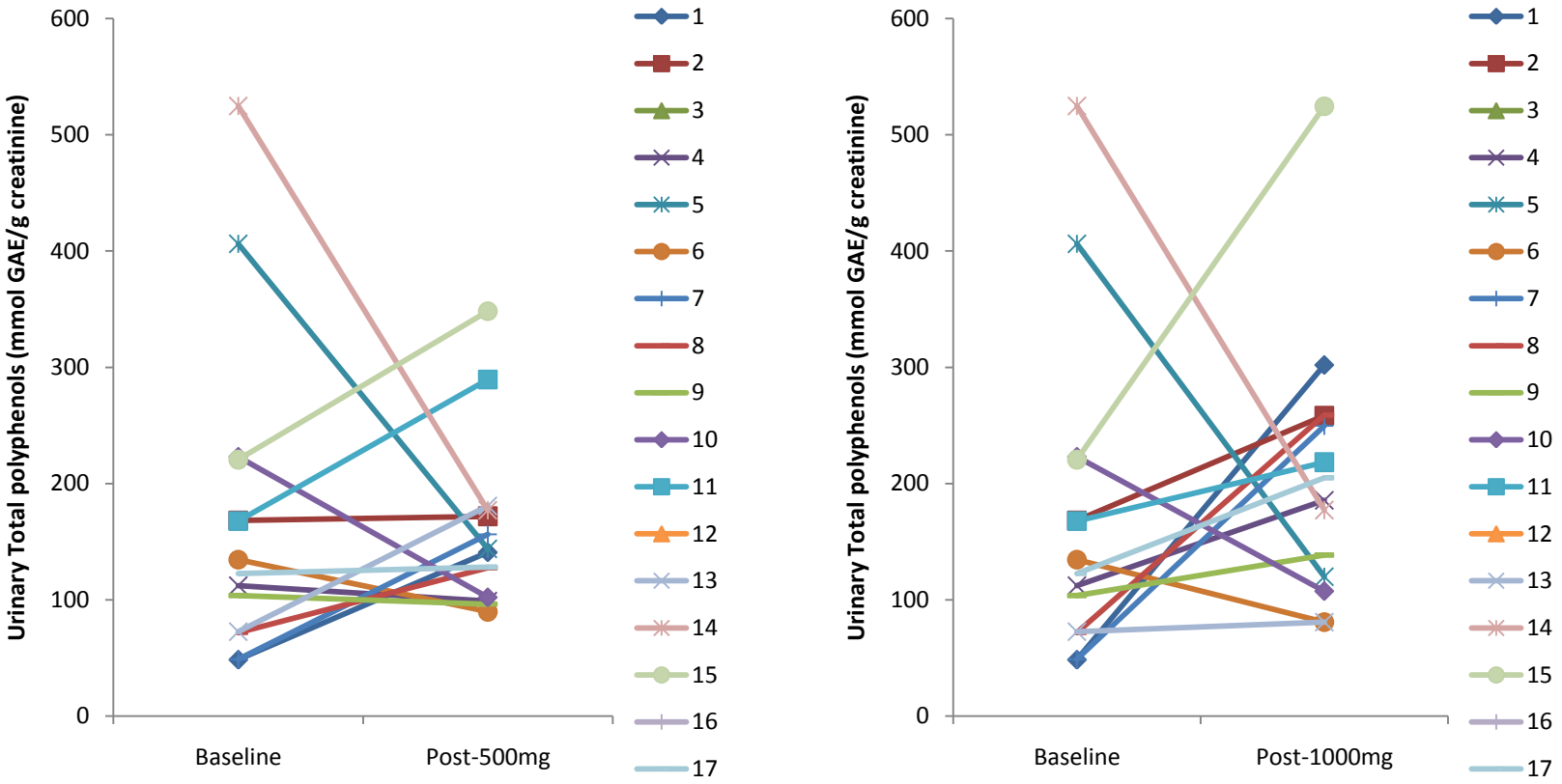
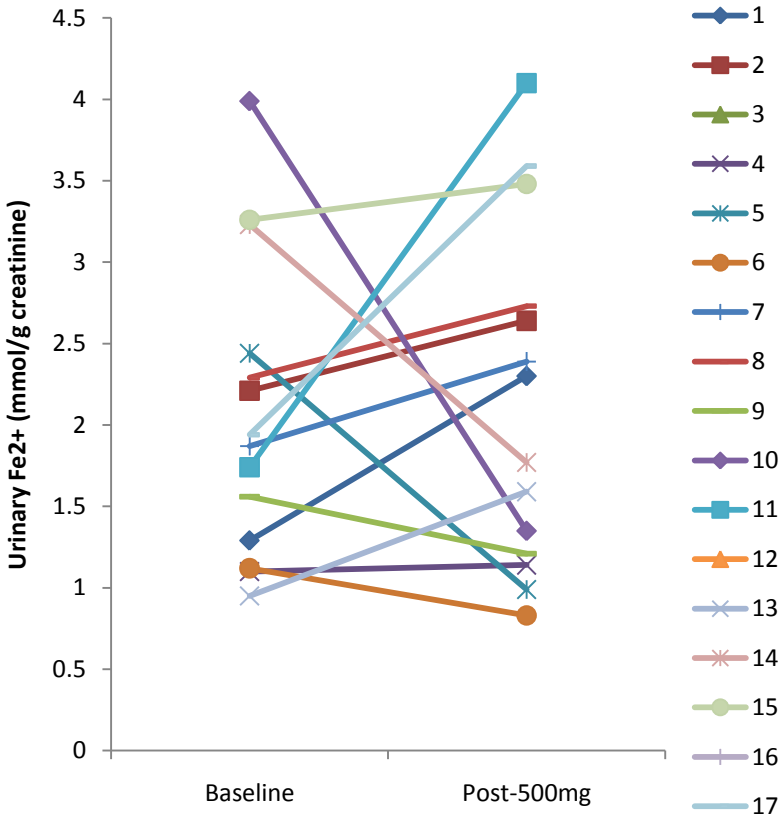
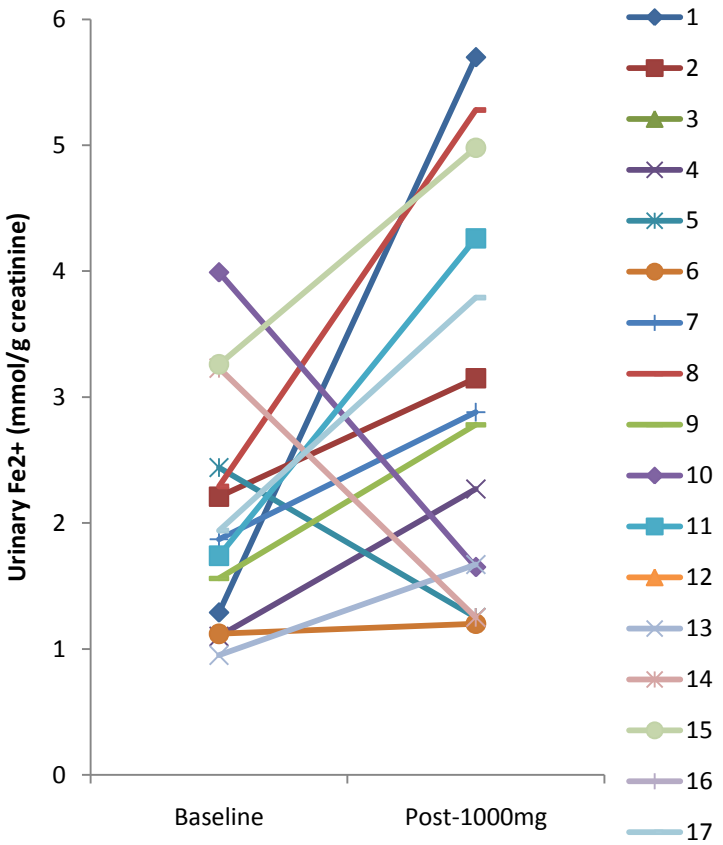


Figure 3.17
Urinary ferric-reducing capcity (Fe^{2+}) at baseline and at the end of 2 weeks of 500mg and 1000mg polyphenols DC for each of 14 volunteers. Lines represent individual reponses. Data are expressed in mmol Fe^{2+} per gram creatinine. (Subjects 5, 10 and 14)





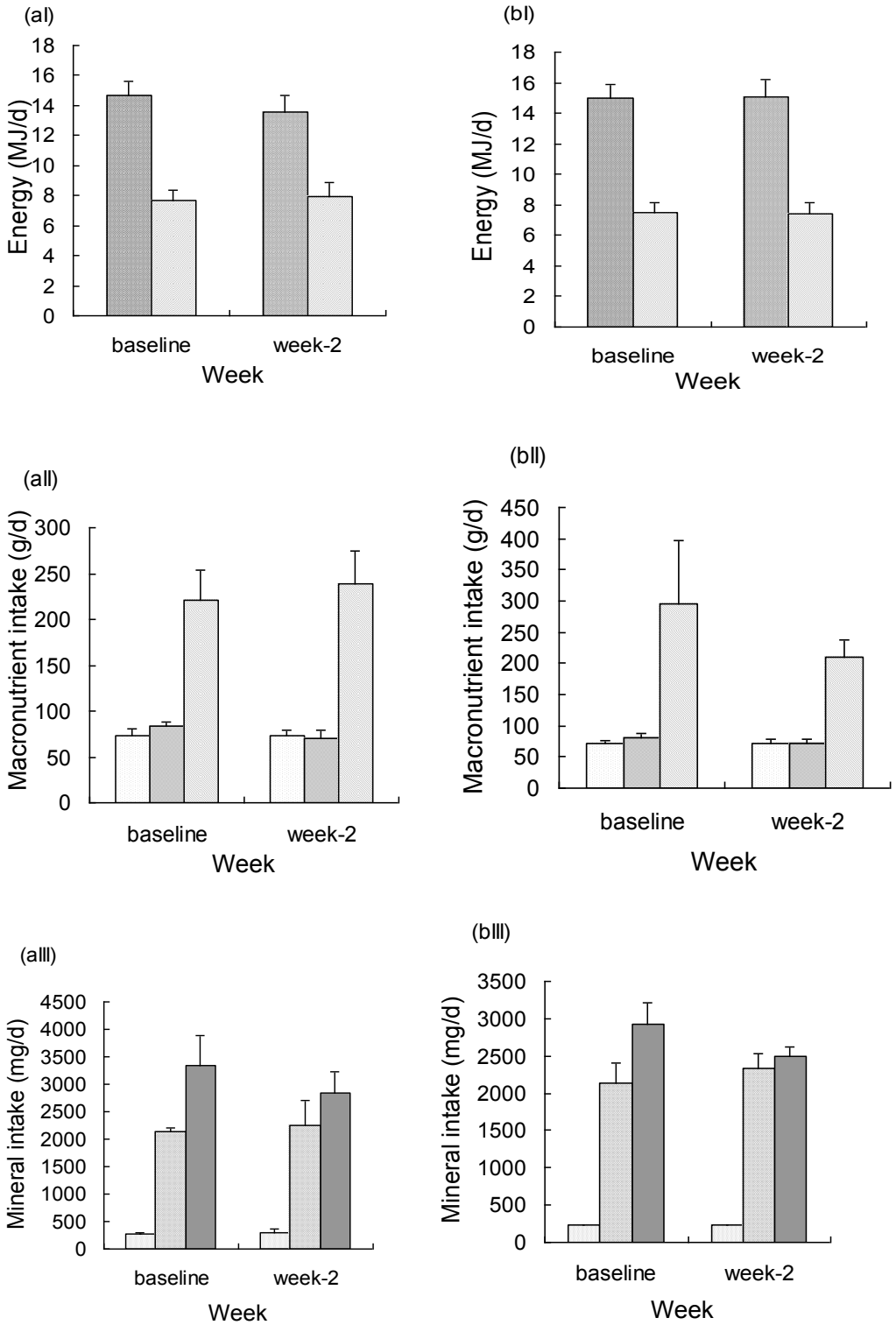


Figure 3.18 Energy (I), Macronutrient (II) and mineral intake (III) at baseline and at the end of each intervention: 20 g dark chocolate with 1000 mg polyphenols (a) and 20 g dark chocolate with 500 mg polyphenols (b). Values are mean, with standard errors represented by vertical bars. (▩), Energy expenditure; (▨) energy intake; (▫) fat intake; (▩) protein intake; (▧) carbohydrate intake; (▫) magnesium intake; (▩) sodium intake; (■) potassium intake.

Table 3.7

Pearson product-moment correlations between changes in urinary glucocorticoids levels and changes in selected parameters

Correlation pair		n	r	P
Δ Urinary free cortisol (nmol/kg/24h)	ΔUrinary free cortisone excretion (nmol/kg/24h)	27	0.599	0.001
	Δ 24h urinary sodium (mmol/g creatinine)	26	0.489	0.011
	Δ physical activity (kJ)	26	-0.384	0.053
Δ Urinary free cortisone (nmol/kg/24h)	Δ cortisol-to-cortisone ratio	27	-0.662	0.000
	Δ cortisone-to-cortisol ratio	27	0.628	0.000
	Δ 24 h urinary sodium (mmol/g creatinine)	26	0.478	0.014

3.6. Main study

3.6.1 Study population: Baseline characteristics

The study included 48 female volunteers (46 Caucasian, 1 Hispanic and 1 Asian). All volunteers completed the trial with the exception of four volunteers who dropped-out following their first visit (Figure 3.19). Of these four volunteers, one was excluded on the basis of not consuming the specified polyphenol-rich DC on 7 consecutive occasions. An additional two volunteers (BMI <25kg/m^2) also dropped-out after completing one part of the study for reasons unrelated to the study. Data from the latter two volunteers was not included in statistical analyses with the exception in baseline comparisons (Figure 3.20 and Figure 3.21). One subject also failed to return their 24h urine collections on 2 occasions. Data from this subject for the remaining non-urinary tests was still included in the analyses. On the basis of remaining volunteers' self-reports and returned empty bags, all chocolate portions were consumed. However, 1 of 1176 polyphenol-rich DC portions and 6 of the 1176 placebo portions were reported to be consumed upto 24h after the specified time-point.

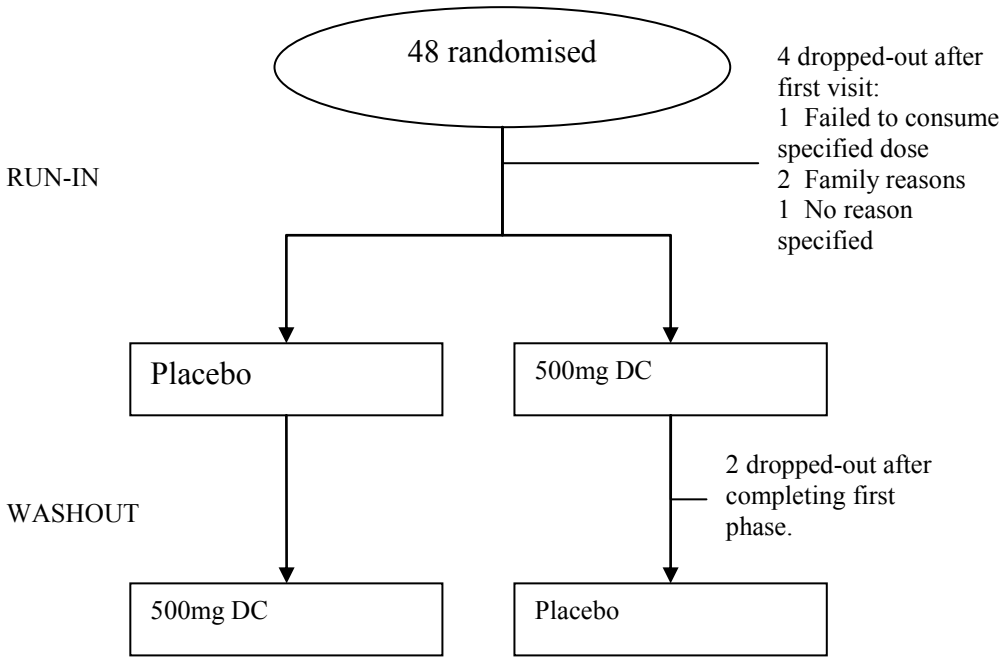


Figure 3.19 Flow of participants through the Main study.

Baseline characteristics of the studied population are shown in Table 3.8. Of the total 42 female volunteers who completed the study, 21 had a BMI< 25kg/m^2, 14 were overweight (BMI= 25-29.99 kg/m^2) and 7 were obese (BMI≥30 kg/m^2). Using WHO criteria for classifying fat distribution, it was observed that within the BMI ≥25kg/m^2 group, 16 participants had peripheral fat distribution (WHR≤ 0.85) and 5 had abdominal fat distribution (WHR> 0.85). One subject from the normal weight group had abdominal fat distribution. In order to assess the risk of metabolic complication, subjects from the overweight and obese group were also classified according to WC. Accordingly, 6 subjects were identified to be at an increased risk of metabolic complications (WC≥ 80cm) while another 11 were deemed to be at a substantially increased risk (WC≥ 88cm). Applying the same classification to the normal weight group showed that 4 subjects had a WC≥ 80cm indicating increased risk of metabolic complications.

Table 3.8 Baseline characteristics of the study population

Variable	BMI < 25 kg/m^2 (n=21)		BMI ≥ 25 kg/m^2 (n=21)		Total (n= 42)	
	Mean	SD	Mean	SD	Mean	SD
Age	30.71	9.80	35.38	11.43	33.05	10.78
BMI	21.69	2.32	28.54	2.98	25.12	4.36
WC	74.48	6.05	89.79	10.07	82.13	11.28
WHR	0.77	0.05	0.81	0.07	0.79	0.06

In contrast to individuals with BMI< 25kg/m^2, overweight and obese subjects had marginally higher levels of fasting insulin (P= 0.053), and significantly greater TC: HDL ratio (P= 0.043), SBP (P <0.0001) and DBP (P <0.0001) (Figure 3.20 and Figure 3.21). Overweight and obese subjects also had significantly higher physical activity level (P <0.0001), WC (P <0.0001), HC (P <0.0001), WHR (P= 0.023) and percentage body fat (P <0.0001). Subjects with BMI< 25kg/m^2 had higher percentage body water than overweight and obese individuals (P <0.0001). No differences in other variables were observed.

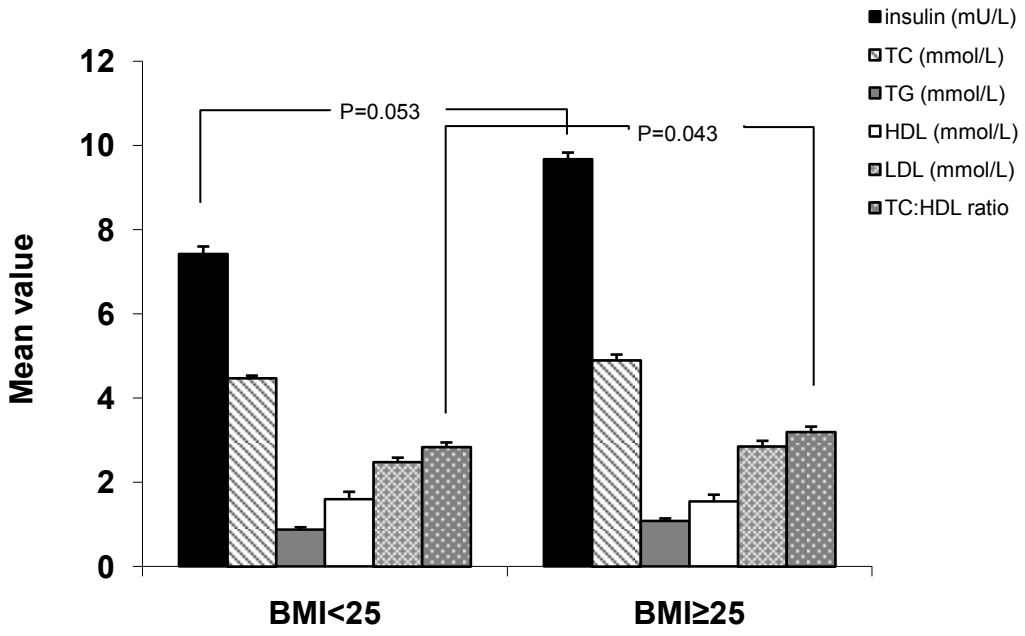


Figure 3.20 Differences in baseline haematological values between the healthy BMI group and the overweight and obese group as tested by an independent sample t-test. Data expressed as mean± SEM. TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC:HDL, total cholesterol-to-high-density lipoprotein ratio (n=44)

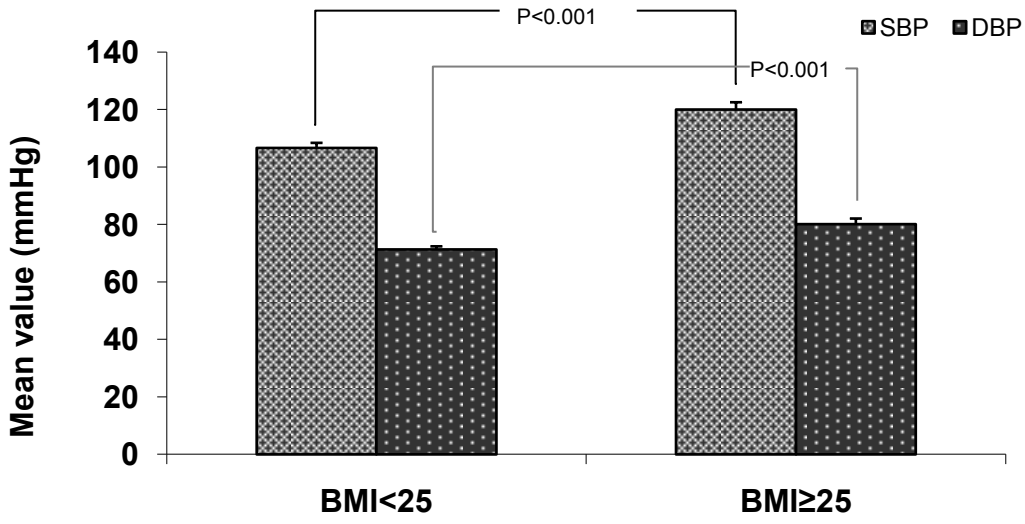


Figure 3.21 Differences in baseline systolic (SBP) and diastolic blood pressure (DBP) between the healthy BMI group and the overweight and obese group as tested by an independent sample t-test. Data expressed as mean± SEM (n=44).

3.6.2 Haematological, biochemical assessment and blood pressure

Three-factor repeated measures ANOVA indicated that none of the variables violated the assumption of homogeneity, with the exception of TG (Leven's tests of equality of error variance P= 0.018). As a result, a more stringent P-value was set for testing differences in this variable ($P \leq 0.01$).

Overall, three-factor repeated measures ANOVA demonstrated no significant effect of time or treatment on lipid profile (Table 3.9). Serum NEFA concentrations were higher at the end of 4 weeks of placebo ingestion as compared to polyphenol-rich DC (Table 3.9). However, this effect did not reach statistical significance (Treatment effect, Wilk's lambda =0.086).

A significant effect of treatment on fasting glucose (P=0.028), fasting insulin (P= 0.030), HOMA-IR (P=0.005), QUICKI (P=0.04), revised-QUICKI (P=0.026), SBP (P= 0.020), DBP (P= 0.008) and salivary cortisol (P=0.006) was revealed. A significant treatment-by-time interaction was also observed for these parameters suggesting that the change from baseline across the two treatment groups (polyphenol-rich DC vs. placebo) was not similar as seen in Figure 3.22 and Figure 3.23. These results were further confirmed when differences from baseline between the DC and placebo group were assessed using a paired-sample t-test. Overall, values for polyphenol-rich DC fasting glucose (P=0.002), HOMA-IR (P=0.001), SBP (P=0.007) and DBP (P= 0.003) were found to be significantly lower after polyphenol-rich DC as opposed to placebo, whereas values for QUICKI (P=0.002) and revised-QUICKI (P=0.015) were significantly higher with polyphenol-rich DC compared to placebo (Figure 3.23). Interestingly, the placebo was found to raise fasting insulin, HOMA-IR and salivary cortisol from baseline by 1.36±4.29 mU/L (P=0.046), 0.37±1.06 (P=0.031) and 15.83±35.74 nmol/l (P=0.007), respectively, as opposed to polyphenol-rich DC which reduced fasting glucose (P=0.012), HOMA-IR (P=0.004) and elevated QUICKI (P=0.042) and revised-QUICKI (P=0.043) but had a negligible effect on fasting insulin (-0.56±2.62 mU/L from baseline) and salivary cortisol (+0.13±7.28 nmol/l from baseline). The placebo also diminished insulin sensitivity from baseline as measured by QUICKI and revised-QUICKI, although the values did not reach statistical significance (P=0.055; p=0.157).

No significant differences in urinary glucocorticoids or mineral excretion were observed through the study period (Table 3.10).

3.6.3 Differences between BMI categories

The three-factor ANOVA demonstrated that participants from the two different BMI categories (BMI< 25 vs. BMI ≥25) differed in their fasting insulin (P=0.026), SBP (P=0.020) and DBP (P=0.001). However, a significant treatment-by-time-by-BMI category interaction was only observed with fasting insulin levels suggesting that only the change in fasting insulin from baseline across the two treatment groups was determined by the BMI category to which the participant belonged to (P=0.029). Consequently, an independent sample t-test was performed to compare the fasting insulin response to treatment across the two BMI categories. The independent sample t-test indicated that overweight and obese subjects responded less favourably to placebo and consequently had higher fasting insulin levels after consumption of placebo (+2.73±5.07 mU/L from baseline) compared to subjects with normal BMI (-0.01±2.82 mU/L from baseline) (P=0.021).

Table 3.9 Change from baseline in fasting insulin and lipid profile given for each of the two body mass index categories and for the total group (*n*=42) as shown by a three-factor ANOVA and confirmed by a paired-sample t-test. Data are expressed as mean±SD.

variable	BMI category	Baseline				Post-treatment				Three-factor ANOVA		Δ (Post-treatment - baseline)				Paired-sample t-test
		Pre-DC		Pre-placebo		Post-DC		Post-placebo		*Wilk's Lambda*		Δ (Post-DC – Pre-DC)		Δ (Post-placebo – pre-placebo)		
		mean	SD	mean	SD	mean	SD	mean	SD	treatment	Treatment x time					*P*
TC (mmol/L)	BMI <25	4.5714	1.07198	4.4810	.77564	4.5286	.81923	4.5619	.98716			-0.04	0.54	0.08	0.81	0.624
	BMI ≥25	4.9000	1.19833	4.8571	1.03854	4.6810	.96572	4.8905	1.18908			-0.22	0.52	0.03	0.73	0.231
	Total	4.7357	1.13521	4.6690	.92512	4.6048	.88785	4.7262	1.09211	0.712	0.249	-0.14	0.53	0.06	0.76	0.244
TG (mmol/L)	BMI <25	.8857	.29204	.8143	.20319	.9333	.33066	.7857	.24349			0.05	0.32	-0.03	0.22	0.411
	BMI ≥25	1.0333	.57562	1.0286	.51783	.9905	.58387	.9524	.43199			-0.04	0.34	-0.08	0.31	0.755
	Total	.9595	.45696	.9214	.40336	.9619	.46954	.8690	.35647	0.051	0.436	0.00	0.33	-0.05	0.27	0.43
HDL (mmol/L)	BMI <25	1.6143	.30379	1.5810	.25420	1.6286	.26673	1.5952	.27473			0.01	0.22	0.01	0.23	1
	BMI ≥25	1.5333	.31517	1.5476	.31562	1.5524	.30760	1.5762	.33302			0.02	0.25	0.03	0.23	0.895
	Total	1.5738	.30847	1.5643	.28355	1.5905	.28696	1.5857	.30168	0.737	0.929	0.02	0.24	0.02	0.23	0.928
LDL (mmol/L)	BMI <25	2.5571	.86636	2.5286	.69148	2.4667	.71438	2.6000	.79875			-0.09	0.30	0.07	0.55	0.290
	BMI ≥25	2.8857	.92428	2.8476	.66153	2.6667	.69162	2.8762	.89213			-0.22	0.46	0.03	0.55	0.147
	Total	2.7214	.90028	2.6881	.68759	2.5667	.70180	2.7381	.84794	0.266	0.072	-0.15	0.39	0.05	0.54	0.069
TC:HDL	BMI <25[3]	2.8619	.50048	2.8762	.53843	2.8238	.49589	2.8952	.50147			-0.04	0.25	0.02	0.22	0.370
	BMI ≥25	3.2333	.72065	3.1571	.39188	3.0571	.59462	3.1429	.55459			-0.18	0.49	-0.01	0.39	0.212
	Total	3.0476	.64097	3.0167	.48636	2.9405	.55351	3.0190	.53704	0.596	0.126	-0.11	0.39	0.00	0.31	0.124
NEFA (mmol/L)	BMI <25	.4705	.16578	.4729	.23111	.4363	.22833	.5471	.26935			-0.03	0.20	0.07	0.27	0.169
	BMI ≥25	.4481	.24064	.4973	.18320	.4430	.23106	.5395	.22262			-0.01	0.26	0.04	0.18	0.506
	Total	.4593	.20441	.4851	.20635	.4397	.22691	.5433	.24409	0.086	0.139	-0.02	0.23	0.06	0.22	0.136

1. N= 21; N=21; total N= 42

2. Significantly difference between the two BMI categories (Three-factor ANOVA and Bonferroni test): P=0.026

3. Three-factor ANOVA and Bonferroni test: P=0.07

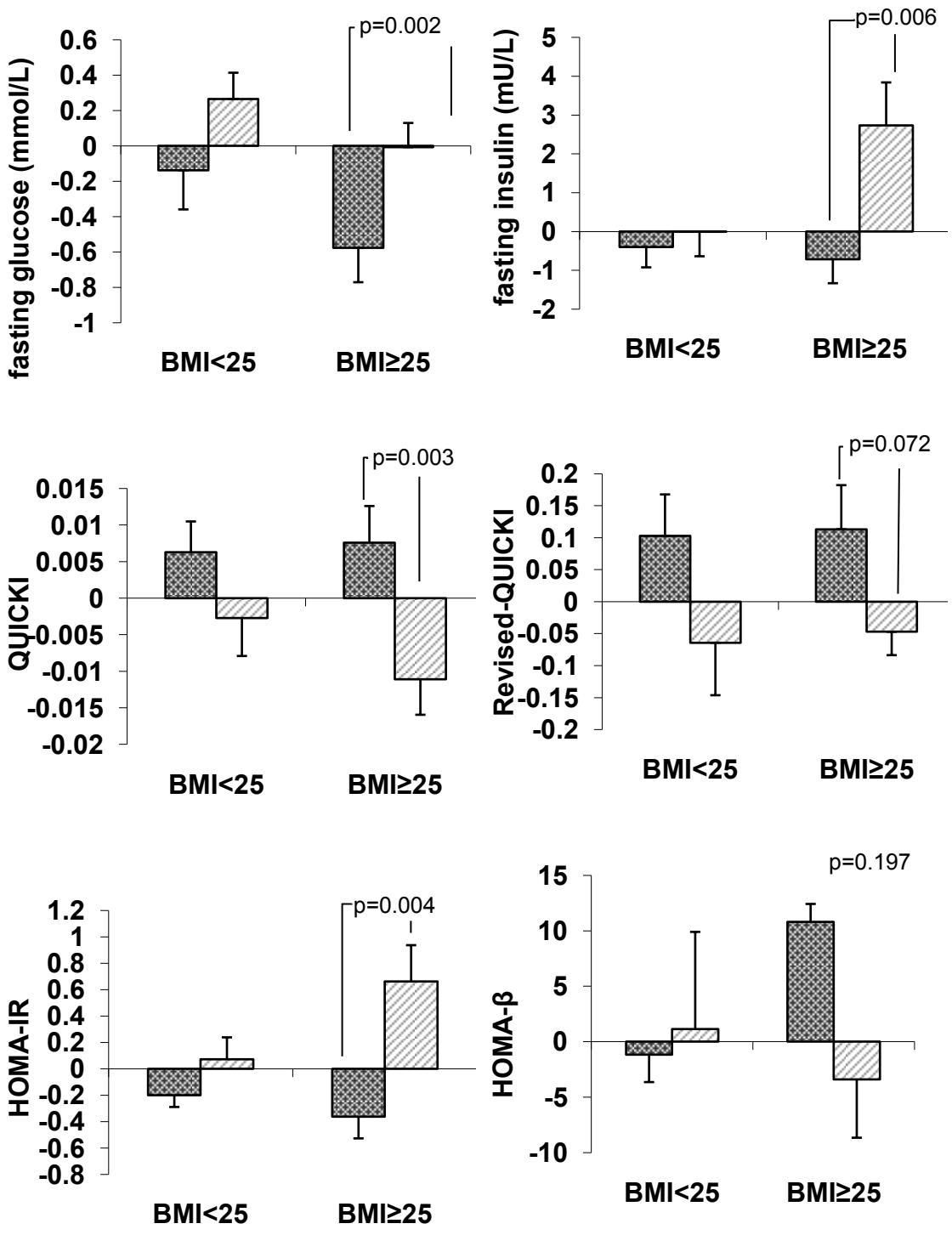


Figure 3.22 Changes in biomarkers of glucose metabolism following polyphenol-rich dark chocolate (▩) and placebo (▨) for each of the lean body mass index group and overweight and obese group. Data are expressed as mean± SEM.

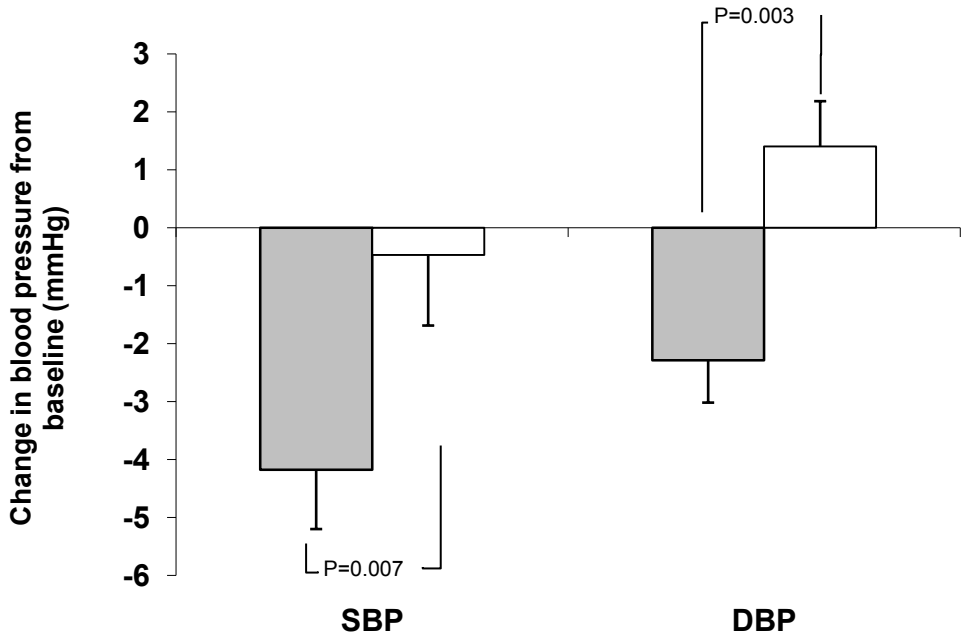


Figure 3.23 Change in systolic (SBP) and diastolic blood pressure (DBP) from baseline following polyphenol-rich DC (■) and placebo (□). Data are expressed as mean± SEM.

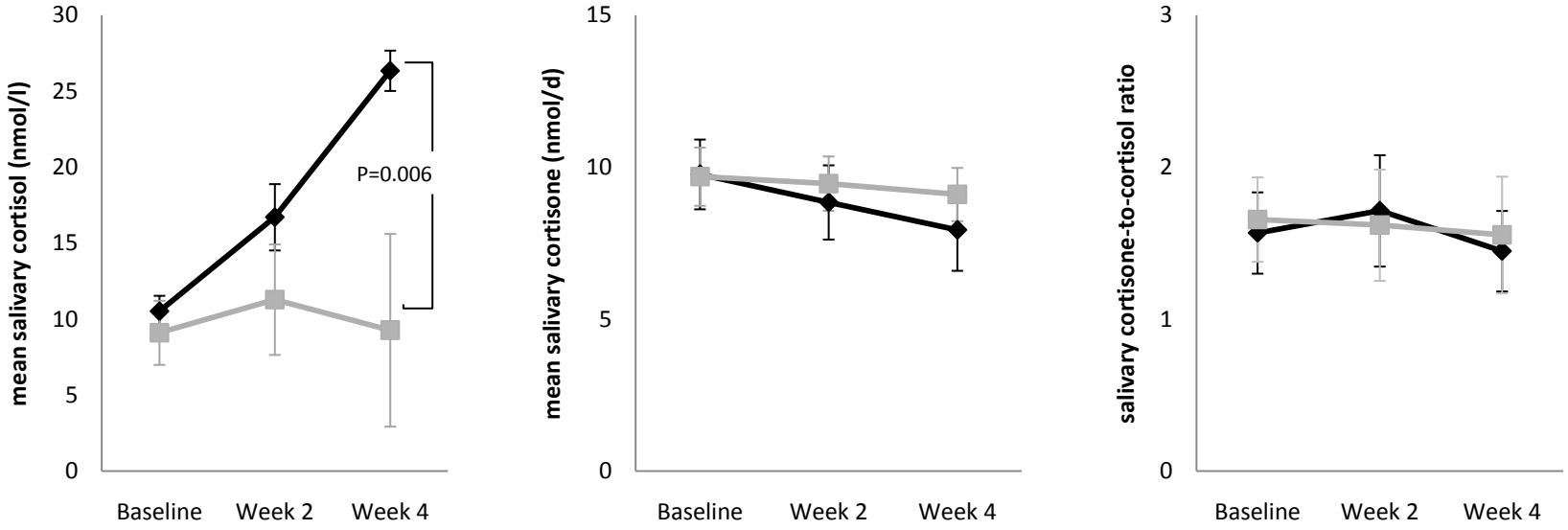


Figure 3.24 Salivary glucocorticoid concentrations at baseline at the end of 4 weeks of polyphenol-rich dark chocolate (■) and placebo (♦) (n=42). Data are expressed as mean of duplicates ± SEM. Note that there was a significant effect of treatment (Wilks' lambda=0.005) and a significant treatment-by-time interaction (Wilks' lambda=0.03) on salivary cortisol with the paired sample t-test showing a greater increase in mean salivary cortisol from baseline within the placebo treatment group as compared to polyphenol-rich DC. Data represents mean of three salivary collections obtained at 9.00-10.00am, 12.00-1.00pm and 4.00-5.00pm.

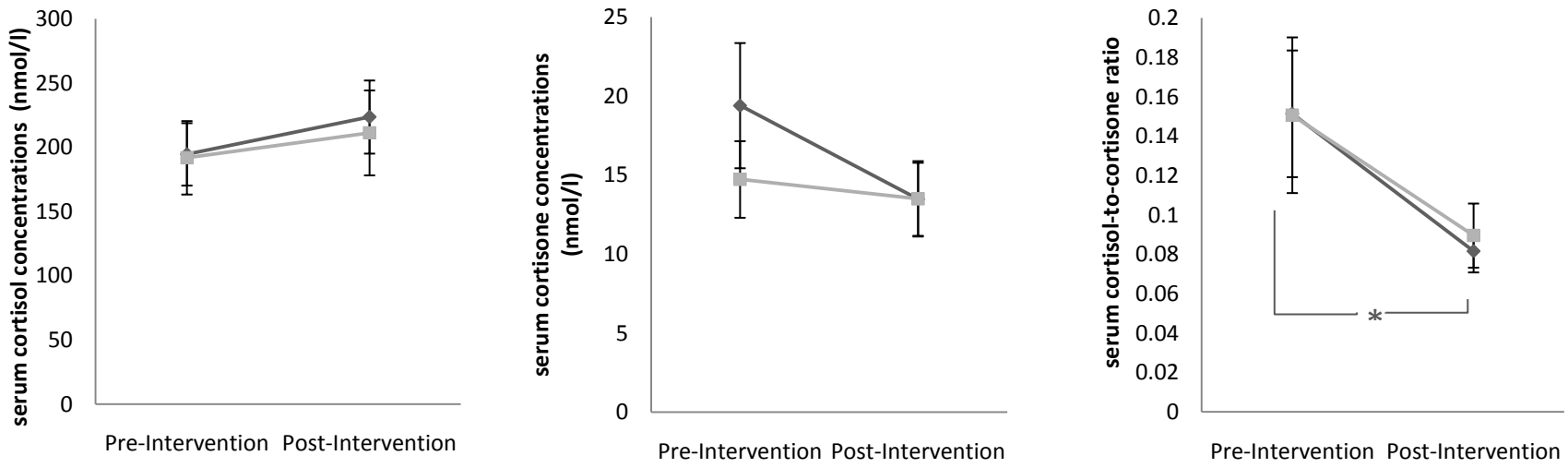


Figure 3.25 serum glucocorticoid concentrations at baseline at the end of 4 weeks of polyphenol-rich dark chocolate (■) and placebo (♦) (n=42). Data are expressed as mean± SEM. Note that there was a significant effect of time on cortisone-to-cortisol ratio (Wilks' lambda=0.021) with the paired-sample t-test showing that the reduction in serum cortisone-to-cortisol ratio within the placebo treatment group was significant (*P*=0.039).

Table 3.10

Results for 24h urine collections (n=41)

(Data are expressed as mean and standard deviations)

	Baseline				Post-treatment				Three-factor ANOVA	
	Pre-DC		Pre-Placebo		Post-DC		Post-placebo		*Wilk's Lambda*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	treatment	Treatment x time
Free cortisol (nmol/d)	35.32	24.86	40.95	36.95	40.20	32.24	42.16	33.75	0.454	0.504
Free cortisone (nmol/d)	62.51	57.22	61.74	61.57	53.83	42.14	61.20	61.77	0.451	0.468
Cortisol-to-cortisone ratio	2.55	2.55	2.51	2.78	2.34	2.69	2.33	3.27	0.922	0.925
Free cortisol (nmol/kg/d)	0.53	0.40	0.60	0.55	0.60	0.51	0.64	0.55	0.468	0.630
Free cortisone (nmol/kg/d)	1.02	1.10	0.98	1.10	0.84	0.71	0.97	1.22	0.502	0.458
Creatinine (mmol/d)	13.27	10.94	14.58	11.20	12.02	9.22	11.63	8.23	0.556	0.240
Magnesium (mmol/d)	3.23	3.40	3.24	3.52	3.25	2.58	4.17	3.97	0.242	0.246
Sodium (mmol/d)	141.67	152.55	147.31	170.65	162.89	135.92	142.90	122.43	0.598	0.447
Potassium (mmol/d)	76.33	105.83	82.61	114.60	92.27	94.18	93.83	82.26	0.683	0.832

3.6.4 Liver enzymes and troponin

Three-factor ANOVA revealed a significant effect of treatment on serum creatine kinase concentration (P=0.023). Accordingly, serum creatine kinase concentrations were found to be significantly higher at the end of 4-week treatment of polyphenol-rich DC as compared to placebo (P=0.023). No changes in aspartate amino transferase or troponin were observed (P>0.05).

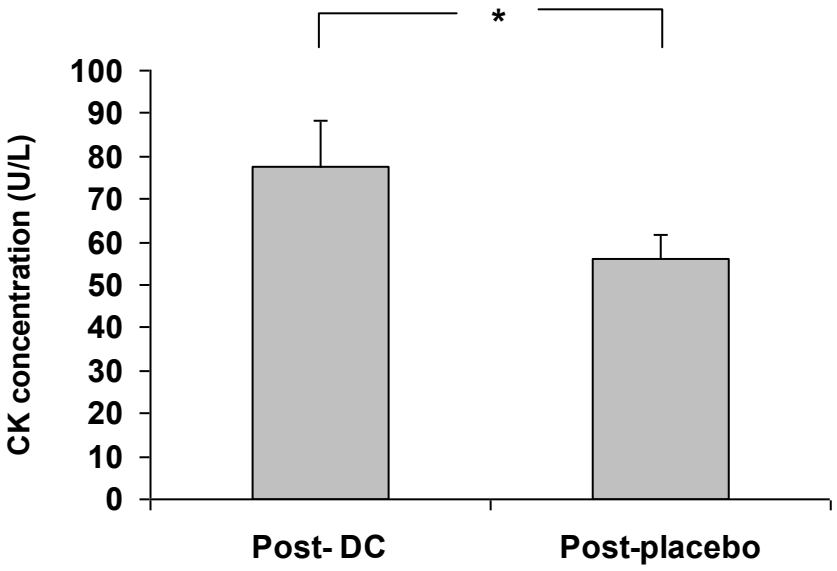


Figure 3.26 Serum creatine kinase concentrations at the end of 4 weeks of 500mg polyphenols dark chocolate and placebo. Data are expressed as mean± SEM. Asterisk denotes significance.

3.6.5 Serum total phenolics content, ferric-reducing capacity of plasma and oxygen radical absorbance capacity

No significant changes in serum phenolics, Fe^{2+} or ORAC were found from baseline or between the placebo and DC group (Table 3.11).

3.6.6 Urinary total phenolics content, ferric-reducing capacity of plasma and oxygen radical absorbance capacity

As shown by the paired-sample t-test, urinary phenolic and Fe^{2+} content increased from baseline by 67.47±182.90 mg/d and 1.51±3.74 mmol/d, respectively, which was significantly different from placebo (FC: P=0.046; Fe^{2+}: P=0.048) wherein a reduction of -28.53±128.11mg/d in FC and of -0.27±0.32 mmol/d in Fe^{2+} was observed. This was further indicated by the significant treatment effect and treatment-by-time interaction obtained by three-factor ANOVA (urinary total phenolics: treatment effect Wilk's lambda= 0.046, treatment-by-time interaction Wilk's lambda=0.005; Fe^{2+}: treatment effect Wilk's lambda= 0.048, treatment-by-time interaction Wilk's lambda= 0.006). No significant treatment-by-time-by-BMI interaction was observed for both phenolic (P=0.815) and Fe^{2+} content (P=0.822) suggesting that changes in these variables were similar across the two BMI categories.

A significant treatment effect (Wilk's lambda= 0.012) and treatment-by-time interaction (Wilk's lambda= 0.003) was also seen with ORAC of urine. This was further confirmed by the paired-sample t-test which showed that the antioxidant capacity as measured by ORAC was increased by 4323.49± 12454.49µmol trolox equivalents/d following DC which was significantly different from the -3602.75±11076.35µmol trolox equivalents/d reduction in ORAC values seen following placebo (P=0.002).

3.6.7 Weight and anthropometry

No significant changes in WC, HC, WHR or percentage body water or fat were observed across the two BMI categories following either DC or placebo (Three-factor repeated measures ANOVA Wilk's lambda > 0.05). However, a significant difference in body weight was detected between the placebo and the active polyphenol-rich DC group at the end of 4 weeks treatment as demonstrated by the three-factor ANOVA (Post-Placebo body weight: 69.93±13.28 kg; post-DC body weight: 69.45±13.01 kg; treatment effect Wilk's lambda = 0.003; treatment-by-time interaction Wilk's lambda= 0.031). Additional analyses using a paired-sample t-test further demonstrated that differences in body weight post-placebo and post-DC were greater within the BMI≥ 25kg/m^2 group (P=0.026) as compared to lean individuals (P=0.894) (Figure 3.29).

Table 3.11

Plasma antioxidant capacity (n=42)

(Data are expressed as mean and standard deviations)

	Baseline				Post-treatment				Three-factor ANOVA	
	Pre-DC[1]		Pre-Placebo		Post-DC		Post-placebo		*Wilk's Lambda*	
	Mean	SD	treatment	treatment	Mean	SD	Mean	SD	treatment	Treatment x time
FRAP[2]	0.59	0.15	0.56	0.13	0.54	0.15	0.60	0.13	0.500	0.009
FC[3]	15.23	3.77	16.09	4.30	14.57	4.77	15.78	3.72	0.069	0.787
ORAC[4]	1699.89	773.99	1600.61	845.61	1474.57	930.80	1509.46	580.58	0.732	0.539

DC[1], dark chocolate; FRAP[2], ferric-reducing ability of plasma[4]; FC[3], Folin-Ciocalteu; ORAC[4], oxygen radical absorbance capacity.

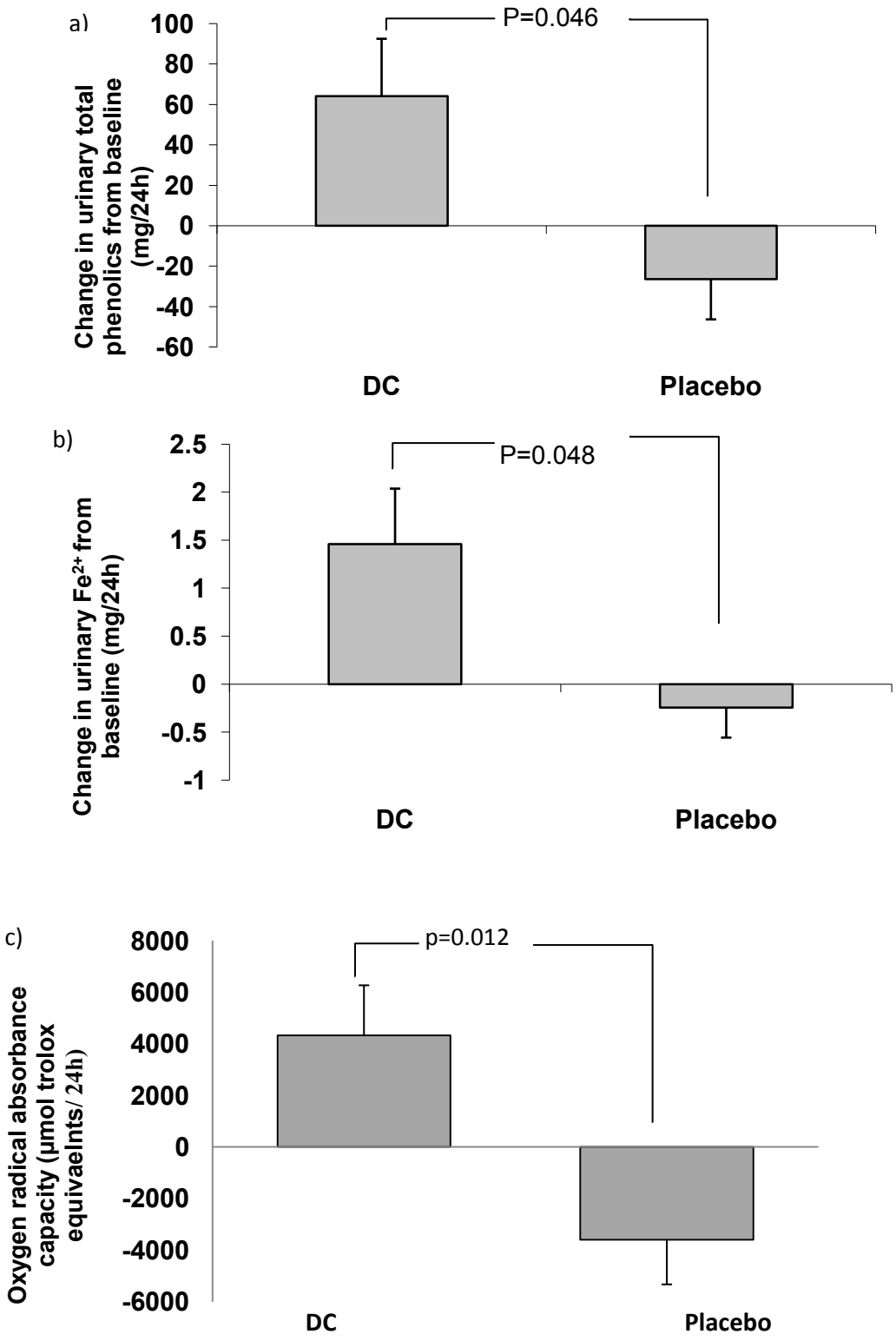


Figure 3.27 Changes in urinary total polyphenols content (a), ferric-reducing capacity (b) and oxygen radical absorbance capacity (c) from baseline across the two body mass index categories following polyphenol-rich dark chocolate and placebo (*n*=41). Asterisk denotes significance. Data are expressed as means± SEM.

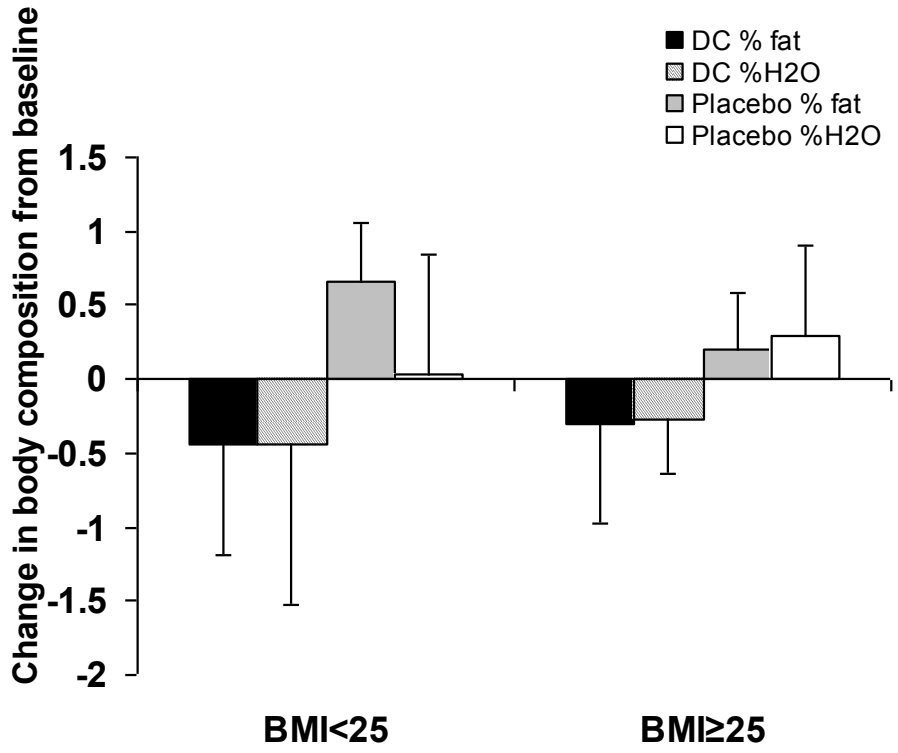


Figure 3.28 Change in body composition from baseline across the two body mass index categories following DC and placebo. Data are expressed as mean± SEM.

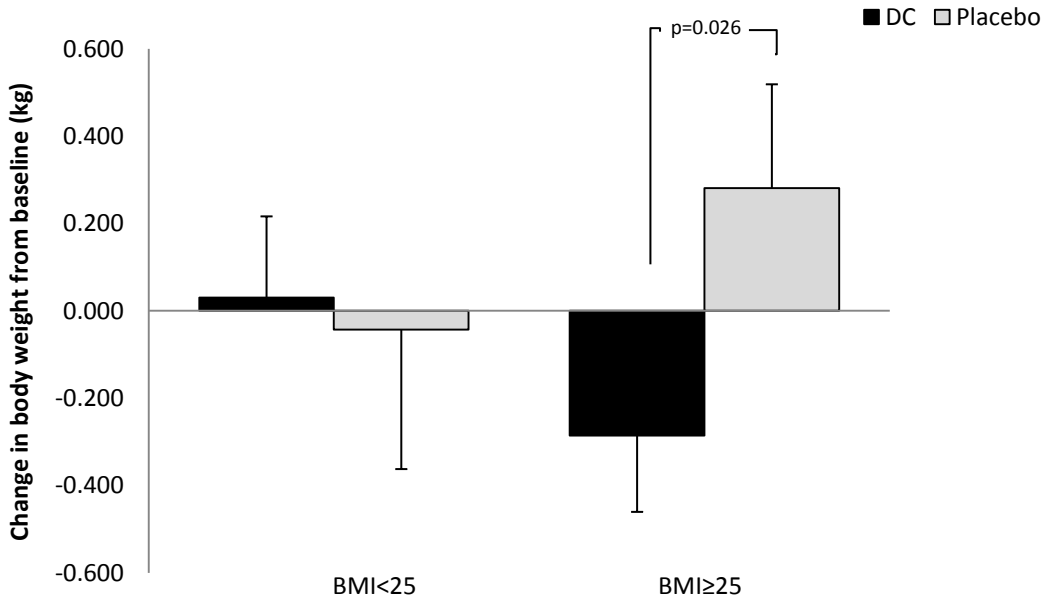


Figure 3.29 Change in body weight from baseline across the two body mass index categories following polyphenol-rich dark chocolate and placebo. Data are expressed as mean± SEM.

3.6.8 Energy Intake and expenditure

Physical activity did not alter significantly through the study period (SPANOVA Wilk's lambda =0.232) (Table 3.12). No significant changes in energy or macronutrient, micronutrient intake were observed across the two BMI categories following either DC or placebo (SPANOVA Wilk's lambda > 0.05) (Figure 3.30).

Table 3.12 Mean energy expenditure at baseline and post-intervention.

	BMI.category	Mean EE[1] (kcal/d)	SD
Baseline	BMI<25	2386.21	467.22
	BMI≥25	3381.37	564.10
	Total	2871.03	716.81
DC[2]	BMI<25	2388.30	388.28
	BMI≥25	3244.60	410.27
	Total	2805.47	585.77
Placebo	BMI<25	2459.41	345.27
	BMI≥25	3378.74	560.38
	Total	2907.29	651.97

EE[1]Energy expenditure

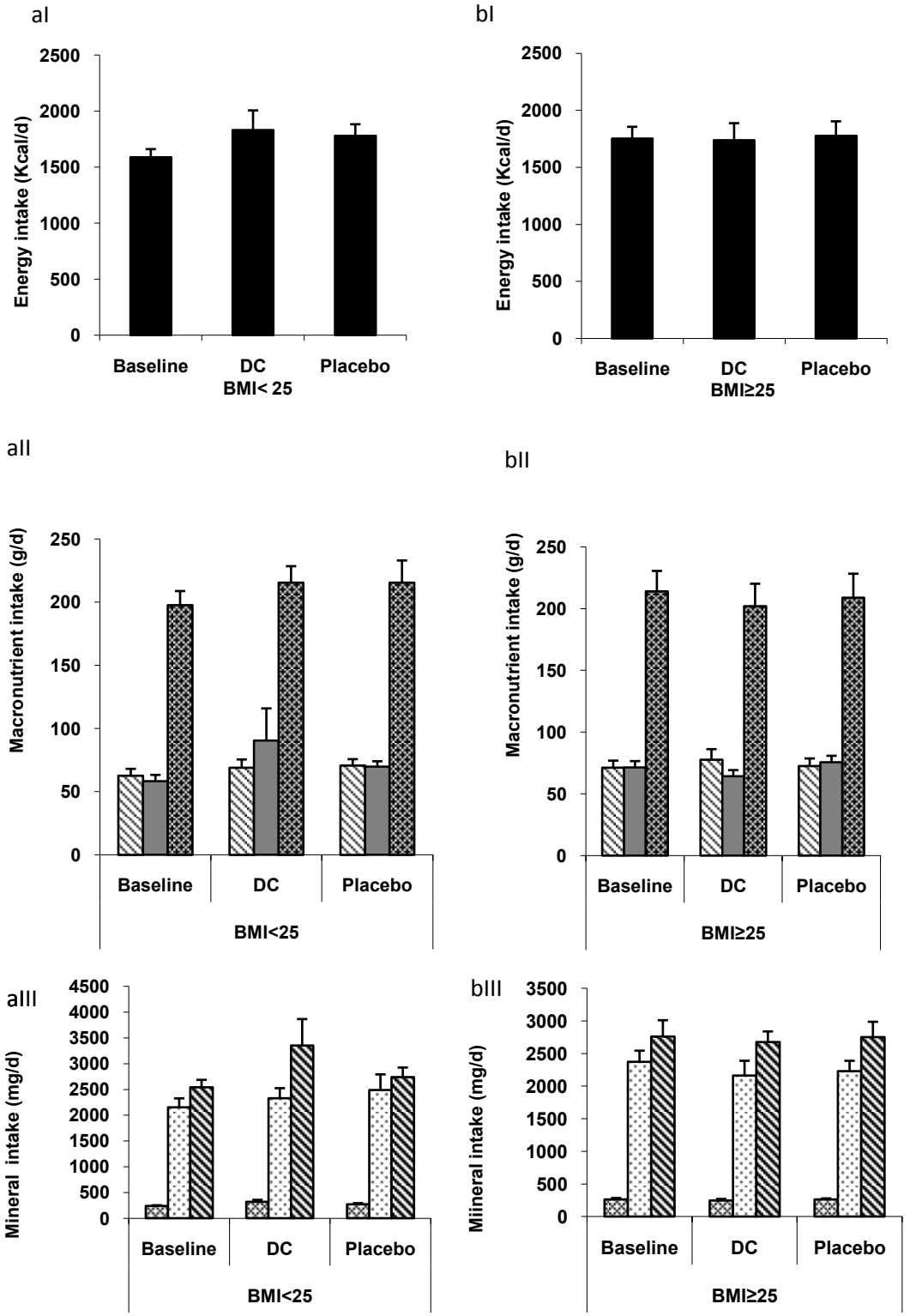


Figure 3.30 Energy (I), Macronutrient (II) and mineral intake (III) across the two body mass index categories: BMI< 25kg/m^2 (a) and BMI≥25kg/m^2 (b). Values are mean, with standard errors represented by vertical bars. (■) energy intake; (▨) fat intake; (▩) protein intake; (▩) carbohydrate intake; (▩) magnesium intake; (▩) sodium intake; (▩) potassium intake).

3.6.9 Correlation between assessed parameters

Urinary cortisone concentrations were significantly correlated with improvements in several metabolic parameters linked to overweight and obesity (Table 3.13). To illustrate, urinary cortisone was positively associated with HDL but negatively with LDL, TC:HDL and WHR. Additionally, urinary cortisone correlated positively with urinary and serum antioxidant capacity as measured by FC and FRAP. Likewise, urinary cortisone-to-cortisol ratio correlated positively with HDL but inversely with LDL, TC: HDL, WHR, SBP and DBP.
By contrast, urinary cortisol was related to higher DBP but surprisingly with lower WHR, HOMA-IR, urinary Fe, salivary cortisol and higher QUICKI and revised-QUICKI.

In saliva (Table 3.14), cortisone was found to be again inversely correlated with LDL, TC:HDL and TC. However, a positive correlation between salivary cortisone and HOMA-IR was observed. High salivary cortisol was marginally associated with lower revised-QUICKI and higher NEFA. Salivary cortisone-to-cortisol ratio correlated negatively with TC, LDL and TC:HDL ratio.

In serum (Table 3.15), cortisone correlated negatively with TC:HDL, SBP and DBP. Surprisingly, serum cortisol correlated negatively with WHR and positively with serum cortisone and urinary cortisone-to-cortisol ratio. Similarly, serum cortisone-to-cortisol was found to be inversely correlated with urinary and serum FC and positively with both salivary cortisone and salivary cortisol.

Table 3.13 Pearson product-moment correlations between changes in urinary glucocorticoid levels and changes in selected metabolic risk factors

Correlation Pair		R	P
Urinary cortisone (nmol/24h)	HDL	0.165	0.046
	LDL	-0.165	0.035
	TC:HDL	-2.53	0.001
	WHR	-0.331	0.000
	SBP	-0.166	0.034
	Urinary FC	0.156	0.046
	Urinary Fe	0.148	0.059
	Serum FC	0.159	0.041
	Serum Fe	0.146	0.062
	Salivary cortisone (nmol/d)	0.252	0.001
Urinary cortisol (nmol/24h)	DBP	0.170	0.030
	HOMA-IR	-0.147	0.061
	QUICKI	0.185	0.018
	Revised-QUICKI	0.142	0.069
	WHR	-0.187	0.016
	Urinary Fe	0.196	0.012
	Salivary cortisol	-0.113	0.154
	Salivary cortisone	-0.212	0.007
	Serum cortisone	-0.205	0.009
urinary cortisone-to-cortisol ratio	HDL	0.149	0.057
	LDL	-0.190	0.015
	TC: HDL	-0.237	0.002
	WHR	-0.159	0.043
	SBP	-0.215	0.006
	DBP	-0.159	0.042
	Salivary cortisone (nmol/d)	0.316	0.000

Table 3.14 Pearson product-moment correlations between changes in salivary glucocorticoid levels and changes in selected metabolic risk factors

Correlation Pair		r	P
Salivary cortisone (nmol/d)	LDL	-0.230	0.003
	TC:HDL	-0.230	0.003
Salivary cortisol (nmol/d)	Revised-QUICKI	-0.144	0.067
	NEFA	0.146	0.0.064
Salivary cortisone-to-cortisol ratio	TC	-0.196	0.013
	HDL	-0.256	0.001
	TC:HDL	-0.263	0.001

Table 3.15 Pearson product-moment correlations between changes in serum glucocorticoid levels and changes in selected metabolic risk factors

Correlation Pair		R	P
Serum cortisone (nmol/l)	TC:HDL	-0.191	0.013
	SBP	-0.170	0.028
	DBP	-0.190	0.013
	Urinary cortisol (nmol/24h)	-0.205	0.009
Serum cortisol (nmol/l)	WHR	-0.244	0.001
	Serum cortisone	0.257	0.001
	Urinary cortisone-to-cortisol ratio	0.187	0.017
Serum cortisone-to-cortisol ratio	SBP	-0.154	0.046
	UFC	-0.176	0.024
	FC	-0.185	0.018
	Salivary cortisol	0.145	0.065
	Salivary cortisone	0.364	0.000
	Urinary cortisone-to-cortisol ratio	0.206	0.017

4. General discussion

4.1. *In vitro* studies:

4.1.1 Variability of extraction methods and antioxidant activity of extracts

The first objective of this thesis was to examine the antioxidant properties of GCBE and TCBE using a variety of extraction methods and a panel of antioxidant assays. The emphasis on antioxidant properties was based on current evidence proposing a central role for oxidative stress in promoting insulin resistance and abnormalities of glucose metabolism. Accordingly, both GCBE and TCBE were found to be rich in phenolic compounds and to act as effective free-radical reducing compounds. In particular, the acetonitrile extract was found to have the highest phenolic content as determined by the FC method as opposed to acidified-water or ethanolic extract. These findings persisted despite using different GCBE samples suggesting that the variation occurred as a result of differences in the ability of acetonitrile, acidified-water and ethanol to extract polyphenols. Similar findings were observed with FRAP assay, wherein the acetonitrile extract exhibited a stronger ferric-reducing ability than the ethanolic and acidified-water extracts.

It is generally well acknowledged that the solvent used for extracting polyphenols determines the degree of polyphenol extraction. Extraction of tea phenolics with ethanol gives a 60% higher phenol content than extraction with water (Gramza *et al.*, 2005). In the case of catechins, this could be explained by the higher solubility of catechins in ethanol than a 50% water-ethanol solvent (Nwuha *et al.*, 1999). In this study three solvents were used: ethanol, acidified water and acetonitrile. The acidified water solvent was selected to reflect the acidic milieu of the stomach, which can affect the absorption and metabolism of polyphenols (G McDougall, personal communications, 2009). The more polar acetonitrile, on the other hand, was chosen because of its ability to dissolve a wide range of compounds as a result of its low acidity (G McDougall, personal communications, 2009). As seen in Table 3.1, there was a large variation between the acidified water extract triplicates as compared to acetonitrile triplicates. These variations could have been due to interferences from sacharrides. Unlike acetonitrile, acidified-water does not remove sacharrides, which could then influence estimates of total phenol content by as much as 50% (Stratil *et al.*, 2007). Generally, a correction value could be applied to account for any such interferences but in this case (Stratil *et al.*, 2007); the acetonitrile extract was selected for the impendent ORAC analysis and for the pancreatic lipase experiment. In relation to DPPH, the ethanolic

extract was chosen since it is known to produce minimal colour interferences (Molyneux, 2004). As for TCBE, the bound fraction was found to contain the majority of phenolic compounds (2952.2µg GAE per ml bound fraction vs. 296.2µg GAE per ml unbound fraction) and was consequently used for lipase assay. Based on the above results, it was also possible to estimate the average total polyphenol intake from GCBE in the preliminary GCBE human study to be 442 mg GAE per day.

The potent reducing capacities of GCBE and TCBE were further reinforced by the DPPH assay. Here data demonstrated that polyphenol-rich GCBE and TCBE possess similar DPPH-free radical scavenging activities to GA and ascorbic acid. With regard to TCBE, such findings are consistent with the potent ROS scavenging activities of the major polyphenols found in TCBE: procyanidin B2, catechin and epicatechin (Yasuda *et al,* 2001). Surprisingly, CGA showed a low proton-donating ability (IC50=39.7µM) despite GCBE having a high DPPH-radical scavenging activity (IC50=2.56µM). This might suggest that CGA may not be the main compound responsible for GCBE's free-radical scavenging activity. The latter could be supported by the findings of Chen and Ho (1997) who have shown a higher DPPH-radical scavenging activity for caffeic acid compared to CGA. Regardless of this observation, the finding that GCBE could inhibit DPPH at concentration as low as 2.558µM is important since this value corresponds to the lower range of CGA found in plasma following consumption of GCBE (Farah *et al.*, 2008a). As a result, it could be argued that despite having a lower IC50 value than ascorbic acid, consumption of GCBE may still be crucial to preventing free radical-induced damage in plasma and tissues.

In addition to their ability to act through the ET pathway, as demonstrated by the afore-mentioned assays, both GCBE and TCBE exhibited potent free-radical scavenging activities via the HAT mechanism. The latter was evident in the high ORAC values seen for these extracts. As stated earlier, HAT-based assays serve as direct measures of antioxidant capacity because they measure the ability of an antioxidant to scavenge the peroxyl radical (Prior *et al.*, 2005). Since the latter is closely linked to lipid peroxidation, the HAT assays are also considered to be physiologically more relevant than ET-based assays (Prior *et al.*, 2005). Nonetheless, Prior and colleagues (2005) argue that a high reducing capacity such as demonstrated by ET-based assays could be important in halting the progression of radical chain reactions and might therefore reflect the ability of an antioxidant compound to maintain redox balance in plasma and tissue. Thus, the combined evidence from both ET and HAT-based assays appear to confirm GCBE and TCBE as potent antioxidants. The

latter will permit the differentiation between the various mechanisms of antioxidant activity (Ou *et al.*, 2001, Prior *et al.*, 2005). Accordingly, the ability of GCBE and TCBE to act both through ET- as well as HAT-based mechanisms may imply that GCBE and TCBE can act as both reducing and free-radicals scavenging compounds. The health implications of such findings lay in the potential contribution of these polyphenol-rich food products to overall antioxidant intake.

To illustrate, analyses of ORAC showed that both GCBE and TCBE have potent antioxidant capacities. In fact, when expressed per 100g, the ORAC values of GCBE and TCBE are found to be equivalent to 1187600µmol trolox equivalents/ 100g and 789100µmol trolox equivalents/100g, respectively. These values rank GCBE and TCBE amongst USDA's 20th highest antioxidant-containing food products (Bhagwat *et al.*, 2007).

To put these figures further into perspective, consider the UK adult NDNS. This survey indicates that on average, a British adult consumes 1073g of tea, 100g of bananas and 118g of apples or pears daily (Office for National Statistics, 2002). When this data is combined with the ORAC values from the USDA database, it appears that compared to the most widely consumed fruits (bananas, apples and pears), a daily intake of one *Theobroma cacao* bean or one GCBE capsule could provide twice the amount of antioxidants in combined apples and pears and over 16 times more antioxidants than bananas (Figure 4.1). Furthermore, when considering that on average a British adult consumes 34g of chocolate daily (Office for National Statistics, 2002), replacing this chocolate with a polyphenol-rich alternative such as the one produced using unroasted *Theobroma cacao* beans, could increase the daily antioxidant intake by a substantial 1393543 µmol trolox equivalents. This invariably suggests that GCBE, and TCBE can be important dietary sources of antioxidants and that due to their popularity and wide consumption, TCBE-derived products such as chocolate and cocoa could act as focal points for dietary intervention aimed at promoting health and increasing antioxidants intake in the population. However, it is important to highlight the fact that the ORAC assay used in the present thesis has not be standardised according to the guidelines set by the international organisation for standardisation. Consequently, the values obtained in the present thesis might differ from the values obtained by USDA. As such, no definite conclusions should be drawn from the above discussion and further studies using a standardised ORAC method will need to be conducted to confirm any statements mentioned herein.

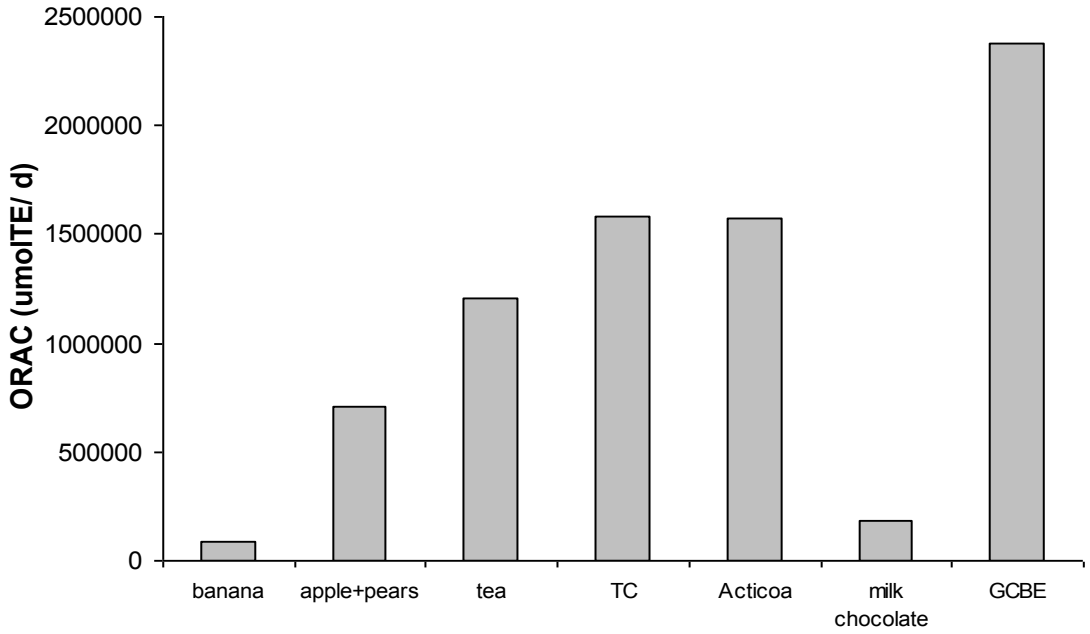


Figure 4.1 Daily contribution of *Theobroma cacao* , Acticoa chocolate, green coffee bean extract in comparison to other polyphenol-rich products consumed in the UK to Oxygen Radical Absorbance Capacity (ORAC) (data adapted from USDA, 2007c). Data are expressed as TE trolox equivalents per day.

4.1.2 Pancreatic lipase

The second objective of the *in vitro* studies was to use the data on total phenol content obtained from the first *in vitro* study to examine the potential inhibitory activities of GCBE and TCBE against pancreatic lipase. A key finding was that GCBE inhibits pancreatic lipase activity dose-dependently whereas similar concentrations of TCBE have a negligible effect on pancreatic lipase activity. In fact, as seen in Figure 3.5, the inhibitory activity of GCBE produced a J-shaped curve of percentage inhibition over concentration of GCBE, with GCBE possessing a total phenolic content of 5mg and 50mg exerting stronger inhibitory activities against pancreatic lipase than GCBE concentrations of 10 and 25mg total phenolics. These findings carry several implications since lipid hydrolysis by pancreatic lipase is a key step regulating dietary lipid absorption and inhibition of this process by pharmacological compounds such as Orlistat is known to reduce post-prandial plasma TG, remnant-like particles cholesterol and FFA (Tan *et al.*, 2002). This could be of particular relevance to obesity, wherein post-prandial lipaemia is associated with an array of obesity-associated complications including insulin resistance and dyslipidaemia.

GCBE is rich in CGA. This phenolic acid has been shown to reach the intestines in high concentrations with only <1% of ingested CGA being hydrolysed in the stomach and the intestine (Lafay *et al.*, 2006a). The latter have been explained by the lack of an esterase enzyme capable of hydrolysing CGA in the stomach or the intestine (Plumb *et al.*, 1999). In fact in an *in situ* rat model of CGA perfusion onto upper intestinal tract (jejunum and ileum), 90.8% of perfused CGA was found to reach the caecum intact (Lafay *et al.*, 2006b). Similarly, Olthof *et al.* (2001a) demonstrated that following 1-4h incubation in duodenal fluids, 95-99% of CGA is recovered. This poor absorption enhances the availability of CGA to intestinal mucosa, the site of pancreatic lipase action, a property exhibited by several known pancreatic lipase inhibitors including (−)-epigallocatechin gallate (Koo *et al.*, 2007) and Orlistat (Zhi *et al.*, 1995). Thus, CGA presents as an ideal candidate that may account for GCBE's inhibitory activity against pancreatic lipase, particularly when considering that caffeine, the second most prevalent constituent of GCBE, has been shown to be a poor inhibitor of pancreatic lipase (Nakai *et al.*, 2005). Moreover, CGA has other beneficial effects on the gastrointestinal tracts including its ability to increase NO bioavailability (Peri *et al.*, 2005). Nonetheless, further research is required to identify the active component of GCBE. This could be achieved by fractionating GCBE constituents and assessing them individually for their inhibitory activity against pancreatic lipase. Synergism between the different constituents of GCBE remains a possibility.

It is important to acknowledge that *in vitro* studies can often overestimate the biological effect of polyphenols since they do not take in account *in vivo* metabolism of polyphenols. As a result, *in vitro* suppression of pancreatic lipase activity does not necessarily imply an *in vivo* effect. Moreover, even if pancreatic lipase activity is reduced, improvements in systemic lipid metabolism are not always observed (Shepard *et al.*, 2000). Nevertheless, it remains plausible that GCBE might exert an inhibitory activity against pancreatic lipase *in vivo* particularly that a recent study in mice has shown that consumption of GCBE reduces hepatic TG and visceral fat accumulation and enhances carnitine palmitoyl transferase activity, a rate-limiting enzyme that catalyses fatty acid transport to mitochondria for β-oxidation (Shimoda *et al.*, 2006). In fact, reductions in hepatic TG content following ingestion of CGA have been observed in the other arm of the metabolic cage study reported in this thesis but conducted by Dickinson (2007) (data not shown). Subsequently, further research is required to examine whether consumption of GCBE could improve post-prandial lipid metabolism or alter the activity of LPL and hepatic triglyceride lipase especially in obesity.

As for TCBE's lack of inhibitory activity against pancreatic lipase, it is consistent with the poor inhibitory potential of non-esterified flavan-3-ols like (-)-epicatechin and catechin (Nakai *et al.*, 2005) which are likely to account for the major fraction of polyphenols in TCBE. However, polyphenols from *Theobroma cacao* possess inhibitory activities against other digestive enzymes such as α-amylase (Queseda *et al.*, 1996). Subsequently, their role in glucose metabolism might be more important than their role in lipid metabolism. The above results are, nonetheless, important since they demonstrate that inhibition of pancreatic lipase does not form one of the underlying mechanisms involved in the reduction of plasma total cholesterol, TG and LDL-cholesterol seen following long-term ingestion of cocoa by obese diabetic mice (Jalil *et al.*, 2009)

Overall, the results from the pancreatic lipase study indicate that GCBE, but not TCBE, is an effective inhibitor of pancreatic lipase activity. Hypothetically such data implies that consumption of GCBE could play a role in improving lipid profile or in attenuating post-prandial dyslipidaemia, areas that deserve further investigation.

4.2. Preliminary green coffee bean extract study

The GCBE trial provided preliminary data on the potential role of GCBE in regulating glucocorticoid metabolism in overweight and obesity. It demonstrated that GCBE can raise cortisone levels in saliva while reducing WC and urinary cortisone production. To our knowledge, few studies have reported such opposing effects with the exception of a recent study conducted by Tsang *et al.* (2009). In the latter study, consumption of anthocyanidin-rich pomegranate juice for 2 weeks yielded similar effects on glucocorticoid metabolism and WC.

As stated earlier, defects in 11βHSD1 activity are closely associated with various features of the metabolic syndrome. In obesity, upregulation of 11βHSD1 enzyme in the liver and adipose tissue is thought to induce hypertension, insulin resistance, dyslipidaemia as well as abdominal fat accumulation (Masuzaki *et al.*, 2001; Paterson *et al.*, 2004). Such effects could invariably increase the risk of CVD and mortality.

Recent studies have identified CGA as a potential therapeutic compound exhibiting strong antioxidant activities *in vitro* (Section 1.11.1.b) and capable of inhibiting 11βHSD1 *in vitro* (Arion *et al.*, 1997, Hemmerle *et al.*, 1997; Arion *et al.*, 1998; Atanasov *et al.*, 2006; Dickinson, 2007) and in animal models (Dickinson, 2007). This study conveys some

evidence to support these reports by demonstrating that GCBE significantly increases salivary cortisone concentrations while exerting some beneficial effects on salivary cortisone-to-cortisol ratio. Based on this data, it is reasonable to speculate the GCBE possesses potential inhibitory activity against 11βHSD1 in humans. Hypothetically such effect should result in improved glucose, lipid and BP homeostasis. Nonetheless, no significant changes in fasting glucose or capillary total cholesterol were observed in this study following 2-week consumption of GCBE. Although these findings are in agreement with previous studies demonstrating no significant effect of GCBE consumption on glucose metabolism in humans (Ochiai *et al.*, 2004; Kozuma *et al.*, 2005), they are in contrast to some of the animal and human studies which have reported beneficial effects of CGA on glucose regulation (Bassoli *et al.*, 2007; Blum *et al.*, 2007; Dickinson, 2007). The lack of a significant effect could possibly be attributed to the presence of 2% of caffeine in the GCBE used in the current study. According to Johnston *et al.* (2003), only the consumption of decaffeinated coffee, rich in CGA, improves glucose homeostasis. Acute ingestion of caffeinated coffee, on the other hand, even if rich in CGA, produces hyperinsulinaemic and hyperglycaemic effects (Johnston *et al.*, 2003, Robinson *et al.*, 2004, Moisey *et al.*, 2008).

In relation to total cholesterol, the lack of a significant effect was not consistent with the results from the *in vitro* study wherein GCBE inhibited pancreatic lipase by 61.5%. This could be attributed to the short duration of the study since changes in total cholesterol can take up to 3 months to occur. This can be partially supported by Kozuma *et al.* (2005) who demonstrated that consumption of GCBE supplying 93mg CGA for 28days reduces total cholesterol. Interestingly, in Kozuma *et al.*'s study consumption of GCBE containing 185mg CGA produced a small yet statistically significant rise in total cholesterol (201.5±28.2mg/dl) as compared to placebo (201.3±31.0 mg/dl). Consequently, it will be interesting to investigate whether different concentrations of GCBE affect total cholesterol in a pattern similar to their J-shaped effect on pancreatic lipase. An alternative explanation for the lack of a significant finding might be attributed to the fact that only changes in total cholesterol were monitored whereas a complete lipid profile was not obtained. In this respect, consumption of GCBE containing 93 and 185mg has been shown to dose-dependently reduce LDL cholesterol without significantly affecting TG and HDL cholesterol (Kozuma *et al.*, 2005).

A significant reduction in WC in the absence of an effect on weight was observed in the preliminary GCBE study. This finding is important since it may reflect an improvement in

body composition. Recently, a double-blind randomized trial conducted by Dellalibera *et al.* (2006) revealed that 60-day consumption of decaffeinated GCBE twice daily induced an increase in muscle mass-to-fat mass ratio. Dellalibera *et al.* (2006) explained their findings by arguing that increased fat utilisation and reduced fat accumulation due to improved glycaemic control might be the underlying mechanism responsible for such improvement in body composition. In the latter study a significant 5.7 % reduction in weight was also observed. No significant changes in percentage body fat were noted in the current study. However considering that no improvement in lipid or glucose metabolism were observed, these data appear to complement each other. In relation to glucose metabolism, GCBE-induced improvements in glucose handling as a result of either reduced glucose absorption or hepatic production are hypothesised to decrease the availability of glucose as an energy substrate, thereby forcing the body to rely on other energy substrates in particular FFA gained from lipolysis (Blum *et al.*, 2007). Such effects if proven to be correct might indicate that polyphenol-induced improvements in glucose regulation and subsequent weight loss could be central to reducing metabolic risk factors in overweight and obesity, particularly when considering that excess weight is known to be associated with insulin resistance, dyslipidaemia and hypertension. The data from the preliminary GCBE study also adds to the body of evidence by providing an additional mechanism by which GCBE can improve body composition. This is evident in the ability of GCBE to influence several parameters of glucocorticoid metabolism. To our knowledge such changes in glucocorticoid metabolism in conjunction with reduced WC have not been reported previously by other studies on polyphenols. These findings were further reinforced in the animal study wherein consumption of CGA for 17 days by mice was shown to influence glucocorticoids in a tissue-specific manner. In the liver, mice given CGA showed a marginally significant reduction in corticosterone whereas corticosterone levels were increased, albeit, non-significantly in the kidney. This pattern was similar to what was observed in human subjects who experienced an increase in salivary cortisone implying possible inhibition of 11βHSD1, and a reduction in urinary cortisone and urinary cortisone-to-cortisol ratio highlighting possible inhibition of renal 11βHSD2. The latter finding, in particular, adds to current controversies. This is because renal 11βHSD2 is an enzyme that catalyzes the conversion of active cortisol to inactive cortisone thereby protecting the kidney against cortisol-induced mineralcorticoid effects (Whitworth *et al.*, 2000). Hypothetically, inhibition of 11βHSD2 would result in increased cortisol concentrations in the kidney. Since cortisol is known to have a high affinity to the mineralcorticoid receptor, the presence of excess cortisol will prevent the binding of aldosterone to the mineralcorticoid receptor through competitive

binding which will then induce symptoms of apparent mineralcorticoid excess including hypertension, hypokalaemia and low aldosterone concentrations. Such symptoms have been previously described in conjunction with the ingestion of liquorice rich in glycyrrhizic acid and grapefruit juice rich in naringenin and in relation to quercetin, tea polyphenols, furosemide and gossypol, all of these polyphenols being known to inhibit 11βHSD2 (Mackenzie *et al.*, 1990; Zhang *et al.*, 1994; Lee *et al.*, 1996; Guo and Reidenberg; 1998, Sardi *et al.*, 2002). In particular consumption of grapefruit juice has been shown to dose-dependently reduce urinary cortisone-to-cortisol ratio in healthy volunteers (Lee *et al.*, 1996) whilst consumption of glycyrrhetinic acid has been related to significant reductions in plasma and urinary cortisone concentrations (MackEnzie *et al.*, 1990). However, contrarily to the latter study, in the present intervention, salivary cortisone increased and was accompanied by a significant fall in BP as well as WC. The design of the current study renders it difficult to elaborate on the mechanism involved in evoking such differential responses in saliva and urine. As a result, further research is warranted to investigate the effect of GCBE or its active component CGA on tissue glucocorticoid metabolism. In particular, the use of HPLC to separate cortisol and cortisone metabolites might provide a useful tool for identifying the specific location within the glucocorticoid pathway that is inhibited by GCBE. This is because changes in cortisol and cortisone metabolite profile can strongly reflect specific changes in 11βHSD1 reductase or dehydrogenase activity as well as changes in 11βHSD2 or α-reductase activity (Please refer to Figure 1.10; p 37.). Although Palermo *et al.* (1996) has previously argued that the measurement of urinary free cortisone and the urinary free cortisol-to-cortisone ratio provides a more sensitive tool for analysing 11βHSD2 activity *in vivo* than the ratio of cortisol-to-cortisone metabolites [(THF+allo-THF)/THE ratio], with both urinary free cortisone and urinary free cortisone-to-cortisol ratio being extensively used in polyphenol research due to their feasibility (see Table 1.9, ; p 99.), Ferrari *et al.* (2001) has demonstrated that intra-individual variability of cortisol-to-cortisone metabolites is lower than that of urinary free cortisol-to-cortisone ratio rendering the former method more sensitive to the detection of changes in 11βHSD2. Moreover, Stewart *et al.* (1999) has used cortisol-to-cortisone metabolites in detecting differences in the activity of 11βHSD1, 11βHSD2 and α-reductase between lean, overweight and obese subjects. Likewise, Stimson *et al.* (2007) has employed urinary cortisol and cortisone metabolite analyses for testing the effect of dietary macronutrients on cortisol metabolism which enabled them to detect selective changes in 5α-reducatse, 5β-reductase, hepatic 11ß-HSD1 and cortisol clearance.

In relation to BP, it is likely that mechanisms other than those involving glucocorticoids have contributed to the reduction in SBP seen following ingestion of GCBE. In this respect, Ochiai *et al.* (2004) has shown that long-term consumption of GCBE improves endothelium function and vasoreactivity as assessed by post-ischemic vasodilator response to reactive hyperaemia method. They attributed these effects to one of the major metabolites of CGA, ferulic acid, a phenolic acid recognised for its strong antioxidant properties and which has been reported to enhance endothelium-dependent NO release in spontaneously hypertensive rats, a model of the metabolic syndrome (Suzuki *et al.*, 2002). In addition to endothelium function, the involvement of the renin-angiotensin-aldosterone system cannot be excluded. This is because like 11βHSD1, 5-α reductase, the enzyme responsible for aldosterone metabolism, requires NADPH, as a co-factor (Dickinson., 2007). Since the inhibitory activity of CGA on cortisol is mediated by indirect inhibition of glucose-6-phospate transporter T1 which limits the availability of NADPH co-factor (Section 1.11.7), CGA might equally be capable of influencing aldosterone metabolism (Dickinson, 2007).

The finding of a reduction in WC, in the absence of an effect on weight, is intriguing and emphasises, yet again, the need to examine the effect of GCBE on tissue glucocorticoid metabolism. This is particularly true when considering that cortisol can increase abdominal fat depots in both men and women (Epel *et al.*, 2000, Wallerius *et al.*, 2003) and that attenuation of adipose tissue 11βHSD1 activity is critical to reducing abdominal fat accumulation and metabolic risk factors (Alberts *et al.*, 2003; Morton *et al.*, 2004b). The effect of GCBE on fat oxidation and lipolysis also warrants further investigation. This could be achieved by measuring glycerol and NEFA or by collecting expiratory breath samples and subsequently calculating carbohydrate and fat oxidation using stoichiometric equations developed by Jeukendrup and Wallis (Venables *et al.*, 2008).

In contrast to the powerful antioxidant properties of GCBE observed *in vitro* (see section 4.1.1) and extensively reported by other researchers (Castelluccio *et al.*, 1995; Chen and Ho, 1997; Olthof *et al.*, 2003), no significant changes in urinary antioxidant capacity were observed in this preliminary study. This could be attributed to the fact that hippuric acid, the major urinary metabolite of CGA has poor antioxidant properties (Olthof *et al.*, 2003). Consequently, quantifying the antioxidant capacity of plasma, rather than urine, or measuring lipid peroxidation might have provided a better reflection of GCBE's *in vivo* antioxidant potential. Recently, Kurihara *et al.* (2003) have noted that polyphenol-rich oolong tea can act via mechanisms involving a reduction in cortisol concentrations and a rise

in antioxidant status to prevent lipid peroxidation. Similar effects are likely to occur with GCBE consumption and merit investigation. The lack of significant change in urinary polyphenol excretion might also indicate that in the case of GCBE, monitoring dietary compliance by measuring total phenolic compounds in urine using FC method might not be ideal.

On the whole, this preliminary study demonstrates that GCBE rich in CGA could potentially be of therapeutical value in the treatment or prevention of the metabolic syndrome through its ability to improve glucocorticoid metabolism and attenuate BP and WC. Consequently, further research using a randomised placebo-controlled design and a larger sample is warranted.

4.3. Animal study

The metabolic cage study was conducted to establish whether differences in the effect of GCBE rich in CGA on salivary and urinary glucocorticoid levels arose as a consequence of a differential effect of CGA on tissue glucocorticoid metabolism. Obesity is known to be characterised by tissue-specific alteration in glucocorticoid metabolism. Consequently investigating the effect of dietary polyphenols on tissue glucocorticoid levels is important in elucidating the mechanisms by which these compounds influence circulating and urinary cortisol and cortisone concentrations. Although the metabolic cage study demonstrated that CGA might influence corticosterone levels in a tissue-specific manner, the apparently insignificant results were somehow disappointing. Moreover, due to lack of specific ELISA assay capable of quantifying the mice cortisone analogue, 11-dehydrocortisosterone, in our laboratory, it was not possible to collect data on the tissue activity of both 11 βHSD1 and 11 βHSD2. Nevertheless, the large effect size observed for both free corticosterone levels in kidney ($Eta^2 = 0.26$) and liver ($Eta^2 = 0.14$) seems suggestive of a strong association between the changes in tissue corticosterone levels and CGA consumption. Thus, the observed negative findings might be ascribed, in part, to the small sample size used in this study. The later could be reinforced by the power calculations that were subsequently conducted using data from the kidney. These showed that a sample of 13-21 animals was required to detect a significant difference in corticosterone levels using $\alpha = 0.1$ and power $(1-\beta) = 0.9$ or $\alpha = 0.05$ and power $(1-\beta) = 0.95$, respectively.

The lack of a significant effect could also be attributed to the use of normal mice which generally have low circulating and tissue glucocorticoid concentrations (Alberts *et al.*, 2005).

The use of obese KKA (y) or *ob/ob* mice might have been more appropriate in this case since these strains have up to 2.8-fold and 16-fold higher circulating corticosterone and up to 5.1 and 8.1-fold higher liver corticosterone levels than normal mice (Alberts *et al.*, 2005). This would have rendered the detection of any changes in corticosterone concentrations more attainable.

The mice study is, nonetheless, important since it demonstrates that CGA may potentially influence corticosterone metabolism differentially in various tissues. In fact in the present study mean corticosterone concentrations in CGA group were found to be higher by 24.8±11.9 mmol/ g tissue in the kidney and lower by 147.2± 170.8mmol/ g tissue in the liver as opposed to the control group. These findings may also imply that apart from influencing 11βHSD1 activity, CGA may affect the two other enzymes involved in glucocorticoid metabolism, 11βHSD2 and α-reductase. However, no northern blotting was undertaken to assess mRNA expression of these enzymes making it difficult to elaborate on the results. Stewart *et al.,* (2009), has recently argued that selective inhibition of hepatic 11βHSD1 may be more relevant to controlling obesity-related complication than inhibition of adipose tissue 11βHSD1. This is because according to Stewart *et al.* (2009), the liver consists the major site of cortisol production. Indeed, systemic rise in corticosterone concentrations have been hypothesised to induce insulin resistance whilst on organ level; excess corticosterone in liver is thought to promote insulin resistance, non-alcohol liver steatosis and weight gain in omental tissue (Livingstone *et al.*, 2000). It thus follows that selective suppression of hepatic 11β-HSD1 could be pivotal to improving insulin sensitivity and reducing hepatic TG accumulation. This could be further reinforced by the significant reduction in hepatic TG levels seen in the CGA-treated mice in the other arm of the present study and which was conducted by Dickinson (2007). Previously, Shimoda *et al.* (2006) reported that mice who consumed 6mg/kg.day CGA for 14 days had more than two-fold reduction in hepatic TG accumulation, this effect being more prominent with CGA consumption than GCBE. Nevertheless, in the latter study only GCBE and not CGA was found to enhance carnitine-palmitoyl transferase. It could, thus, be postulated that the reduction in liver TG content seen by Shimoda *et al.* (2006) and Dickinson (2007) could be partially due to suppression of 11β-HSD1 activity in liver. This hypothesis is further supported by the finding that CGA can dose-dependently inhibit 11βHSD1 conversion of cortisone to cortisol in murine liver microsomes (Dickinson, 2007). However, given the results of the present *in vivo* trial, further research using a larger sample size and obese mice, will be required to confirm this hypothesis.

The present study also carries some implications to some of the current research being conducted on the effect of polyphenols on 11βHSD1 activity in humans (Song *et al.*, 1992; Lee *et al.*, 1996; Guo *et al.*, 1998; Sardi *et al.*, 2002). This is because most of these studies rely on urinary cortisone-to-cortisol ratio as a measure of 11βHSD1 activity. As argued by Hardy *et al.* (2008), urinary cortisol can reflect both systemic as well as kidney glucocorticoid metabolism, hence this measure does not offer a true reflection of tissue glucocorticoid concentrations, which is more relevant to obesity. In fact, urinary free cortisol does not even reflect cortisol production rates since it accounts for less than 1% of total cortisol metabolites (Andrew *et al.*, 1998). As a result, caution needs to be exercised when interpreting urinary cortisol results. Moreover, if a better picture/profile of cortisol metabolism is to be gained from such studies, several measurements including saliva and blood might need to be employed.

In summary, CGA might potentially influence tissue corticosterone concentrations differentially in kidney and liver. However, due to the nature of the results, no direct conclusions could be drawn. Further studies using a larger and more representative mice strain are needed to test this hypothesis.

4.4. Preliminary dark chocolate study

The preliminary DC study compared and contrasted the effect of two polyphenol-rich DC doses on capillary fasting glucose, total cholesterol, BP and urinary glucocorticoids. The feasibility of conducting a long-term DC intervention was also determined. Overall, this short-term trial demonstrated that polyphenol-rich DC reduces fasting blood glucose levels and BP in overweight and obese individuals. These findings are consistent with previous observations that polyphenol-rich DC intake improves insulin resistance, insulin sensitivity, fasting glucose levels and BP in healthy individuals (Grassi *et al.*, 2005a), hypertensives (Grassi *et al.*, 2005b), glucose-intolerant hypertensives (Grassi *et al.*, 2008), and obese subjects (Davison *et al.*, 2008). The results are also in agreement with studies on diabetic obese mice where reductions in blood glucose and fructosamine levels were reported following consumption of cacao liquor procyanidins (Tomaru *et al.*, 2007).

Enhanced vascular function is thought to be the main mechanism by which DC polyphenols improve glucose and BP homeostasis (Karim *et al.*, 2000; Fisher *et al.*, 2003; Taubert *et al.*, 2003; Grassi *et al.*, 2005a-b, Taubert *et al.*, 2007; Balzer *et al.*, 2008; Faridi *et al.*, 2008;

Grassi *et al.*, 2008; Davison *et al.*, 2008), although other mechanisms like decreased and delayed carbohydrate digestion and absorption might also be involved (Quesada *et al.*, 1996; McDougall *et al.*, 2005). This study investigated whether polyphenol-rich DC could alter cortisol metabolism and whether improvements in glucose and BP seen in obese individuals following DC consumption are linked to improved cortisol metabolism. The hypothesis was based on that cortisol plays an important role in glucose and BP homeostasis, probably through a mechanism involving increased ROS production and decreased NO bioavailability, and that in obesity several alteration in cortisol metabolism are observed which are, in turn, linked to increased insulin resistance and hypertension. The study demonstrates that both 500 mg and 1000 mg polyphenol DC decrease 24h urinary free cortisol and cortisone levels. However, these reductions were not significant and were not associated with reductions in fasting blood glucose or BP. Such findings differ from previous findings wherein polyphenols increased (Song *et al.*, 1992; Lee *et al.*, 1996; Guo *et al.*, 1998; Sardi *et al.*, 2002) or decreased cortisol levels (Arion *et al.*, 1997; Hemmerle *et al.*, 1997). The lack of significance could be related to a number of factors. For instance, the sample size might have not been sufficiently large to detect a significant change. In this case, using several parameters of cortisol metabolism including its measurement in urine, saliva and blood might have helped detect any such effect. Additionally, the study population consisted mainly of subjects with peripheral obesity rather than those with abdominal obesity who exhibit more prominent abnormalities in cortisol metabolism as indicated by the association between high WC or WHR and high urinary cortisol or cortisone-to-cortisol ratio (Fraser *et al.*, 1999; Vicennati and Pasquali, 2000). Similarly, differences in sodium intake were not controlled for and could have acted as confounding factors (Chamarthi *et al.*, 2007). This could be observed in the association between changes in urinary free cortisol or cortisone and changes in sodium excretion, and the association between changes in sodium intake and changes in cortisol-to-cortisone ratio. Dietary factors have been reported to influence cortisol metabolism. High-fat low-carbohydrate diets stimulate cortisol regeneration by 11βHSD1 while reducing cortisol inactivation in liver (Stimson *et al.*, 2007). Sodium loading, on the other hand, decreases plasma cortisol levels by enhancing cortisol elimination (Litchfield *et al.*, 1998) possibly via a mechanism involving increased hepatic blood flow (Kerstens *et al.*, 2001). The latter may explain the association between increased urinary free cortisol excretion and urinary sodium levels. However, subjects did not report significant changes in sodium intake during the study, which overall suggests that DC polyphenols influence glucose and BP homeostasis mainly via the NO pathway.

This study demonstrates that DC with 500 mg polyphenols is equally effective in reducing fasting blood glucose levels in overweight and obese individuals as 1000 mg polyphenol DC with a similar macronutrient composition. Furthermore, the results indicate that DC polyphenols reduce blood glucose levels after 2 weeks of commencing a polyphenol-rich DC diet. These findings are important since in relation to glucose metabolism, inconsistencies still exist regarding the treatment duration and dose required to achieve a glucose-lowering effect. For example in their pilot study Stote *et al.* (2007) failed to show any significant improvement in glucose levels, insulin resistance, and insulin sensitivity following 5 d of twice daily consumption of procyanidin-rich cocoa beverage containing 22 to 900 mg procyanidins by insulin-resistant men and women. Similarly, Taubert *et al.* (2007) failed to demonstrate any improvement in glucose or insulin levels following 18 weeks of daily ingestion of 6.3 g DC containing 30 mg polyphenols. Conversely, Davison *et al.* (2008) showed reduced insulin resistance following consumption of a cocoa beverage containing 902 mg flavanols twice daily for 12 weeks in overweight and obese subjects. Together these findings suggest that a longer duration and a higher dose of polyphenols could be required to achieve a significant reduction in glucose levels. This study reinforces this hypothesis while demonstrating that increasing polyphenol dose does not necessarily results in further reductions in glucose and BP level. In relation to BP, 20 g DC with 500 mg polyphenols was found to reduce SBP and DBP to a similar extent as 20 g DC with 1000 mg polyphenol. Two explanations could be provided for the observed similarities in response to 500mg and 1000mg polyphenols DC. First, that increasing polyphenol dose does not necessarily confer additional benefits in relation to glucose metabolism or BP possibly because a plateau effect is reached with increasing polyphenol dose. Second, that there exists a large inter-subject variations in the absorption and metabolism of polyphenols rendering certain subject more responsive to the actions of polyphenol-rich DC than others. In agreement with the latter concept, differences in the ability to absorb or metabolise polyphenols have been reported by several studies (see review by Manach *et al.*, 2004). Procyanidins, in particular, have been shown to be differentially absorbed and metabolised by humans mainly because their large polymeric structure interferes with their absorption, thereby necessitating the hydrolysis of their polymeric structure into their respective trimers, dimers or monomers (Manach *et al.*, 2004). Since these processes are regulated by various enzymes, inter-individual variations in the expression and the activity of these enzymes may account for differences in polyphenol metabolism (Manach *et al.*, 2004). Even if procyanidins are effectively metabolised to the more absorbable dimers, the dimers, themselves, can undergo further hydrolysis into monomers or isomerisation under both acidic and alkaline conditions (Zhu *et al.*, 2002a).

This has led Zhu *et al.* (2002a) to conclude that 'the amount and type of flavanols and procyanidins in the gastrointestinal tract following the consumption of cocoa can be influenced by the stability of these compounds in both acidic and alkaline environments'. The food matrix is another major determinant of polyphenols bioavailability and, concurrent ingestion of DC polyphenols and carbohydrates is known to enhance DC polyphenols' absorption (Schramm *et al.*, 2003). This might indicate that subjects consuming greater quantities of carbohydrates could absorb DC polyphenols more effectively than subjects with lower carbohydrate intake, although such concept remains to be clarified. Regardless of the explanation, the pilot study provides interesting findings since few studies have compared the effect of different DC polyphenol doses on glucose, lipid and BP homeostasis for up to 2 weeks. As such the preliminary DC study highlights the need for a better understanding of the bioavailability of DC polyphenols since it will permit identifying and establishing maximum and minimum levels of DC polyphenol intake needed to exert a biological effect. This could be important since a reduction in the polyphenol-content of chocolate also implies reduced bitterness (Luna *et al.*, 2002; Counet *et al.*, 2004), which could render the chocolate more palatable and acceptable to the general population.

It is noteworthy that one of the main strengths of the preliminary study was the use of 20g DC providing 500-1000mg polyphenols. Most previous studies have been criticised for using large quantities of DC. In fact, Grassi *et al.*(2005a-b) used a daily dose of 100g DC containing 500mg polyphenols. According to Desch *et al.*(2009), long-term consumption of such large quantities of DC is likely to result in weight-gain, thereby counteracting any beneficial effect of DC polyphenols on BP. Since few studies have examined the short-term effect of ingestion of small portions of polyphenol-rich DC, this study is amongst the first to demonstrate that consumption of 20 g DC (500 mg polyphenols) produces comparable reduction in BP to 100g DC with 500mg polyphenols (7 and 3 mmHg reduction in SBP and DBP, respectively) (Grassi *et al.*, 2005a). Such findings may suggest that reducing the portion of DC while maintaining a similar total phenolic content results in equivalent reductions in BP. This could provide several advantages since reducing the portion of DC would permit delivery of high quantity of polyphenols in a less energy dense form, which is essential if DC is to be included as part of a healthy balanced diet.

In relation to total cholesterol, no significant changes in total cholesterol were observed following 2-week consumption of either 500mg or 1000mg polyphenols DC. This is in contrast to Fraga *et al* (2005) and Grassi *et al.* (2005b). However, such results are to be

expected since in the present study subjects had normal baseline total blood cholesterol levels as compared to Grassi *et al.* (2005b) (baseline total cholesterol in Grassi *et al*'s study was 5.4±0.6 mmol/L). Moreover, Grassi *et al.* (2005b) suggested that both the catechin and the fat component of DC account for its beneficial effect on total cholesterol. Similar assumptions were made in relation to stearic acid in DC (Ding *et al.*, 2006). Since in the present study a lower DC portion was used, the lack of significant change in total blood cholesterol could be related to the lower levels of linoleic, oleic and stearic acids present in this DC.

In the present preliminary study, no correlation between reported energy intake and physical activity was detected. This finding is similar to the findings of Davison *et al.* (2008) who argued that obese individuals can underreport energy intake and over-report physical activity. Likewise, in the present study, no significant changes in urinary antioxidant capacity were observed. This lack of significance could be attributed to the large inter-individual differences in baseline urinary polyphenols excretion. In fact, despite efforts to educate volunteers about the importance of excluding polyphenol-rich products during the run-in phase, three subjects were found to have higher polyphenol levels in urine at baseline as compared to post-500mg and post-1000mg polyphenols DC treatment. These outliers accounted for the initial lack of statistically significant changes in urinary antioxidant capacity. Exclusion of these outliers produced a dose-dependent increase in urinary antioxidant capacity consistent with previous observations of a dose-dependent association between polyphenol intake and urinary antioxidant capacity (Richelle *et al*., 1999; Wang *et al.*, 2001).

Overall, the present short-term preliminary study confirms previous reports of improved fasting glucose levels and BP following DC consumption while demonstrating that these effects are unlikely to be mediated through changes in cortisol metabolism. Nonetheless, further studies using cortisol measurements in urine, saliva and possibly blood might be necessitated. Research aimed at identifying the optimal dose of polyphenols required to improve glucose metabolism and the mechanisms involved in such hypoglycaemic properties is also warranted.

4.5. Main study

As stated in section 1.13., one of the aims of the current thesis was to identify the minimum polyphenol dose capable of producing hypoglycaemic and hypotensive effects and to

subsequently investigate the long-term response of overweight and obese individuals to this polyphenol dose. The importance of differentiating between the effects of 500mg and 1000mg polyphenols was based on the emerging evidence relating possible pro-oxidant effects to intake of high doses of procyanidins (Sakano *et al.*, 2005) and the controversial findings concerning the glucose-lowering effects of high cacao polyphenol intake (Stote *et al.*, 2007; Balzer *et al.*, 2008; Davison *et al.*, 2008). Overall, the main study demonstrated that 4-week consumption of 500mg polyphenols DC can improve BP and glucose metabolism as indicated by the reduction in fasting glucose, HOMA-IR and the rise in QUICKI and revised-QUICKI. Such findings confirmed the observations of the short-term preliminary DC study and were consistent with previous studies on healthy individuals, hypertensives and glucose-intolerant hypertensives. The data was also in accordance with the most recent findings of Davison *et al.* (2008) who reported beneficial effects of polyphenol-rich cocoa on cardiometabolic markers in subjects with BMI $\geq$25kg/m^2. Most importantly, the current study demonstrates that overweight and obese subjects respond more effectively to the hypoglycaemic and hypotensive properties of polyphenol-rich DC than their lean counterparts. To our knowledge, this is the first study that allowed direct comparison of the effect of polyphenol-rich DC on glucose, lipid and BP across the two BMI categories. Such differences in response may be attributed to the raised metabolic risk factors seen in individuals with BMI$\geq$25kg/m^2 and which were predicted in the current study by the higher baseline WHR, WC, TC: HDL ratio and BP compared to lean individuals. Support for this explanation could be gained from Grassi *et al.* (2005a-b, 2008) who documented greater reductions in SBP and DBP in hypertensive patients compared to healthy normotensive volunteers.

By contrast to previous human studies (Grassi *et al.*, 2005a-b; 2008), no significant changes in fasting insulin or insulin secretion (HOMA-β) were observed in this study. This may be expected since in animals, some studies have failed to report any improvement in insulin in some models of obesity but not in others, which could indicate that the underlying cause of insulin resistance may dictate a subject's responsiveness to polyphenol-rich DC. Alternatively, the use of a more robust study design which included a DC placebo rather than a white chocolate placebo might have eliminated any possible effect of varying magnesium and theobromine content between the active DC and the placebo. This implies that, unlike other studies which did not control for differences in macronutrient and micronutrient content, any changes in the assessed parameters in our study can be directly related to the presence of DC polyphenols rather than other DC components. The strength of such design

could be further viewed in that DC placebo permits effective blinding of volunteers, which may minimise expectation bias.

In the present intervention, reduction in SBP and DBP of 4.17mmHg and 2.29mmHg, respectively, were observed. These values are consistent with a recent meta-analysis of 10 randomised controlled trials involving 297 volunteers which demonstrated that, on average, polyphenol-rich DC and cocoa reduce SBP by 4.5mmHg and DBP by 2.5mmHg, respectively (Desch *et al.*, 2009). However, when comparing changes in BP in the main study to the preliminary DC study, it can be observed that the magnitude of reduction in SBP and DBP were lower at the end of the 4-week main DC intervention than at the end of the 2 weeks of the preliminary study (SBP -6.98mmHg from baseline, DBP -5.62mmHg from baseline). This is partially in agreement with the afore-mentioned meta-analysis wherein Desch *et al.* (2009) demonstrated greater reductions in SBP and DBP in short-term 2-week trials as opposed to long-term 4-18 week interventions suggesting a possible adaptation occurring to high doses of polyphenol over time. The study by Taubert *et al.* (2007) is particularly interesting in this respect since it is the only study to have monitored BP at different time-point of the intervention (i.e. at baseline, 6, 12 and 18 weeks of polyphenol-rich DC ingestion). In that study no adaptation to polyphenols was observed and SBP and DBP continued to fall steadily from baseline through week 6 to week 12 and week 18 of the trial (Taubert *et al.*, 2007). However, the lack of adaptation could be attributed to the use of a small quantity of DC (6.3g) containing 30mg polyphenols which might indicate that with lower polyphenol doses a cumulative effect of dose on BP occurs over time (Taubert *et al.*, 2007). By contrast, with larger doses such as used in the present study, maximum reduction in BP might be achieved rapidly after which the effect might begin to subside until the maximum possible long-term reduction is reached.

Although, the lack of significant improvement in lipid profile is in contrast to previous studies (Mathur, 2002, Mursu *et al.*, 2004, Grassi *et al.*, 2005b, Baba *et al.*, 2007a-b), these findings are consistent with other researchers who have failed to observe any changes in lipid profile after ingestion of polyphenol-rich DC (Taubert *et al.*, 2003, Engler *et al.*, 2004, Grassi *et al.*, 2005a). In general, studies that reported changes in lipid profile used 75-100g DC containing 500mg polyphenols (Mursu *et al.*, 2004, Grassi *et al.*, 2005b, 2008) which provided up to 5 times more cocoa butter than the present study. It is, therefore, likely that cocoa butter, with its high monounsaturated fat content, accounted for some of the improvements in lipid profile. Nonetheless, Baba *et al.* (2007a-b) and others (Mathur *et al.*,

2002) have also reported a reduction in LDL concentrations and a rise in HDL levels after consumption of liquid cocoa, containing no cocoa butter, suggesting that polyphenols *per se* can influence lipid profile. Hence, the lack of significant effect in this present study could only be attributed to the normal lipid levels of this study's population. Genetic polymorphisms, likewise, cannot be excluded (Ordovas, 1999).

It is important to stress that the present long-term dietary intervention adds to current research by indicating that overweight and obese individuals might be affected more adversely by the ingestion of polyphenol-poor DC. This is observed in the significant rise in fasting insulin, HOMA-IR and the marginally significant decline in QUICKI following placebo, which could suggest that common chocolate might increase the risk of hyperinsulinaemia and insulin resistance in overweight and obese individuals. In support of this finding, Brand-Miller *et al.* (2003) have shown that compared to alternate flavourings, the addition of common cocoa powder into foods increases postprandial insulin secretion. In fact, according to Brand-Miller *et al.* (2003), products containing commercially-available chocolate increase insulin secretion to a greater extent than products with a similar fat content like potato chips and croissants. Likewise, Holt *et al.* (1997) have reported that chocolate confectionery (Mars Bar) and chocolate-flavoured breakfast cereal (Coco Pops) can have an insulin index 50-60% higher than their glycolic index. This led Brand-Miller *et al.* (2003) to argue that stearic acid in cocoa butter or amino acids in cocoa might account for the enhanced insulin response of cocoa products. The current study provides some insight into the findings of the above studies as will be discussed later. Regardless of mechanisms, the combination of hyperinsulinaemia and unfavourable lipid profile (raised TC: HDL) as seen in the overweight and obese group in the present study might render this population group more prone to the development of CVD. It is noteworthy that the findings concerning the changes in HOMA-IR and QUICKI following placebo have not been reported by other studies. Such discrepancies might be attributed to differences in the products used as most other studies that investigated the effect of DC polyphenols on glucoregulatory biomarkers used white chocolate containing milk powder and butyric fat as placebo (Grassi *et al.*, 2005a-b, 2008).

Despite the negative correlations seen between urinary, salivary and serum cortisone as well as urinary cortisone-to-cortisol ratio and several parameters of the metabolic syndrome including LDL, TC: HDL, SBP, DBP and WHR, no significant changes in glucocorticoid metabolism were observed within the polyphenol-rich DC group. This was consistent with

findings of the preliminary DC study. However, a significant rise in salivary cortisol and a significant reduction in cortisone-to-cortisol ratio were observed following ingestion of placebo. Although at present it is difficult to elaborate on the observed changes in glucocorticoid metabolism within the placebo group, a plausible explanation could be related to the placebo's effect on fasting insulin. Fasting insulin and HOMA-IR are known to be strongly and positively correlated with 5α-reducase activity (Tomlinson *et al.*, 2008). Under normal physiological conditions and those of insulin resistance, upregulation of this enzyme helps reduce cortisol availability in the liver, a mechanism aimed towards sustaining hepatic insulin sensitivity (Tomlinson *et al.*, 2008). However, in obesity, enhanced 5α-reductase activity stimulates hepatic clearance of cortisol causing a compensatory activation of HPA, which in turn leads to excess cortisol secretion (Livingstone *et al.*, 2000; Westerbacka *et al.*, 2003). As a result, it is possible to postulate that upregulation of 5α-reductase via insulin might be the underlying mechanism by which the placebo raised salivary cortisol secretion. Emerging evidence concerning the role of dietary macronutrients in regulating 5α-reductase and HPA activity could provide a further explanation for the effect of placebo on salivary cortisol and serum cortisone-to-cortisol ratio while supporting the involvement of 5α-reductase. Accordingly, several studies in mice and rats have shown that high-fat feeding stimulates basal and stress induced HPA activity (Morton *et al.*, 2004a; Drake *et al.*, 2005). This effect has also been observed in humans following infusions of insulin or lipid (Wake *et al.*, 2006) and following ingestion of a mixed-meal (Basu *et al.*, 2006) or a high-fat low-carbohydrate diet (Basu *et al.*, 2006; Wake *et al.*, 2006; Stimson *et al.*, 2007). In the study by Stimson *et al.* (2007), healthy obese men were given a high-fat low-carbohydrate diet for 4 weeks. At the end of the study period, an increase in cortisol regeneration by 11βHSD1 and a reduction in α- and β-reductase-induced cortisol inactivation was observed. Although this evidence only provides preliminary data on the relation between macronutrients and cortisol metabolism, there is a possibility that a similar effect could have occurred in the current study wherein the placebo provided an additional 7.34g of fat to the diet per day which over 4 weeks and in the absence of adequate antioxidant intake might have resulted in enhanced cortisol secretion. Biochemically, such response permits the shift in substrate oxidation towards enhanced fat utilisation and diminished glucose oxidation (Andrew and walker 1999). Albeit a normal physiological response, in obesity, hypersensitivity of HPA axis means excess cortisol could be secreted which could have detrimental effects on the metabolic state, particularly when considering that raised cortisol levels induce oxidative stress (see section 1.8.3) and promote abdominal TG accumulation in the presence of hyperinsulinaemia via upregulation of LPL activity (Ottosson *et al.*, 1994). The finding that

the placebo stimulated cortisol secretion whereas polyphenol-rich DC did not, could be important as it might suggest a potential role for polyphenols in counteracting the effect of high-fat diet-induced cortisol secretion. Thus, improving processing methods of cocoa beans could be vital to the development of novel functional foods that can protect the population against overweight and obesity-related metabolic abnormalities. The above findings also highlight one major public health implication. That is if the consumption of placebo, which is equivalent to the polyphenol-poor chocolate products currently available on the market, increases cortisol generation and if the latter effect occurs via enhanced 5α-reductase activity, which in turn, may influence body composition and insulin resistance (see Tomlinson *et al.*, 2008), then it could be hypothesised that the current commercially-available chocolate products may act as part of the driving force that promote the current overweight and obesity epidemic both through their energy-density and detrimental impact on cortisol metabolism (Figure 4.2). This statement may also help explain the hyperinsulinaemic-effects of commercial chocolate and chocolate-products reported by Holt *et al.* (1997) and Brand-Miller *et al.* (2003) as the relation between 5α-reductase and insulin is reciprocal. That is as well as being influenced by insulin, 5α-reductase might influence insulin levels (Tsilchorozidou *et al.*, 2003). If this hypothesis is proven to be correct then it might provide a more perturbing picture considering the popularity of these food-products across various population subgroups. However, further research will be required to elucidate the points mentioned above. Moreover, the active component responsible for the placebo's stimulatory effect on cortisol will need to be identified since as yet it remains unclear whether the adverse response to polyphenol-poor DC is evoked as a consequence of its high fat content or due to the presence of specific hyperinsulinaemic and hypercortisolaemic compounds in placebo. As such further studies that compare the effect of polyphenol-rich DC, polyphenol-deficient DC and a food product containing a similar fat content but an alternative fat to cocoa butter on fasting insulin and salivary cortisol levels may be required.

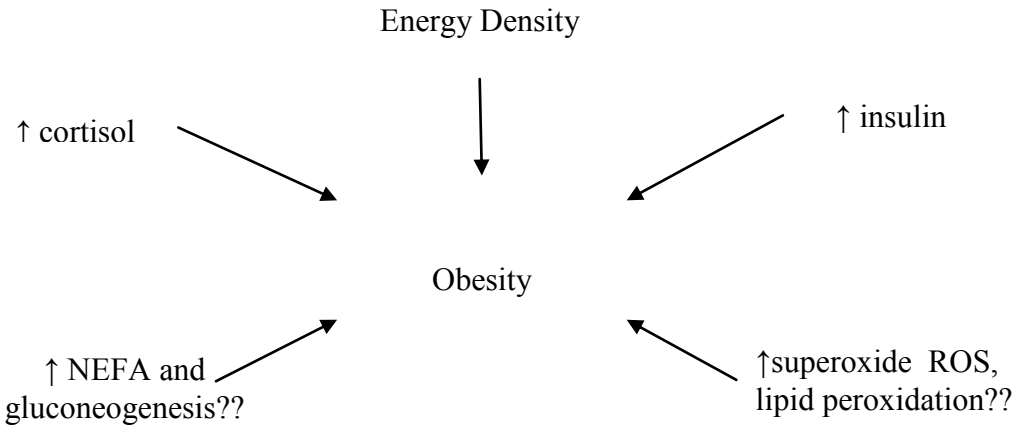


Figure 4.2 Proposed novel hypothesis by which the high energy density and potential hyperinsulinaemic, cortisolaemic effects and possibly gluconeogenic and oxidative stress-inducing properties of polyphenol-deficient DC may promote weight gain.

In support of the hypothesis depicted in Figure 4.2, body weight was found to be significantly higher at the end of 4 weeks of placebo treatment as compared to polyphenol-rich DC. Similarly, the concentration of serum NEFA was increased, albeit marginally, following consumption of placebo but not polyphenol-rich DC. Moreover, a significant positive correlation between serum NEFA and salivary cortisol concentrations was observed. In general, 70-75% of cortisol circulates in blood bound to corticosteroid-binding globulin with only 5–10% of cortisol being found in the active unbound while 15–20% is bound to albumin (Andrews and Walker, 1999). When the levels of free cortisol increase, due to saturation of corticosteroid-binding globulin in the morning or during conditions of stress or abdominal obesity, two outcomes are to be expected (Andrews and Walker, 1999; Purnell *et al.*, 2004). First, fat is redistributed from peripheral to abdominal fat depots leading to a raised WHR (Pasquali *et al.*, 1993; Westerbacka *et al.*, 2003). Second, hepatic gluconeogenesis is stimulated (Khani and Tayek, 2001).

In the first instance, although no changes in fat distribution as measured by WC, HC and WHR were seen in this trial, body weight was significantly higher at the end of 4 weeks of placebo ingestion as compared to polyphenol-rich DC wherein a significant reduction from baseline was observed. As with changes in salivary cortisol and fasting insulin, changes in body weight occurred selectively within the overweight and obese group. Such changes in

body weight have not been reported by previous studies which can be attributed to the use of lean individuals, the short duration of the former trials (see Table 1.5 to Table 1.8) and the use of tailored iso-caloric diets (Grassi *et al.*, 2005a). Arguably, the changes in body weight within the overweight and obese group might have influenced the hemodynamic (BP) and metabolic variables (glucoregulatory biomarkers) assessed in this trial, thereby explaining the significant differences in these parameters between the placebo and polyphenol-rich DC groups. This may suggest that despite the lack of significant changes in energy intake or physical activity as measured by the diet and physical activity diaries, and despite efforts to ensure that both treatment products provided similar amounts of energy, macronutrients and micronutrient, non-compliance, under-reporting and changes in dietary intake within the overweight and obese group cannot be excluded. However, it is important to note that changes in body weight occurred selectively following administration of placebo or polyphenol-rich DC while no significant changes between the two baselines periods were detected. In animal models, ingestion of cocoa has been reported to increase thermogenesis (Matsui *et al.*, 2005). Improved glycaemic control has also been postulated to be one of the underlying mechanisms by which polyphenols promote weight reduction (Dellalibera *et al.* 2006; Blum *et al.*, 2007). Based on these findings, it is not possible to elaborate on the direction of the cause-and-effect relation as changes in body weight can occur as both a cause and a consequence of changes in insulin sensitivity (Potenza *et al.*, 2007). In the present thesis, the application of dichloromethane extraction onto serum samples and the use of salivary cortisol permitted the quantification of the active free form of cortisol (Vining *et al.*, 1983; Gozansky *et al.*, 2005). Hence, the combination of increased salivary cortisol, reduced serum cortisone-to-cortisol ratio and raised fasting insulin makes it tempting to speculate that these changes might have accounted for the rise in body weight within the placebo group. Moreover, the fact that all of these changes were specific to the BMI $\geq$25kg/m^2, with the exception of serum cortisone-to-cortisol ratio which decreased across both BMI groups, and were not seen within the lean group supports such concept, although no definite conclusions should be drawn at present.

The association between changes in body weight, fasting insulin and glucocorticoid metabolism could be further reinforced by the observed rise in serum NEFA concentrations in the placebo group. Cortisol is known to stimulate NEFA release which is then correlated with enhanced gluconeogenesis (Khani and Tayek, 2001; Djurhuus *et al.*, 2002). This enhanced gluconeogenesis has been hypothesised to induce hyperglycaemia and hyperinsulinaemia leading to insulin resistance and eventually type II diabetes (Barthel and

Schmoll, 2003). By contrast, inhibition of early morning rise in cortisol using pharmacological compounds such as metyrapone reduces NEFA and glycerol concentrations (Samra *et al.*, 1996). In the present trial, fasting glucose levels were not increased following ingestion of placebo. However, fasting insulin and HOMA-IR were raised within the placebo group which is consistent with the metabolic effects of raised circulating NEFA (refer to Figure 1.8 in Introduction). Moreover, salivary cortisol concentrations were found to correlate positively with serum NEFA concentrations and inversely with insulin sensitivity as measured by revised-QUICKI. Thus, the combined evidence from the present long-term trial appears to favour the hypothesis concerning the detrimental effects of DC deficient in polyphenols and highlights the importance of elucidating the differential effects of polyphenol-rich DC and polyphenol-deficient DC on overweight and obese individuals.

Although not investigated in the present trial, it is well recognised that DC and cocoa polyphenols exert their beneficial effect via modulation of NO bioavailability. Accordingly, Schroeter et al. (2006) have demonstrated higher urinary nitrate+nitrite excretion among Kuna Indians consuming a flavanol-rich cocoa-based diet as compared to Kuna Indians consuming a western-diet. Dose-dependent rise in the concentration of plasma nitros(yl)ated species (RNO), also referred to as the NO pool and which consists of S- and N-nitrosoproteins and iron-nitrosyl complexes, have also been reported 2h following acute ingestion of 100ml cocoa drink containing 176–185mg flavanols (70-74mg monomers, 20-22mg epicatechin, 106-111mg procyanidins) (Heiss et al., 2005). Likewise, increased plasma S-nitrosoglutathione have been documented with chronic consumption of polyphenol-rich DC but not white chocolate (Taubert et al, 2007). Recently, two isoforms of NOS have been identified in white adipose tissue: eNOS and inducible-NOS (iNOS) (Ribiere et al., 1996; Kapur et al., 2000). This has led to a rise in the number of studies investigating the potential modulatory or regulatory activities of NO in adipose tissue. Accordingly, studies on isolated adipocytes have indicated that NO can modulate the processes of lipolysis and lipogenesis. In fact, incubation of adipocytes, isolated from male Sprague-Dawley rats, with increasing concentrations of the NO donors, the nitrosothiols, has been shown to produce dose-dependent stimulation of basal lipolysis as indicated by the rise in glycerol release (Gaudiot et al., 1998). NO has also been demonstrated to enhance differentiation of brown (Nisoli et al., 1998) as well as white adipose tissue (Yan et al., 2002), although in the case of the latter this effect was only achieved in the presence of an adipocyte differentiation stimulus (Yan et al., 2002). NO is also known to regulate the gene expression and activity of several enzymes involved in lipogenesis (glycerol-3-phosphate

acyltransferase, SREBP-1c and phosphoenolpyruvate carboxykinase) and lipolysis (hormone-sensitive lipase) (Jobgen et al., 2006). This evidence in conjunction with the changes in body weight seen in the main DC study emphasise the need for elucidating the precise metabolic and biochemical pathways that can be modulated by polyphenols, specifically in relation to overweight and obesity. Moreover, such evidence adds weight to the hypothesis that polyphenols, in particular cocoa and DC polyphenols, can act as important dietary targets for the prevention of obesity-associated chronic conditions (Figure 4.3).

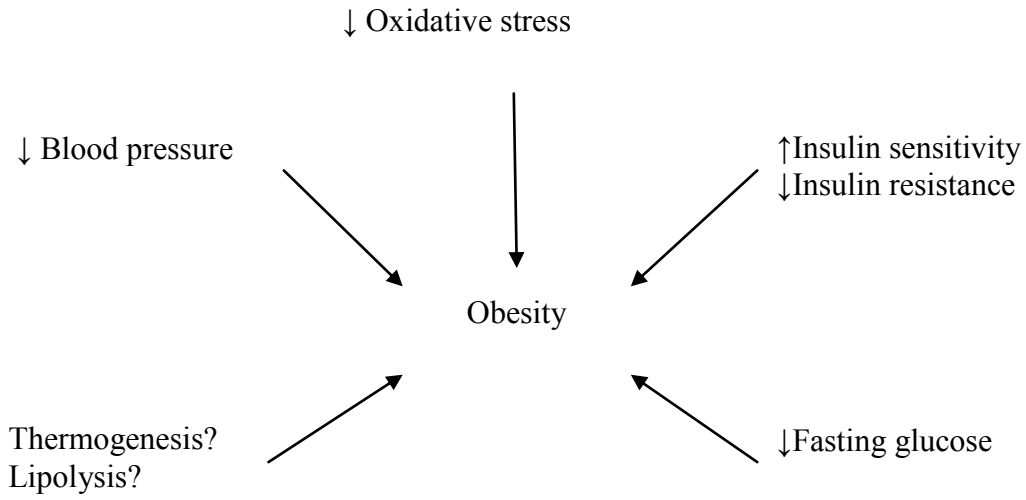


Figure 4.3 Proposed mechanisms by which polyphenols from dark chocolate can protect against obesity-related complications. Changes in thermogenesis and fat oxidation are areas that deserve further investigation given the findings of animal studies and the potential role of NO in modulating lipolysis and adipose tissue differentiation.

Finally, it remains to be stated that although commonly-consumed chocolate may adversely affect body weight and other metabolic parameters in overweight and obese individuals, as observed in this thesis and previous studies, this does not argue against the use of small or moderate amounts of polyphenol-rich DC or cocoa as part of a healthy balanced diet in the prevention of obesity-related complications. Nonetheless, it is important to highlight that in a recent study by Desch *et al.* (2010), consumption of polyphenol-rich DC by overweight and obese individuals over 3 months was shown to induce a significant rise in body weight. Although, in the latter study, patients did not receive a control diet, rendering comparisons between the effect of polyphenol-poor DC and the effect of polyphenol-rich DC difficult, such observations imply that research on the potential application of polyphenol-rich DC or cocoa in the context of overweight and obesity should be pursued further in order to establish the long-term metabolic effects of polyphenol-rich DC or cocoa consumption.

In the present main DC study, no significant differences in plasma or urinary antioxidant capacities as measured by FC, FRAP and ORAC were observed between the lean and overweight/ obese group. Similarly, no significant changes in plasma antioxidant capacity were detected during the intervention period which is in agreement with other studies (Vlachopoulos *et al.* 2005, Taubert *et al.*, 2007). Epicatechin, the main DC polyphenol, is known to be rapidly absorbed and metabolised in the body because of its basic structure. As a result, its concentration in plasma alongside plasma antioxidant capacity usually return to baseline 6h following the consumption of polyphenol-rich DC (Richelle *et al.*, 1999, Wang *et al.*, 2000). Since in the present study blood was drawn at fasting 12h following the ingestion of DC, it is unsurprising that no changes in plasma antioxidant capacity were detected. Urine, on the other hand, was collected on the last day of DC consumption. Accordingly, an increase in urinary FC, FRAP and ORAC was found following consumption of polyphenol-rich DC but not placebo. In fact using estimates from FC, urinary polyphenol levels were found to increase by 29.23% from baseline. Likewise, based on the mean rise in urinary total polyphenol concentrations from baseline (64.11mg GAE/d), urinary recovery of polyphenols in the present study could be estimated to be 12.8%. In general, researchers have reported wide variations in urinary recovery of polyphenols. For instance, Ito *et al.* (2005) has noted a 1.9% recovery of epicatechin following consumption of a cocoa beverage containing 289mg catechin and procyanidins. Baba *et al.* (2000) showed a 25-30% recovery of epicatechin following ingestion of cocoa or DC. Most of these studies employed HPLC coupled with electrospray ionisation mass-MS or a combination of HPLC and LC-MS to detect phenolic compounds under controlled dietary conditions making it difficult to compare the data from these studies with the present study since HPLC and LC-MS permit more accurate quantification of polyphenols in urine than FC. The only study that employed the FC method for polyphenol quantification demonstrated a 55% increase in urinary polyphenols levels from baseline and an average polyphenol recovery of 7.44% (Roura *al.*, 2006). Assuming that in the present study, participants consumed half the polyphenol dose used in Roura *et al.*'s study, these findings may reflect that a dose-dependent effect of increasing polyphenol intake on urinary polyphenol excretion exists, which is in agreement with former studies (Richelle *et al.*, 1999; Wang *et al.*, 2001) and the preliminary DC study.
Unfortunately, due to limited resources no direct measurement of lipid peroxidation was undertaken in plasma or urine. This would have provided additional support for the hypothesis summarised in Figure 4.2 if the placebo-DC was found to increase lipid peroxidation at the end of the placebo period. Moreover, assessment of lipid peroxidation would have provided some insight as to whether the increase in cortisol and insulin

resistance seen following placebo was associated with elevated plasma or urinary lipid peroxides. This would have been particularly important considering that a positive correlation between urinary cortisone and serum and urinary antioxidant capacities were observed in this thesis, which is consistent with a role for glucocorticoids in inducing oxidative stress (see section 1.8.3). In this respect, quantification of F2-isoprostanes in plasma or urine might have been useful but unfeasible. F2-isoprostanes are compounds formed from ROS-induced peroxidation of arachidonic acid. Because their levels in human biological fluids are considerably higher than other prostanoids (Morrow *et al.*, 1995), they can be accurately and easily quantified in conditions of low as well as high oxidative stress (Young, 2005, Longmire *et al.*, 1994). F2-isoprostanes are also directly involved in the pathologies of many diseases and consistent evidence exists to demonstrate a strong association between their levels in obesity and obesity-related co-morbidities including insulin resistance (Keaney *et al.*, 2003, Urakawa *et al.*, 2008). As a result, F2-isoprostanes are considered by far the best marker of lipid peroxidation and are consequently deemed much superior to measurement of malondialdehyde using TBARS assay, another assay used for determination of lipid peroxidation but which is considered to reflect less accurately the process of lipid peroxidation within biological fluids (Morrow *et al.*, 1995). Nevertheless, the results from the urinary antioxidant assays (Figure 3.27) seem suggestive of oxidative stress as the urinary values for total phenolics and FRAP appear to be lower at the end of 4 weeks of placebo than at the end of 4 weeks of polyphenol-rich DC. Notably, urinary ORAC values were significantly reduced from baseline after placebo (Figure 3.27). Added to this, is the evidence from the *ex vivo* and *in vivo* animal and human studies conducted by Vinson *et al.* (2006) which clearly demonstrate the pro-oxidant and pro-atherogenic properties of cocoa butter and which suggest that cocoa polyphenols can blunt the pro-oxidant properties of cocoa butter (see p 72. of Chapter 1). A key question that arises from such results is the potential implications of such findings and the potential application of cocoa polyphenols in counteracting obesity-related and high-fat diet-induced oxidative stress?

In conclusion, the above long-term DC study provides evidence for a potential role for moderate consumption of polyphenol-rich DC in improving glucose and blood pressure levels in overweight and obese females. However, given the potential adverse metabolic effects observed with polyphenol-poor DC and the associated changes in weight, further research will be required to elaborate on the findings of this human trial.

4.6. Limitations

There are several limitations to the human studies conducted as part of this thesis. For instance in the pilot studies, capillary blood was used to measure glucose and total cholesterol. Although the use of either capillary blood or venous blood does not pose any issues in relation to total cholesterol (Warnick *et al.*, 1994; Sblendorio *et al.*, 2008), differences between capillary whole blood glucose and venous blood glucose are known to exit (Stahl *et al.*, 2002; Colagiuri *et al.*, 2003; Boyd *et al.*, 2005). For instance, Colagiuri *et al.* (2003) has shown that fasting glucose concentrations can be higher if measured using venous plasma compared to capillary blood whereas 2h post glucose tolerance test glucose levels are lower when measured using venous plasma as opposed to capillary blood. Consequently, although a good correlation may exists between capillary blood glucose and venous blood (Boyd *et al.*, 2005), caution needs to be exerted when comparing the results from the DC pilot study to the main DC study and other studies that used venous blood for measuring glucose.

Additionally, a number of problems were observed with the study design. For instance, baseline data were only obtained once in the pilot studies, thus failing to take in account day-to-day fluctuations in glucose, cholesterol, BP and urinary glucocorticoids levels. This proved to be particularly problematic in relation to BP, as certain subjects had low baseline BP and were therefore expected to increase regardless of the intervention. Monitoring BP once during each treatment period also posed numerous difficulties as it only reflected BP levels at a particular period during the day, rather than the entire intervention period. The latter could also explain why Taubert *et al.* (2003) and Grassi *et al.* (2005a-b) observed greater reductions in BP, as they used more reliable methods, including daily BP monitoring and 24-hour ambulatory sphygmomanometers.

The lack of control or appropriate placebo in the pilot GCBE study also implies that no direct conclusions could be drawn from this intervention and that further studies using a placebo-controlled design or a parallel-group design are needed to confirm the results from the GCBE trial. As indicated in section 1.13.3, an open-label single-subject experimental AB design is only ideal for feasibility studies that can then direct future research and justify the need for more rigorous research designs. As such, conducting a bioavailability study using several DC and GCBE doses would have been probably more useful for calculating doses and designing dosage regimens. Moreover, conducting a bioavailability study would have permitted examining the pharmacokinetics of epicatechin and CGA as well as assessing

for a correlation between the different metabolites and plasma oxidative capacity. This, however, was not feasible.

Another limitation to the current studies is the use of free-living volunteers whose diet was not closely controlled. Although all volunteers were instructed to limit the intake of polyphenol-rich products and to consume a similar diet on the days prior to undertaking the measurements, large intra and inter-individual variations in urinary polyphenol excretion were seen in both the GCBE and DC interventions. This was further exacerbated by the use of FC and FRAP assays for estimating polyphenols levels in plasma or urine as a method of measuring compliance. This is because both these assays lack specificity and the presence of any reducing compound could interfere with the readings produced by these tests. As a result given adequate resources, it would have been best to quantify polyphenols in urine using HPLC or LC-MS. Apart from permitting more precise monitoring of compliance, these methods can enable accurate measurement of the recovery of polyphenols which can in turn assist in studying inter-subject differences in polyphenol metabolism.

In relation to the main study, a crossover design was used. As discussed earlier, cross-over studies are generally designed to measure the efficacy of a treatment under controlled conditions while a parallel-group design measures a treatment's effectiveness under less astringent conditions. As a result, the efficacy of a drug does not necessarily determine its effectiveness and broader long-term studies are needed to confirm the effectiveness of an intervention (Pittler and White, 1999). This suggests that regardless of the efficiency of cross-over trials, the use of parallel-design studies remains indispensable for confirming the findings of cross-over trials and ensuring their generalisation (Sever *et al.*, 1989, Cleophas and Tavenier, 1995). Cross-over designs are also susceptible to carry-over effects (Cleophas, 1993) and as such are likely to underestimate the effect of treatment (Cleophas, 1990). There are also issues with the use of placebo for blinding or for controlling for macronutrient and micronutrient differences between the active treatment period and non-active treatment period. This is because differences between treatment and placebo even if significant do not essentially imply clinical relevance (Cleophas, 1995). In respect to the latter much remains to be understood with regard to the effect of the pattern of DC consumption on health. This is because the therapeutic value of DC can be limited by the short-half lives and rapid elimination of DC polyphenols. Hence the extent to which DC-induced improvements in fasting glucose and BP are maintained beyond 12h following DC withdrawal need to be examined. A key question that remains to be elucidated is whether

non daily intermittent consumption of DC is of any therapeutical value to overweight and obesity?

The use of Tanita scales for monitoring body composition also acted as a limitation. Although the manufacturer claims that the readings obtained using these scales for percentage body fat are within 5% of the gold standard, DEXA method (Tanita, 2009), Lazzer and colleagues (2003) have argued that foot-to-foot bioelectrical impedance and DEXA are two non-interchangeable methods and that despite showing great limits of agreement, Tanita scales can often underestimate fat mass. Most importantly the use of foot-to-foot bioelectrical impedance shows large inter-subject variability in fat mass estimates (Lazzer *et al.*, 2003). Subsequently, future research aimed at investigating the effect of GCBE on body composition should focus on using more robust techniques for measuring body composition. This is particularly important when considering some of the changes in anthropometrical indices seen in the preliminary GCBE and the main DC study.

4.7. Implications

The ability of GCBE to reduce WC in individuals with BMI $\geq$25kg/m^2 while simultaneously increasing salivary cortisone concentrations and salivary cortisone-to-cortisol ratio has important implications to the prevention of obesity-related health risks and the treatment of obesity. This is because WC is an important predictor of excess adiposity (Katzmarzyk *et al.*, 2006), obesity-related complications (Zhu *et al.*, 2002b; Janssen *et al.*, 2004) and all-cause mortality (Visscher *et al.*, 2001; Bigaard *et al.*, 2005; Katzmarzyk *et al.*, 2006) whilst excessive cortisol regeneration by 11βHSD1 as indicated by a lower salivary cortisone-to-cortisol ratio is known to be associated with abdominal fat accumulation, insulin resistance, hypertension and dyslipidaemia. Conversely a smaller WC and inhibition of 11βHSD1 are often linked to improved metabolic profile and reduced metabolic risk factors. This could be further reinforced by the findings from the main DC study wherein urinary cortisone correlated inversely with several parameters of the metabolic syndrome including LDL, TC:HDL, SBP and WHR while a positive correlation was observed between urinary cortisone, HDL and serum and urinary antioxidant capacities (refer to Table 3.13). As such, increasing polyphenol intake in the overweight and obese population in addition to conferring antioxidant protection against ROS might be pivotal to reducing metabolic risk factors in this population via modulation of WC and glucocorticoid metabolism.

Currently, most strategies aimed at reducing WC focus on caloric restriction and increasing physical activity (Dumesnil *et al.*, 2001; Koh-Banerjee *et al.*, 2003; Mayo *et al.*, 2003; Valsamakis *et al.*, 2004). These strategies do not produce selective reduction in WC and any changes in WC generally result from an overall drop in body weight. This is evident in that exercise although capable of evoking large reductions in weight which are preferentially linked to decreased WC (Mayo *et al.*, 2003), is still not proven to produce a dose-dependent decrement in WC (Ross and Janssen, 2001). In contrast, if dietary factors such as polyphenols are proven to selectively diminish WC and improve body composition independently of any effect on weight, then their use in weight loss programmes might confer additional health benefits. In this respect, more recent data suggest that certain macronutrients have the capacity to modulate WC, in the absence of caloric restriction. Accordingly, an increment of 12g in fibre intake has been reported to be associated with a 0.63cm reduction in WC while the intake of trans fats (2% of total energy intake) is linked to a 0.77-cm waist gain over 9 years (Koh-Banerjee *et al.*, 2003). Although the mechanism of such an effect has not been explored so far, it is likely to involve inhibition of 11βHSD1. Support for such concept arises from the preliminary findings of this thesis and from observations that, in women, cortisone is inversely related to WC (Cooper *et al*., 2004).

It is important to note that consumption of GCBE in humans and of CGA in mice reduced cortisone levels in urine and the kidney, respectively. Hypothetically such changes in cortisone concentrations could be reflective of 11βHSD2 inhibition. Inhibition of 11βHSD2 is known to be associated with increased kidney exposure to the mineralcorticoid effects of cortisol which causes hypertension. However, since no rise in BP was observed in this study, it is difficult to elaborate on the implications of such tissue-specific effects of GCBE and CGA. Alternatively it could be speculated that inhibition of 11βHSD2 in the kidney will reduce the availability of the cortisone substrate, thereby abrogating hepatic 11βHSD1's ability to generate cortisol from cortisone (Seckl and Walker, 2001) (see section 1.8.2 and Figure 1.11; p. 47).

As for the DC interventions, the hypoglycaemic and insulin sensitising properties of polyphenol-rich DC can be of relevance to conditions of impaired glycaemic control such as glucose intolerance and diabetes wherein the loss of glucose homeostasis is reflected in increased risk of vascular diseases. In relation to diabetes, consistent evidence exists from animal research using different models of diabetes suggesting a potential role for cocoa in improving short-term and long-term glycaemic response (Ruzaidi *et al.*, 2005, Tomaru *et al.*,

2007, Jalil *et al.*, 2008), diabetes-related cardiometabolic risk factors such as dyslipidaemia (Ruzaidi *et al.*, 2005, Jalil *et al.*, 2008) as well as diabetes-induced complications like cataracts (Osakabe *et al.*, 2004). Only two studies have so far examined the effect of chocolate or cocoa consumption on glycaemic control in diabetics. The first study was conducted on diabetic adolescents. It demonstrated that milk chocolate yields a lower glucose peak and glucose AUC than a conventional diabetes snack (Cedermark *et al.*, 1993). The second study that was carried out on medicated type-II diabetics showed that daily intake of cocoa containing 963mg flavanols reversed vascular dysfunction in this population without adversely affecting their glycaemic control (Balzer *et al.*, 2008). Benefits to glucose-intolerant hypertensives have also been reported (Grassi *et al.*, 2008). In light of this evidence and the findings of the present study, it appears that further research is warranted in this field especially using sugar-free DC or cocoa, in which the effect of polyphenols would not be attenuated by the sugar content.

The clinical importance of the hypotensive properties of DC can be viewed in epidemiological studies demonstrating that a 3.7mmHg decrement in SBP is linked to a 15-year reduction in CVD and mortality (Buijsse *et al.*, 2006) whereas, a 2mmHg reduction in DBP reduces the prevalence of hypertension by 17% and the risk of coronary artery disease and stroke by 6% and 15%, respectively (Cook *et al.* 1995). Moreover, essential hypertension forms 90% of all hypertension cases (Kumar and Clarke, 2005), with weight gain being the most common cause of this disease (Fletcher *et al.*, 1999). Given the inadequacy of pharmacological strategies in such cases, the addition of food with hypotensive properties such as DC could help prevent or reduce the occurrence of hypertension in overweight individuals. Such hypothesis is reinforced by the fact that chocolate decreases BP even in populations with high sodium intakes (McCullough *et al.*, 2006).

Conversely, the lack of significant improvements in lipid profile in the main DC study, albeit disappointing, highlights an important observation and that is including a moderate portion of DC as part of a balanced diet does not adversely affect lipid metabolism, regardless of the fat content of DC. However, long-term studies will be required to monitor the effect of long-term consumption of DC on body composition and weight gain.

To our knowledge, the findings of increased creatine kinase following 4-week consumption of polyphenol-rich DC but not placebo have not been reported by other studies. Although no

explanation could be provided at present, it is possible to speculate that because of the nature of polyphenols as plant toxins (Duthie *et al.*, 2003); it is likely that the liver in its attempt to metabolise and eliminate these products raises serum creatine kinase. However, skeletal muscle can also account for the raised serum creatine kinase (Apple *et al.*, 1985) alongside other high energy demand tissues wherein creatine kinase helps regulate energy metabolism (Wallimann *et al.*, 1992). Consequently, the cause of the raised creatine kinase values and the implications of such rise should be investigated further and the major organ contributing to this effect identified.

4.8. Directions for future research

Data from *in vitro* antioxidant studies highlight the need for examining the contribution of various food products to polyphenol intake in the UK. There is also a need for identifying major dietary sources of polyphenols in the average UK adult diet as these can reflect focal points for designing appropriate dietary strategies aimed at reducing the prevalence of chronic diseases. Additionally, the *in vitro* studies revealed that GCBE possesses a strong antioxidant capacity and although no significant increase in urinary total phenolics was observed in the preliminary GCBE study, further research on the effect of GCBE on plasma antioxidant capacity is warranted. Assessing the pharmacokinetics of CGA from GCBE and the impact of food matrix on the bioavailability of CGA from GCBE is also deserved as so far only one study has examined the bioavailability and metabolism of CGA from GCBE. Understanding the bioavailability of CGA and its metabolites is important in determining *in vivo* antioxidant properties of GCBE as well as in clarifying the contribution of each of CGA or its metabolites to the hypotensive properties of GCBE noted in the preliminary GCBE study. The pros and cons of supplementing coffee formulations with GCBE as suggested by Oka (2007) need to be evaluated as well. Similarly, the advantages of incorporating TCBE in the diet and identifying various nutritional application of this bean in addition to the well-known uses in cocoa powder and chocolate production merits further exploration.

Based on the pancreatic lipase experiment, attention should also be drawn to the potential role of GCBE in modulating the activity of pancreatic lipase and other lipases in humans and the possibility of improved lipid metabolism as a consequence of such actions. However, more importantly, a large randomised placebo-controlled trial is warranted to investigate the effect of GCBE on body composition and glucocorticoid metabolism. It is well established that inhibition of 11βHSD1 lies at the centre of the current therapeutic targets aimed at

treating obesity and the metabolic syndrome (Wake and Walker, 2004; Anagnostis *et al.*, 2009) and in recent years several selective 11βHSD1 inhibitors have been developed (Wake and Walker, 2004; Anagnostis *et al.*, 2009). However, the role of diet in modulating glucocorticoid metabolism remains unclear. Similarly, in respect to the aetiology of HPA axis hyperactivity in obesity and the metabolic syndrome, although a role for chronic stress and low birth weight have been speculated (Wake *et al.*, 2003), involvement of dietary factors have not been investigated. In connection with diet, some data exists to suggest that dietary macronutrients can influence glucocorticoid metabolism in obesity independently of weight loss (Stimson *et al.*, 2007). As such the role of dietary macronutrients and polyphenols in the aetiology and/or treatment of HPA hyperactivity emerge as an interesting field of future research. In particular structure-activity relations should be clarified. If polyphenols are indeed proven to influence glucocorticoid metabolism, then their effect on growth hormone and testosterone will need to be examined as well. This is because both these hormones are known to oppose the actions of cortisol on TG accumulation (see Bjorntorp,1998). Moreover, administration of testosterone is reported to reduce fasting glucose, insulin resistance, total cholesterol and WC (Kapoor *et al.*, 2006). For future studies, the use of decaffeinated GCBE such as Le Svetol® is recommended as to exclude any potential interference from the caffeine component of GCBE (Dellalibera *et al.*, 2006).

In relation to DC, it is important to note that geographical origin can influence the composition of cocoa phenolics resulting in certain varieties of cocoa to contain up to 4 times more polyphenols than other varieties. Natsume *et al.* (2000) has even demonstrated that although the ratio of the various procyanidin components might not vary, the amount of procyanidins is markedly influenced by geographical origin. The concentration of epicatechin and catechin monomers is also affected by geographical origin. These differences could in turn account for some of the inconsistencies in the findings between different researchers. As a result, it might be good practice in future for studies to state the origin of the cocoa beans used in their trials in an attempt to identify the factors that determine cocoa's and DC's health properties.

It remains plausible that some of the health-promoting properties of cacao polyphenols may be concealed in the long-term by the energy density and high-fat content of DC (Desch *et al.*, 2009) and that, as a result, consumption of cocoa powder may incur greater benefits to overweight and obese individuals than ingestion of polyphenol-rich DC. In this respect, it remains to be clarified whether minor differences in the polyphenol composition between

cocoa and DC products affect their biochemical properties, particularly that, to date, studies that have used polyphenol-rich cocoa as an alternative to DC have produced conflicting results on glycaemic control and BP.

In addition to polyphenols, DC and cocoa also contain high concentrations of methylxanthines such as theobromine, caffeine, theophylline and paraxanthines (Eteng and Ettarah, 2000) and minerals like magnesium. Theobromine, in particular, has been shown to improve lipid profile in rats (Eteng and Ettarah, 2000). This may suggest that inconsistencies in the findings relating to lipid profile could be attributed to difference in theobromine content of the various DC and cocoa products used in research, since most previous studies have not controlled for theobromine content. However, so far, little data exists concerning the contribution of methylxanthins to DC-induced improvements in lipid profile. Since, theobromine and theophylline have half-lives of 6.2 and 7.2h, respectively (Lelo *et al*., 1986), which are considerably longer than the half-lives of epicatechin, the role of these compounds in cocoa and DC's hypolipidaemic, hypotensive and hypoglycaemic properties remains to be examined.

As for minerals, it is well known that 100g DC can provide up to 300mg magnesium which is equivalent to 100% of recommended daily allowance of magnesium (Meisel, 2005). As a result, previous studies demonstrating beneficial effects of polyphenol-rich DC consumption have been criticised for not controlling for magnesium intake (Meisel, 2005). This is because previous researchers compared the effects of polyphenol- and magnesium-rich DC to a white chocolate placebo which is deficient in both polyphenols and magnesium (Grassi *et al.*, 2005a-b; 2008). Consequently, the beneficial properties of polyphenol-rich DC might have been exaggerated. The present study has the advantage of using DC placebo which contained a similar magnesium content as the polyphenol-rich DC. Consequently, any hypotensive or hypoglycaemic properties could be directly attributed to the polyphenols in DC and not magnesium. Nonetheless, it remains to be clarified whether combining high polyphenol intake with a diet rich in magnesium could produce an accumulative or synergetic effect on BP and glucose levels (Meisel, 2005).

The results of the main study in conjunction with the afore-mentioned *in vitro* and preliminary studies also highlight one major gap in the current scientific literature and that is the scarcity of studies that estimate total polyphenol intake in the general population and that assess differences in polyphenol intake amongst the different population subgroups.

Furthermore, it remains unclear whether there exists differences in the polyphenol intake and plasma antioxidant status between overweight and obese individuals and lean individuals which then predisposes the former group to oxidative stress. Such investigations will be focal to identifying the pathophysiological link between oxidative stress and systemic inflammation in obesity since polyphenols in addition to possessing antioxidant properties, have the capacity to modulate several biomarkers of inflammation. Indeed, emerging evidence suggests a central role for oxidative stress in the pathophysiology of chronic conditions like obesity, diabetes, hypertension and CVD while demonstrating that increased antioxidant intake can improve antioxidant status while reducing complications and mortality due to these conditions (Gey *et al.*, 1993; Sundaram *et al.*, 1996; Lopes *et al.*, 2003; Maxwell *et al.*, 2003; Kashyap *et al.*, 2005; Crujeira *et al.*, 2006). Overweight and obese individuals, in particular, exhibit a low antioxidant status and dysfunctional antioxidant defence mechanism as observed in reduced plasma α-tocopherol and β-carotene levels and diminished superoxide dismutase and glutathione peroxidase activity (Beltowski *et al.*, 2000; Ozata *et al.*, 2002; Molnar *et al.*, 2004) making it a high priority to examine the polyphenol intake of this population subgroup. However, according to Molnar *et al.* (2004), obese individuals are not likely to consume lower quantities of antioxidants; neither are they more predisposed to having defects in the absorption of antioxidants compared to lean individuals. Nonetheless and as discussed throughout this thesis and suggested by Molnar *et al.* (2004), the presence of hypertriglyceridaemia, hyperinsulinaemia and subsequent oxidative stress are likely to exhaust the antioxidant reserves in overweight and obese individuals leading to lower antioxidant availability and a higher demand for antioxidants to preserve redox balance in these individuals. This once again confirms the need for maintaining adequate polyphenol and micronutrient intake in the overweight and obese population while possibly reducing the intake of pro-oxidant food products such as fat.

5. General conclusion

This thesis embarked on exploring the potential role of polyphenols in preventing overweight and obesity-related complications. It supports the hypothesis that polyphenols from GCBE and DC can differentially improve glucose and BP levels in overweight and obese individuals without significantly affecting lipid profile. It also provides insight into novel mechanistic pathways by which polyphenols can potentially protect against obesity and its associated complications and which involve inhibition of pancreatic lipase and possibly 11βHSD1.

The following points outline the general conclusions and implications of this thesis:

1. GCBE and TCBE are rich in polyphenols and possess strong antioxidant capacities. These products could act as focal points of intervention as both DC and coffee are widely consumed on a daily basis in the UK.
2. GCBE, but not TCBE, can inhibit pancreatic lipase dose-dependently *in vitro*. This is of particular relevance to overweight and obesity since reducing fat absorption constitutes a key target for counteracting overweight and obesity and their associated co-morbidities.
3. In healthy overweight and obese individuals, short-term consumption of GCBE can improve BP and glucocorticoid regulation. This effect is likely to be mediated via the differential effect of CGA on tissue corticosterone concentrations as demonstrated by the animal study.
4. Short-term consumption of 20g DC containing 500mg or 1000mg polyphenols reduces fasting glucose and BP with both 500mg and 1000mg polyphenol DC yielding a similar effect on fasting glucose and BP.
5. Long-term ingestion of 20g DC containing 500mg polyphenols improves glucoregulatory biomarkers and BP without significantly affecting lipid profile. These metabolic effects appear to be more prominent in overweight and obese females than lean females and could be related in part to improved antioxidant capacity. The role of glucocorticoids as one of the underlying biochemical pathways by which polyphenol-rich DC exerts its beneficial metabolic effects remains to be clarified.
6. Polyphenol-poor DC has the potential to adversely affect insulin, cortisol and body weight regulation in overweight and obese females.

Key research areas to be considered in future in relation to overweight and obesity include:

1. Investigating *in vivo* effect of polyphenols, in particular GCBE, on fat absorption. Herein, the effect of polyphenols on pancreatic lipase could be examined. Measurement of time-course changes in total cholesterol or TG following acute consumption of a high-fat meal could also be used to assess the effect of GCBE on fat absorption.
2. Identifying and examining the effect of polyphenols on 11βHSD1 activity. To achieve this, a series of *in vitro*, animal and human studies can be conducted. I*n vitro,* inhibition of 11βHSD1 by polyphenols could be assessed in hepatic microsomes. In animals, administration of a diet rich in a specific phenolic compound or a polyphenol-rich product followed by cortisone injection would permit accurate measurement of cortisone conversion to cortisol, hence 11βHSD1 activity. The use of mice strains such as KKA (y) or *ob/ob* mice is recommended. In humans, application of HPLC for accurate quantification of cortisol and cortisone metabolites is recommended. This will enable identification of selective changes in the activity of the enzymes that regulate cortisol metabolism including 11βHSD1, 11βHSD2 and α-reductase following consumption of a polyphenol-rich product such as GCBE.
3. Investigating the effect of polyphenols on thermogenesis, substrate oxidation and lipolysis. This could be attained using indirect colorimetry in combination with measurements of NEFA and glycerol. Quantification of TG in cultured adipocytes would also permit estimation of lipolytic activity. Lipoprotein lipase and hormone-sensitive lipase activity could also be estimated by incubating polyphenol extracts with isolated adipocytes and subsequently measuring NEFA and glycerol.
4. A potential role for polyphenols in regulating appetite and energy intake also warrants investigation.

It remains to cite Duthie *et al.* (2003) that although deficiencies in polyphenol intake are not accompanied with any apparent symptoms of deficiency as is the case with the more traditional antioxidants, their ability to influence and prevent disease processes highlights the importance of ensuring adequate intake of these antioxidants amongst high risk populations such as the overweight and obese population. Moreover, the fact that certain classes of polyphenols possess the potential to counteract some of the underlying endocrinological abnormalities characteristic of overweight and obesity provides novel insights into the relation between diet and health. With the ever-increasing global burden of the overweight

and obesity epidemic, the need to prevent overweight and obesity-related chronic conditions through appropriate dietary strategies and public health strategies is becoming ever-more important. However, the diversity and complexity of these compounds implies that much remains to be elucidated concerning the mechanisms by which these compounds influence health.

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7. Appendices

7.1. Appendix 1 Precision and accuracy of assays

7.1.1 Validation of Folin-Ciocalteu method

7.1.1.a Standard curve for determination of total phenolic content

Linear regression produced a calibration curve of Y =0.0343x + 0.0422, r^2= 0.9975, where Y reflects abs in nm and X the concentration of GA.

7.1.1.b Assay precision and accuracy

Assay precision was determined replicate analysis (n=4) of six known concentrations of GA (Roura *et al.*, 2006). The limit of detection (LoD) and limit of quantification (LoQ) for the assay were 0.003 and 0.011mM, respectively.

Table 7.1 Evaluation of precision and accuracy of Folin-Ciocalteu method

Calibrator concentration mg/l	Mean measured concentrations mg/l n(4)	Precision, RSD[1] (%)	Predicted concentration (%)
5	4.236152	4.248305	87.34694
10	9.877551	1.476215	97.7551
20	20.6793	3.420414	100.9548
30	31.24781	1.339651	105.1555
40	40.57726	2.166507	99.91254
100	49.13411	0.168029	98.1516

RSD[1], relative standard deviation

7.1.2 Validation of ferric-reducing capacity of plasma method

7.1.2.a Standard curve for determination of ferric-reducing capacity of plasma

Linear regression produced a calibration curve of Y =0.0343x + 0.0422, r^2= 0.9977, where Y reflects abs in nm and X the concentration of Fe^{2+}.

7.1.2.a Assay precision and accuracy

Assay precision was determined by replicate analysis (n=4) of five known concentrations of ferrous sulphate standards. The limit of detection (LoD) and limit of quantification (LoQ) for the assay were 0.003 and 0.011mM, respectively.

Table 7.2 Evaluation of precision and accuracy of ferric-reducing capacity of plasma assay

Calibrator concentration mg/l	Mean measured concentrations mg/l n(4)	Precision, RSD[1] (%)	Predicted concentration (%)
1	1.101156	2.677926	110.1156
2	1.973988	5.197011	98.69942
4	3.971098	2.301195	99.27746
6	6.306358	2.433795	105.106
8	8.00289	1.839638	100.0361

RSD[1], relative standard deviation

7.1.3 Validation of oxygen radical absorbance capacity method

7.1.3.a Standard curve for determination of ORAC

Linear regression produced a calibration curve of Y =0.190x + 1.233, r2= 0.996, where Y reflects abs in nm and X the concentration of trolox.

7.1.3.b Assay precision

Assay precision was determined by replicate analysis (n=4) of five known concentrations of Trolox standards. The limit of detection (LoD) and limit of quantification (LoQ) for the assay were 1.142 and 3.807 μM trolox equivalents, respectively.

Table 7.3 Evaluation of precision and accuracy of oxygen radical absorbance capacity method

Calibrator concentration mg/l	Mean measured concentrations mg/l n(4)	Precision, RSD[1] (%)	Predicted concentration (%)
6.25	6.648198	2.573648	106.3712
12.5	13.70795	1.853424	109.6636
25	26.66962	0.463117	106.6785
50	48.81343	0.21282	97.62685
100	87.52354	0.21869	87.52354

RSD[1], relative standard deviation

7.1.4 Validation of 2,2-diphenyl-1-picrylhydrazyl radical

7.1.5 Standard curve for determination of 2,2-diphenyl-1-picrylhydrazyl radical

Linear regression produced a calibration curve of Y =0.0048x + 0.0454, r^2= 0.9988, where Y reflects abs in nm and X the concentration of DPPH in medium.

7.1.5.a Assay precision

Assay precision was determined by replicate analysis (n=4) of five known concentrations of DPPH. The limit of detection (LoD) and limit of quantification (LoQ) for the assay were 0.003 and 0.011mM, respectively.

Table 7.4 Evaluation of precision and accuracy of 2,2-diphenyl-1-picrylhydrazyl radical assay

Calibrator concentration mg/l	Mean measured concentrations mg/l n(4)	Precision, RSD[1] (%)	Predicted concentration (%)
5	4.395833	0.113744	87.91667
10	11.16667	0.044776	111.6667
20	20.85417	0.028771	104.2708
40	40.22917	0.027343	100.5729
60	59.70833	0.021773	99.51389

RSD[1], relative standard deviation

7.1.6 Cortisol ELISA validation data

Cross-reactivity was not that significant with many interfering steroids: Cortisone=o.68%, Corticosterone= 0.06%, Deoxy-cortisol= 1.1%, Prednisolone=24%, Testosterone= 0.4%, other steroids were < 0.5%.

Intra-assay precision ranged from 3.65% to 6.12%, and Inter-assay precision data ranged from 4.74% to 8.66%.

Recovery studies for a range of cortisol levels from 2.6 – 40.8 ng/ml were 89.8% to 107.7%.

Sensitivity of the assay defined as the minimum detection limit was 0.1 ng/ml.(0.276 nmol/L)

7.1.7 Cortisone ELISA validation data

Cross reactivity of our affinity purified cortisone antibody with cortisol was reduced to 0.25% (see Table).

The validity of cortisone ELISA was confirmed by the good correlation obtained before and after an HPLC fractionation step (Y= 1.09X - 0.21, R^2=0.98).

Intra and Inter-assay imprecision were 5.5 – 11.7% and 8.7 – 12.8% CV respectively.

Minimum detection limit of cortisone ELISA was 28 pg/mL (77.7pmole/L)

Table 7.5: Cross-reactivity data for purified Anti-Cortisone antibody

Steroid	% Cross-reactivity
Cortisone	100
Cortisol	0.252
11-Deoxycortisol	0.105
Aldosterone	0.011
17α OH-Progesterone	0.014
Progesterone	0.0002
Deoxycorticosterone	0.006
Corticosterone	0.0025
Testosterone	0.0005
DHEA	0.0004
DHEA sulphate	0.0002
Androstenedione	0.006
18-OH-deoxycorticosterone	0.0002
5β dihydroaldosterone	0.0003
5 β Tetrahydrocorticosterone	0.0004
Dihydropregnenolone	0.0005
5α Dihydroaldosterone	0.0003
Tetrahydrodeoxycorticosterone	0.0003
5 β Dihydrocortisol	0.004
5α Dihydrocorticosterone	0.002
Tetrahydroaldosterone	0.0005

7.1.8 Assays accuracy and precision

7.1.8.a Glucose Liquid (Sentinel Diagnostics, UK)

Measuring range: 2 - 400 mg/dL.
Intra-Assay Precision: 338.2 mg/dL, SD 3.30, CV% 0.98.
Inter-Assay Precision:

	Mean	Within Run		Run to Run		Total	Total
	mg/dl	SD	CV%	SD	CV%	SD	CV%
L1	102	1.10	1.07	2.11	2.07	2.38	2.33
L2	198	1.26	0.64	1.44	0.73	1.92	0.97
L3	245	2.25	0.92	1.96	0.80	2.98	1.22

Sensitivity: 2 mg/dL.
Accuracy: N = 60, r = 0.99908, y = 0.9883 x - 0.30816

7.1.8.b Insulin (Mercodia, Sweden)

Sensitivity Detection limit is 1 mU/l (6 pmol/l) calculated as two standard deviations above Calibrator 0.
Recovery Recovery upon addition is 94-113% (mean 104%).
Precision Each sample was analyzed in six replicates on six different occasions.

Sample	Obtained value (mU/l)	CV		
		within assay %	Between assay%	Total assay%
1	11	3.4	3.6	5.0
2	36	4.0	2.6	4.7
3	80	2.8	2.8	4.0
4	154	3.2	2.9	4.4

7.1.8.c NEFA (Wako Chemicals, Neuss, Germany)

Accuracy ± 15% of the known concentration.

Sensitivity When purified water is assayed, the absorbance is not more than 0.140. When a standard of given concentration (oleic acid 1 mEq/L) is assayed, the absorbance is 0.100 - 0.380.
Precision When a sample is assayed not less than 5 times in a run, CV of absorbance is not more than 1.5%.
Measurable range 0.01 - 4.00 mEq/L NEFA. (In the case of using the standard procedure)

7.2. Appendix 2 Consent form and information sheets

Queen Margaret University
EDINBURGH

QMUC Research Ethics Sub-Committee
Standard Consent Form

Effect of polyphenols on glucoregulatory biomarkers, BP and lipid profile in overweight and obese subjects

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I agree to participate in this study.
I have read and understood the subject information sheet and this consent form. I have had an opportunity to ask questions about my participation.
I understand that I am under no obligation to take part in this study.
I understand that I have the right to withdraw from this study at any stage for no reason.

Name of subject: _____
Signature of subject: _____
Signature of investigator: _____
Date: _____

Further information is available from:

Name of Investigator: Suzana Almoosawi
Address: Postgraduate student
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Queen Margaret University
EDINBURGH

Information sheet for potential subjects

Effect of polyphenols on glucoregulatory biomarkers, BP and lipid profile in overweight and obese subjects (Pilot study)

My name is Suzana Almoosawi and I am Public Health Nutrition PhD student currently undertaking my research project in the Department of Dietetics, Nutrition & Biological Sciences at Queen Margaret University. In view of the current interest in the protective role of dark chocolate and green coffee extract against cardiovascular disease, I wish to carry out my research project in this area. More precisely my research will examine the effect of green coffee extract intake on fasting glucose, blood pressure, total cholesterol and urinary mineral, cortisol and cortisone excretion in overweight and obese people.

Green coffee extract are rich sources of antioxidants, known as polyphenols. It has been shown to protect against hypertension and weight-gain. Moreover, it possesses the potential to reduce risk of diabetes because it contains high levels of a polyphenol known as chlorogenic acid. Though current manufacturing processes destroy much of coffee beans' antioxidants, a number of polyphenol-rich green coffee extracts are still available on the market, whose properties deserve to be investigated. Indeed, if positive health attributes are to be found in these products, then their role in the primary prevention of hypertension, diabetes and cardiovascular diseases could be significant. Such findings will be of particular relevance to populations at risk of these diseases, such as the overweight population.

If you wish to take part in this study, you have to be healthy with a body mass index equal to or above 25, non-smoker, not currently taking any blood pressure or cholesterol-lowering drugs. You should also have a normal physical activity level and your intake of cocoa, dark chocolate, green tea and coffee should be low.

The study will consist of a baseline week + 2 weeks intervention. You will have to be available on two days two weeks apart for an average of 15 minutes (see, timetable attached at the end of this document).

You will be asked to follow your usual diet throughout the study, but to avoid consuming polyphenol-rich beverages and foods. You will find a list of these products and a list of alternative products that you can consume at the back of this document (see Table-7.6). During the week before the intervention, you will be asked to avoid eating any type of chocolate, chocolate products and coffee. During this baseline week you will also be required to complete a 3-day diet diary (two weekdays & one weekend), a physical activity questionnaire, a 24-urine collection (see timetable at the end of this document) and 3 saliva collections.

Following the baseline week, you will be assigned to receive a green coffee bean extract. You will be asked to follow the diet for 14days. You will be provided with FREE Quest's green coffee extract. The doses will consist of 2 tablets of green coffee extract; one capsule to be taken in the morning between 8-10am with breakfast or preferably 200ml water and white bread and another capsule to be taken with your evening meal or preferably with 200ml water and white bread between 8-10pm. During the intervention week you will also

be asked to complete another diet diary and physical activity questionnaire, in addition to completing a 24-urine collection and 3 saliva samples at the end of 14 days period.

The following measurements will also be assessed during the study: height, weight, body mass index, waist and hip circumferences, blood pressure, fasting glucose, total cholesterol, urinary magnesium, sodium, potassium, cortisol and cortisone (see, timetable attached at the end of this document).

Before, examining your blood pressure, you will be asked to sit comfortably for 15 minutes. Your blood pressure will then be measured three times with 5 minutes interval using an electronic sphygmomanometer. This should therefore take approximately 15 minutes. The 12-hour fasting glucose and cholesterol measurements will be obtained by finger-prick blood samples. This procedure should not cause any distress as finger prick samples are obtained quickly.

You will be free to withdraw from the study at any stage for any reason. All data collected will remain anonymous and your name will be replaced with an identification number that could only be identified by the researcher.

If you would like to consult a person who is not involved in this research for independent advice, please contact Dr Jane McKenzie. Her contact details are provided below.

If you have any further queries concerning this research, please do not hesitate to ask. Alternatively, if you have read and understood the information provided in this document and you would like to participate in this study, please sign the attached consent form.

Contact Details of the investigator

Name of Investigator: Suzana Almoosawi
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Table 7.6 List of polyphenol-rich foods and beverages to be avoided by the GCBE group (adapted from Clifford, 1999)

Foods rich in polyphenols (chlorogenic acids)	Chlorogenic acid content (mg) per kg food		Alternatives
	Total CGAs	Caffeoylquinic acids (CQA)*	
Apples, raw (per kg)	30-60	62-385	
Artichoke	-	180-450	
Aubergines	-	600	
Blackberries	70	-	Raspberries Strawberries Redcurrants gooseberries
Blackcurrants	140	-	
Blueberries	-	500-2000	
Cherries	-	150-600	
Pears, raw	-	60-280	
Beverages rich in polyphenols (chlorogenic acids)	**Total chlorogenic acids or caffeoyl-quinic acid content (mg) per litre beverage or per 200ml coffee**		
	Total CGAs	Caffeoylquinic acids (CQA)	
Apple juice	-	Up to 208	
Bilbbery wine	50	-	
Cider	-	11-480	
Coffee			
Instant brew	-	50-150	
Weak brew, very dark roast	20	-	
Strong brew, very pale roast robusta	675	-	
Arabica	70-200	-	
Robusta	70-300	-	
Pear juice	-	240	
Tea Green			

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My name is Suzana Almoosawi and I am Public Health Nutrition PhD student currently undertaking my research project in the Department of Dietetics, Nutrition & Biological Sciences at Queen Margaret University. In view of the current interest in the protective role of dark chocolate against cardiovascular disease, I wish to carry out my research project in this area. More precisely my research will examine the effect of dark chocolate on fasting glucose, blood pressure, total cholesterol and urinary mineral, cortisol and cortisone excretion in overweight and obese people.

Dark chocolate is a rich source of antioxidants, known as polyphenols. Recently, a number of studies have indicated that polyphenol-rich dark chocolate could reduce the risk of hypertension, diabetes and coronary artery disease. Though current manufacturing processes destroy much of chocolate's antioxidants, some polyphenol-rich chocolate is still available on the market, whose properties deserve to be investigated. Indeed, if positive health attributes are to be found in these products, then their role in the primary prevention of hypertension, diabetes and cardiovascular diseases could be significant. Such findings will be of particular relevance to populations at risk of these diseases, such as the overweight population.
If you wish to take part in this study, you have to be healthy with a body mass index equal to or above 25, non-smoker, not currently taking any blood pressure or cholesterol-lowering drugs. You should also have a normal physical activity level and your intake of cocoa, dark chocolate and green tea should be low.

The study will last approximately 6 weeks. You will have to be available for an average of 15 minutes once every week, except for Week-3 where no measurements will be taken (see, timetable attached at the end of this document).

You will be asked to follow your usual diet throughout the study, but to avoid consuming polyphenol-rich beverages and foods. You will find a list of these products and a list of alternative products that you can consume at the back of this document (see Table-7.7). During the week before the intervention, you will be asked to avoid eating any type of chocolate and chocolate products. During this baseline week you will also be required to complete a 3-day diet diary (two weekdays & one weekend), a physical activity questionnaire and a 24-urine collection (see timetable at the end of this document).

On Week-0, you will be randomly assigned to one of the two intervention groups: low dark chocolate or high dark chocolate. You will be asked to follow each diet for 14days followed by 1-week washout period after which you will be asked to cross-over to the 2nd diet and so on until you complete all four diets. You will be provided with FREE dark chocolate for each of the intervention weeks. The doses will be in the range of 20g of dark chocolate. During the dark chocolate diet, you will be asked to distribute the chocolate dose throughout the day. For example, you could have some of the dark chocolate instead of your usual breakfast, and then eat the remaining chocolate every 2-3 hours. You will be asked to

complete another diet diary and physical activity questionnaire during Week-1 and 4 in addition to completing 24-urine collection at the end of each of these weeks.

The following measurements will also be assessed during the study: height, weight, body mass index, waist and hip circumferences, blood pressure, fasting glucose, total cholesterol, urinary magnesium, sodium, potassium, cortisol and cortisone (see, timetable attached at the end of this document).

Before, examining your blood pressure, you will be asked to sit comfortably for 10 minutes. Your blood pressure will then be measured three times with 2 minutes interval using an electronic sphygmomanometer. This should therefore take approximately 6 minutes. The 12-hour fasting glucose and cholesterol measurements will be obtained by finger-prick blood samples. This procedure should not cause any distress as finger prick samples are obtained quickly.

You will be free to withdraw from the study at any stage for any reason. All data collected will remain anonymous and your name will be replaced with an identification number that could only be identified by the researcher.

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If you have any further queries concerning this research, please do not hesitate to ask. Alternatively, if you have read and understood the information provided in this document and you would like to participate in this study, please sign the attached consent form.

Contact Details of the investigator

Name of Investigator: Suzana Almoosawi
Address: Postgraduate student
Public Health Nutrition
Department of Dietetics, Nutrition & Biological Sciences
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Queen Margaret University Drive
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EH21 8UU
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Contact Details of the independent adviser

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Email: JMcKENZIE@qmu.ac.uk
Telephone: 0131 474 0000

Table 7.7 List of polyphenol-rich foods and beverages to be avoided by the cocoa group (adapted from the USDA database for flavonoid content of foods, 2007a).

Foods rich in polyphenols (epicatechin)	Epicatechin content (mg) per 100g food product	Alternatives
Apples		Strawberries
Fuji	5.21	Quinces
gala	4.71	
golden with peel	3.79	
granny smith	3.60	
Apple sauce	5.41	
Apricots	5.47	
Blackberries	4.66	
Blueberries	13.69	
Broadbeans, boiled	7.82	
Cacao beans	99.18	
Cherries, canned	4.31	
Cherries, raw	6.97	
Chocolate, dark	41.5	White chocolate
Chocolate, milk	10.45	
Grapes, black	8.68	Grapes, red Grapes, white
Nectarines, raw	2.54	Avocado
Peaches, raw	2.34	
Pears	1.74	
Plums, raw	3.2	
Raspberries	4.07	
Soybeans	37.41	
Beverages rich in polyphenols (epicatechin)		
Red wine	3.28	White wine
Green tea	8.29	Black tea

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Information sheet for potential subjects

Effect of polyphenols on glucoregulatory biomarkers, BP and lipid profile in overweight and obese subjects

---

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Dark chocolate is a rich source of antioxidants, known as polyphenols. Recently, a number of studies have indicated that polyphenol-rich dark chocolate could reduce the risk of hypertension, diabetes and coronary artery disease. Though current manufacturing processes destroy much of chocolate's' antioxidants, a number of polyphenol-rich chocolates are still available on the market, whose properties deserve to be investigated. Indeed, if positive health attributes are to be found in these products, then their role in the primary prevention of hypertension, diabetes and cardiovascular diseases could be significant. Such findings will be of particular relevance to populations at risk of these diseases, such as the overweight population.
If you wish to take part in this study, you have to be healthy, non-smoker, not currently taking any blood pressure or cholesterol-lowering drugs. You should also have a normal physical activity level and your intake of cocoa, dark chocolate and green tea should be low.

The study will last approximately 11 weeks. You will have to be available for an average of 20 minutes once every 2 weeks (see, timetable attached at the end of this document).

You will be asked to follow your usual diet throughout the study, but to avoid consuming polyphenol-rich beverages and foods. You will find a list of these products and a list of alternative products that you can consume at the back of this document (see Table 7.8). During the week before the intervention, you will be asked to avoid eating any type of chocolate or chocolate products. During this baseline week you will also be required to complete a 3-day diet and physical activity diary (two weekdays & one weekend), 3 saliva collection (one before breakfast, 1 before lunch, 1 before dinner) and one 24-urine collection (see timetable at the end of this document).

On Week-0, you will be randomly assigned to one of the two intervention groups: placebo then polyphenol-rich dark chocolate, or polyphenol-rich dark chocolate then placebo. You will be asked to follow each diet for 4 weeks followed by 2-week washout period after which you will be asked to cross-over to the 2nd diet and so on until you complete both diets. You will be provided with FREE Barry Callebaut dark chocolate for each of the intervention weeks. The doses will be 20g for both intervention. During the intervention, you will be asked to distribute the chocolate dose throughout the day. For example, you could have 5g in the morning before breakfast, 5g before lunch, 5g before dinner and 5g at 8 or 10pm. You will be asked to complete another a 3-day diet and physical activity diary and 3 saliva

collection (one before breakfast, 1 before lunch, 1 before dinner) during Week-2, and 8. You will also be requested to complete 3 saliva collection (one before breakfast, 1 before lunch, 1 before dinner) and a 24-urine collection at the end of each intervention.

Overall, the following measurements will be assessed during the study: height, weight, body mass index, waist and hip circumferences, percentage body fat, blood pressure, fasting glucose, insulin, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides, salivary and urinary cortisol and cortisone. Before taking your blood pressure, you will be asked to sit comfortably for 15 minutes. Your blood pressure will then be measured three times with 5 minutes interval using an electronic sphygmomanometer. This should therefore take approximately 15 minutes. The 12-hour glucose, insulin and lipids measurements will be obtained using venous blood samples. This procedure may cause some discomfort but you will only be required to undergo it 4 times within the 11 weeks. You will also be paid 5£ for each venous blood samples you will provide.

You will be free to withdraw from the study at any stage for any reason. All data collected will remain anonymous and your name will be replaced with an identification number that could only be identified by the researcher.

If you would like to consult a person who is not involved in this research for independent advice, please contact Dr Jane McKenzie. Her contact details are provided below.

If you have any further queries concerning this research, please do not hesitate to ask. Alternatively, if you have read and understood the information provided in this document and you would like to participate in this study, please sign the attached consent form.

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Table 7.8 List of polyphenol-rich foods and beverages to be avoided by the cocoa group (adapted from the USDA database for flavonoid content of foods, 2007a).

Foods rich in polyphenols (epicatechin)	Epicatechin content (mg) per 100g food product	Alternatives
Apples		Strawberries
Fuji	5.21	Quinces
gala	4.71	
golden with peel	3.79	
granny smith	3.60	
Apple sauce	5.41	
Apricots	5.47	
Blackberries	4.66	
Blueberries	13.69	
Broadbeans, boiled	7.82	
Cacao beans	99.18	
Cherries, canned	4.31	
Cherries, raw	6.97	
Chocolate, dark	41.5	White
Chocolate, milk	10.45	chocolate
Grapes, black	8.68	Grapes, red
		Grapes, white
Nectarines, raw	2.54	Avocado
Peaches, raw	2.34	
Pears	1.74	
Plums, raw	3.2	
Raspberries	4.07	
Soybeans	37.41	
Beverages rich in polyphenols (epicatechin)		
Red wine	3.28	White wine
Green tea	8.29	Black tea

7.3. Appendix 3 Screening questionnaire

Queen Margaret University
EDINBURGH

Screening questionnaire

Effect of dark chocolate and green coffee bean extract on glucoregulatory biomarkers, BP and lipid profile in overweight and obese subjects

---

Personal history

1. Please tick the appropriate box

Male	Female
☐	☐

Date of birth day……..month……..year……..

2. Please tick the appropriate box about your employment situation

☐ In a full-time job
☐ In a part-time job
☐ Unemployed, seeking work
☐ Unemployed because sick or disabled
☐ Housewife
☐ Full-time student

Family history

3. Did your mother or father have heart disease before they were 60 years old?

☐ Yes ☐ No ☐ don't know

4. How many brothers and sisters did you have in your family (not counting yourself)?

……brothers and sisters

5. Did any of your brothers or sisters have heart disease before they were 60 years old?

☐ Yes ☐ No ☐ don't know

Medical history

6. Have you ever been told by a doctor that you have, or have had any of the following? Tick **Yes** or **No** for each condition.

Yes	No	
□	□	Angina
□	□	Heart attack (coronary thrombosis, mycardial infarction)
□	□	High blood pressure
□	□	Stroke
□	□	Diabetes
□	□	High chloesterol

7. Are you taking any medication for high blood pressure?

☐ Yes ☐ No

If yes, please write the names of the medicine(s) you are taking.

…….

8. Are you taking any medication for high cholesterol?

☐ Yes ☐ No

If yes, please write the names of the medicine(s) you are taking.
……

9. Are you now taking aspirin regularly?
☐ Yes ☐ No if no, go to question
If yes, is it for your heart
☐ *Yes* ☐ *No* ☐ *don't know*
If it is for your heart, why did you start taking it?
☐ *The doctor told you to take it*
☐ *Ou decided for yourself*
☐ *Other reason, please give details……*

11. Are you taking an other medication at present?
☐ Yes ☐ No
If yes, write the name of the medicine(s) and what you are taking them for (if you know).
……
12. Are you regularly taking any vitamins, mineral or food supplements at present?
☐ Yes ☐ No

If yes, give the type of supplement, brand name, and how often you take each one.
Type Brand (and strength) Frequency

---

13. Do you smoke cigarettes, now?
☐ Yes, regularly
☐ No
☐ Ocassionally (usually less than one a day)

Your diet
1. How many cups of coffee do you drink? …….cups per day
……...cups per week
Is this ☐ ground coffee ☐ instant coffee
Is it decaffeinated? ☐ Yes ☐ No

2. How many cups of tea do you have? …….cups per day
……...cups per week
3. How many cups of green tea do you have? …….cups per day
……...cups per week
4. How many cups of cocoa or hot chocolate do you have? …….cups per day
……...cups per week
5. How often do you eat dark chocolate? …….cups per day
……...cups per week

7.4. Appendix 4 Nutritional composition of dark chocolate

BARRY CALLEBAUT

Product Specification

Article : CHD-Q65ACTICOA-000
Legal denomination : Chocolate
Commodity code : 1806.2010

Typical composition
Cocoa mass ; Sugar ; Anhydrous milkfat ; Fat-reduced cocoa powder ; Emulsifier: soya lecithin ; Natural vanilla

Delivery form : Liquid
Order quantity 21,000 KG (or multiply of this)

Analytical data - chemical

MOISTURE	max 1.00 %		IOCCC1(1952)
TOTAL FAT	36.7 %	+ /-1,5	IOCCC14(1972)
POLY HYDROXYPHENOLS	5.6 - 6.4 %		HPLC

Analytical data - physical
Particle size : max. 3 % of the dry fatfree substance is > 30 micron. IOCCC38(1990)

Analytical data - microbiological	n	c	m	M	Ref.Method
TOTAL PLATE COUNT	5	2	1000/g	5000/g	ISO4833
YEASTS	5	2	10/g	50/g	ISO7954
MOULDS	5	2	10/g	50/g	ISO7954
ENTEROBACTERIACEAE	5	2	0/g	10/g	ISO21528-2
COLIFORMS	5	2	0/g	10/g	ISO4832
E.COLI	5	0	0/g	0/g	ISO7251
SALMONELLAE	15	0	0/25g	0/25g	ISO6579

Dimensions
Not specified

Shelf life and recommended storage conditions
1 month(s) after production date
Storage Temperature : 45 - 60 ℃
In order to avoid separation, the product should be stirred !
Store the product in a clean, dry (relative humidity max.70 %), and odourless environment.

Nutritional data for 100g (by calculation based on literature data)

ENERGY VALUE	509 kcal	VITAMIN B5	RDA	8.9 %
ENERGY VALUE	2,129 kJ	VITAMIN B6	PYRIDOXIN	0.1 mg
TOTAL PROTEIN	6.7 g	VITAMIN B6	RDA	3.3 %
MILK PROTEIN	0.0 g	VITAMIN B12	CYANO-COBALAMINE	0.0 µg
AVAILABLE CARBOHYDRATES	37.2 g	VITAMIN B12	RDA	0.0 %
SUGARS (MONO- DISACCHARIDES)	32.7 g	VITAMIN C	L-ASCORBIC ACID	0.0 mg
POLYOLS	0.0 g	VITAMIN C	RDA	0.0 %
STARCH	4.0 g	VITAMIN D	CALCIFEROL	1.6 µg
TOTAL FAT	36.7 g	VITAMIN D	RDA	31.0 %
SATURATED FAT	23.1 g	VITAMIN D	(IU)	6.2
MONO UNSATURATED FAT	12.3 g	VITAMIN E	ALPHA-TOCOPHEROL	2.9 mg
POLY UNSATURATED FAT	1.3 g	VITAMIN E	RDA	29.3 %
TFA (PLANT ORIGIN)	0.0 g	VITAMIN E	(IU)	2.0
TRANS FATTY ACID (TFA) TOTAL	0.2 g	VITAMIN H	BIOTIN	0.0 mg

CHOLESTEROL	11.9 mg	VITAMIN M FOLIC ACID	15.2 µg
ORGANIC ACIDS	0.81 g	SODIUM	7.0 mg
DIETARY FIBRE	8.4 g	PHOSPHORUS	259.3 mg
TOTAL ALKALOIDS	1.0 g	PHOSPHORUS RDA	32.4 %
ALCOHOL	0.0 g	IRON	19.3 mg
POLY HYDROXYPHENOLS	5.7 g	IRON RDA	137.6 %
VITAMIN A RETINOL	39 µg	MAGNESIUM	167.1 mg
VITAMIN A RDA	4.7 %	MAGNESIUM RDA	55.7 %
VITAMIN A (IU)	131	ZINC	2.3 mg
PROVITAMIN A BETA-CAROTENE	6 µg	ZINC RDA	15.4 %
VITAMIN B1 THIAMIN	0.1 mg	IODINE	0 µg
VITAMIN B1 RDA	0.0 %	IODINE RDA	0.0 %
VITAMIN B2 RIBOFLAVIN	0.1 mg	CALCIUM	46.3 mg
VITAMIN B2 RDA	8.3 %	CALCIUM RDA	5.8 %
VITAMIN B3/PP NIACINE/NICOTIN	1.0 mg	CHLORIDE	13.4 mg
VITAMIN B3 RDA	5.7 %	POTASSIUM	842.1 mg
VITAMIN B5 PANTOIC ACID	0.5 mg	ASH CONTENT	2.4 g

Additional allergens info

MILK PROTEINS	1	AZO-COLOURS **	0
LACTOSE	1	TARTRAZIN (E102)	0
EGG PRODUCTS	0	CINNAMON	0
SOY PROTEINS	1	VANILLIN	1
SOY OIL	1	CORIANDER	0
LUPIN	0	CELERY	0
GLUTEN	0	UMBELLIFERAE	0
WHEAT	0	ORANGEYELLOW S (E110)	0
RYE	0	AZORUBIN (E122)	0
BEEF	0	AMARANT (E123)	0
PORK	0	COCHINEAL RED A (E124)	0
CHICKEN	0	ALLURA RED AC E129	0
FISH	0	PATENT BLUE E131	0
CRUSTACEAN AND SHELL-FISH	0	INDIGOTINE E132	0
MOLLUSCS	0	ANNATO-LYCOPENE E160D	0
MAIZE	0	TRAGACANTH E413	0
COCOA	1	GUM ARABIC	0
YEAST	0	SORBIC ACID (E200-> E203)	0
LEGUMINOUS PLANTS	0	HYDROLYSED VEGETABLE PROTEIN	0
HAZELNUTS, ALMONDS	0	ALCOHOL	0
OTHER NUTS *	0	ASPARTAME	0
HAZELNUT OIL,ALMOND OIL	0	ANIMAL PRODUCTS	0
PEANUTS	0	HONEY	0
PEANUT OIL	0	ADDED SALT	0
SESAME PRODUCTS	0	GARLIC	0
SESAME OIL	0	CAFFEIN	1
MUSTARD	0	BHA/BHT (E320/E321)	0
GLUTAMINATE (E620 -> E625)	0	SACCHAROSE	1
SULPHITE (E220 -> E227)	0	FRUCTOSE	1
BENZOIC ACID (E210-> E213)	0	SUITABLE FOR VEGETARIANS	1
PARABENE (E214-> E219)	0	SUITABLE FOR VEGANS	0

Legend : 1 = present / suitable 0 = absent / not suitable

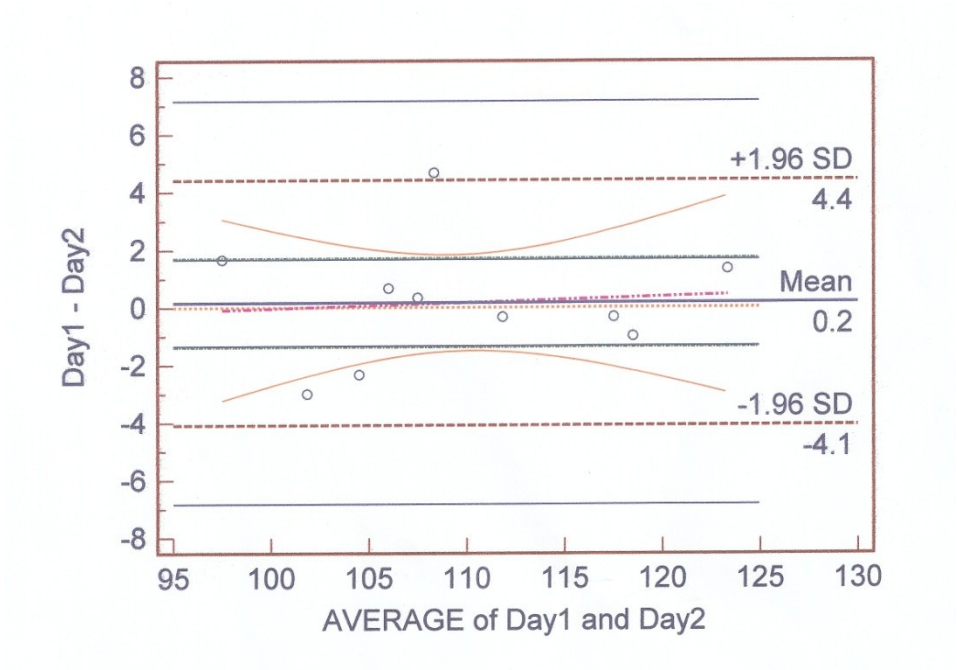
Azo-colours** : E102,E110,E122,E123,E124,E151,E154 and E155

Other nuts * : walnuts, pecan nuts, cashew nuts, pistachio nuts, brazil nuts, macadamia nuts, chestnuts.

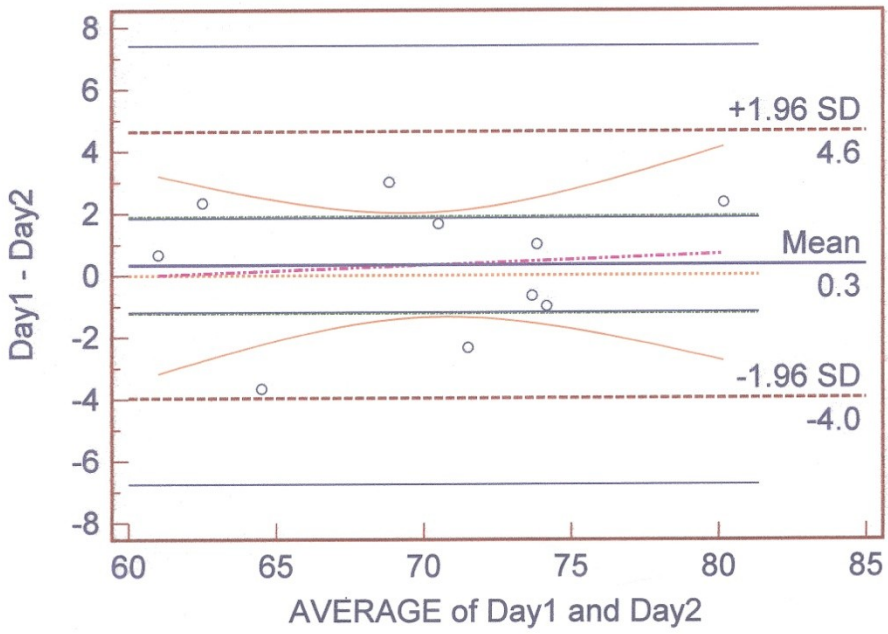
7.5. Appendix 5 Inter-day variability in blood pressure measurements

Figure 7.1 Bland-Altman plots for systolic (a) and diastolic blood pressure (b) showing limits of agreement and mean inter-day differences between blood pressure measurements on two different occasions (Day 1 and Day 2) taken from the same group of subjects (n=10) 1 week apart. Values are mean of three readings.

(a)



(b)



7.6. Appendix 6 Conferences and Publications

1. Almoosawi S, Fyfe L, Ho C, Al-Dujaili E. (2010). The effect of polyphenol-rich dark chocolate on fasting capillary whole blood glucose, total cholesterol, blood pressure and glucocorticoids in healthy overweight and obese subjects' *British Journal of Nutrition*;103(6):842-50
2. S Almoosawi, GJ McDougall, L Fyfe, EAS Al-Dujaili. Investigating the inhibitory activity of green coffee and cacao bean extracts on pancreatic lipase. *Nutrition Bulletin* (Under review)
3. Almoosawi S, Fyfe L, Ho C, Al-Dujaili E. (2009). Polyphenol-rich dark chocolate: Effect on fasting capillary glucose, total cholesterol, blood pressure and glucocorticoids in healthy overweight and obese subjects' In: Nutrition Society., 2009, Surrey University. (Unpublished)
4. Almoosawi, Suzana and Fyfe, Lorna and Al-Dujaili, Emad A S (2009) 'The effect of green coffee bean extract rich in chlorogenic acid on antioxidant status of healthy human volunteers In: Nutrition Society., 2009, Surrey University. (Unpublished)
5. S Almoosawi, A Dickinson, L Fyfe, CJ Kenyon & EAS Al-Dujaili (2009). Effect of green coffee bean extract and chlorogenic acid consumption on 11βHSD activity in humans and mice. *Endocrine Abstracts* 19: P112
6. Suzana Almoosawi; Catherine Tsang; Lorna Fyfe; Emad EAS Al-Dujaili and Davidson I (2010) 'Effect of polyphenol-rich dark chocolate on cardiovascular risk factors and glucocorticoids in healthy overweight and obese subjects' In Scottish cardiovascular forum 13th annual meeting, 2010, University of Glasgow (Unpublished).
7. Almoosawi S, Tsang C, Fyfe L, Al-dujaili EAS and Davidson I (2009). Effect of polyphenol-'rich dark chocolate on glucoregulatory biomarkers, blood pressure, lipids and glucocorticoids in healthy overweight and obese subjects' In 4th international conference on polyphenols and health, 2009, Harrogate. (Unpublished)
8. The effect of polyphenol-rich dark chocolate on glucoregulatory biomarkers, lipid profile, blood pressure and glucocorticoids in healthy overweight and obese subjects. *Molecular Nutrition and Food research* (manuscript under preparation).

British Journal of Nutrition (2009), page 1 of 9
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The effect of polyphenol-rich dark chocolate on fasting capillary whole blood glucose, total cholesterol, blood pressure and glucocorticoids in healthy overweight and obese subjects

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Numerous studies indicate that polyphenol-rich chocolate reduces fasting blood glucose, blood pressure (BP) and total cholesterol in healthy individuals and hypertensives with or without glucose intolerance. The aim of the present study was to investigate the effect of two doses of polyphenol-rich dark chocolate (DC) on fasting capillary whole blood glucose, total cholesterol and BP and to examine whether improvements in these parameters are associated with changes in adrenocorticoid excretion in overweight and obese individuals. The study used a randomised, single-blind, cross-over design where fourteen overweight and obese subjects were randomised to either take 20 g DC with 500 mg polyphenols then 20 g DC with 1000 mg polyphenols or vice-versa. Participants followed each diet for 2 weeks separated by a 1-week washout period. It was observed that the 500 mg polyphenol dose was equally effective in reducing fasting blood glucose levels, systolic BP (SBP) and diastolic BP (DBP) as the 1000 mg polyphenol dose suggesting that a saturation effect might occur with increasing dose of polyphenols. There was also a trend towards a reduction in urinary free cortisone levels with both groups although it did not reach statistical significance. No changes in anthropometrical measurements were seen. We suggest that more research is required to investigate the mechanism(s) by which polyphenol-rich foods influence health.

Dark chocolate: Glucose: Blood pressure: Cholesterol: Glucocorticoids: Obesity

Epidemiological studies link high polyphenol intake with reduced risk of oxidative stress-related diseases like diabetes, hypertension and CVD[1–3]. In particular, consumption of cocoa and dark chocolate (DC) has been shown to improve endothelium function, insulin sensitivity, blood pressure (BP) in healthy individuals, hypertensives with or without glucose intolerance[4–6] and obese subjects[7]. Cocoa and DC are rich sources of polyphenols providing on average more polyphenols per serving than red wine, green tea or black tea[8]. These polyphenols confer potent antioxidant properties to cocoa and DC[8,9] in addition to the ability to regulate NO[4–7].

Obesity is known to be associated with insulin resistance and elevated BP[10]. One of the underlying factors linked to these cardiovascular risk factors is abnormal cortisol metabolism[11,12]. Cortisol is a counterregulatory hormone that is essential in the long-term maintenance of blood glucose[13] and which could also unfavourably influence BP and lipid profile[12–15]. When present in excess, cortisol induces overproduction of reactive oxygen species[16,17] leading to reduced endothelial NO synthase expression[18]. In obesity, particularly abdominal obesity, postprandial hypercortisolism and enhanced peripheral metabolism of cortisol, characterised by increased urinary cortisone-to-cortisol ratio, are observed which are linked to insulin resistance and increased fasting insulin[11]. Increased expression of subcutaneous adipose tissue 11β-hydroxysteroid dehydrogenase type 1 has also been reported, which is known to impair glucose-stimulated insulin secretion[19]. Since improved NO bioavailability is the main mechanism by which DC polyphenols reduce endothelium dysfunction, insulin resistance and hypertension[4–7], this preliminary study aimed to assess and compare the effect of DC containing two different doses of polyphenols on fasting capillary whole blood glucose levels, total cholesterol, BP, urinary free cortisol and cortisone excretion in healthy overweight and obese subjects. The other objective was to observe whether improvements in fasting blood glucose, total cholesterol and BP could be correlated with changes in urinary free cortisol or cortisone excretion. A secondary objective was to monitor Mg intake and excretion since DC is known to contain large quantities of Mg, which, in turn, could influence BP, insulin action and the metabolic syndrome[20–22].

Abbreviations: BP, blood pressure; DBP, diastolic BP; DC, dark chocolate; FG, fasting glucose; SBP, systolic BP.

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Methods

Study design

The study used a randomised, cross-over design where each subject acted as their own control. Following a 1-week run-in phase, eligible subjects were randomly assigned to one of the two polyphenol doses: 500 mg polyphenols DC or 1000 mg polyphenols DC. Participants followed each intervention for 2 weeks, after which they were crossed-over to the next intervention separated by a 1-week washout period (Fig. 1). The study included healthy non-smoker volunteers, aged 19–50 years with BMI $\geq 25\,kg/m^2$[23], no history of diabetes, hypertension or CVD. People taking dietary supplements, BP or cholesterol-lowering drugs, or those with soya and nut allergies were excluded. Smokers were excluded to minimise confounding factors since nicotine consumption is known to enhance hypothalamic–pituitary–adrenal axis activity, hence resulting in elevated cortisol levels[24,25]. Participants gave written consent, completed a lifestyle questionnaire before being screened for fasting blood glucose, total cholesterol, BP and BMI to determine their eligibility. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by Queen Margaret University Ethics Committee.

Diet

Table 1 provides a summary of the nutrient composition of the two Acticoa DC used in the present study, which were kindly supplied by Barry Callebaut (Lebbeke, Belgium). The 500 mg dose was previously shown to reduce fasting glucose (FG) levels and BP by Grassi *et al.*[4,5] and Taubert *et al.*[26]. However, due to the great variation in epicatechin and catechin levels between the chocolate used in the present study and the one used by Grassi *et al.*[4,5], a higher DC dose of 1000 mg was also chosen. This 1000 mg dose was selected to provide similar quantities of polyphenol to what is consumed by the Kuna population of Panama, who are known to consume large quantities of cocoa and to have low incidence of hypertension[27]. This dose will also provide about 43·2 % of the epicatechin and catechin dose used by Grassi *et al.*[4,5]. Subjects were instructed to distribute DC doses throughout the day to achieve a high steady-state concentration. They were also instructed to maintain their usual diet throughout the study but to refrain from polyphenol-rich foods and beverages that supply ≥15 mg/kg epicatechin and ≥4 mg/l epicatechin[28–30]. Subjects completed a 3-d (two weekdays and one weekend) diet and physical activity[31] diary during the run-in phase and at the end of each dietary intervention. The Photographic Atlas of Food Portion Sizes was used to assist subjects in describing their portion sizes[32]. Diet diaries were validated by interviewing the subjects using a validated questionnaire[33]. The diet diaries were analysed and energy, fat, protein, carbohydrate and magnesium intake were estimated using Windiet software (Windiet Research, Univation Ltd, Robert Gordon University, Aberdeen, UK). Compliance with the study's protocol was assessed by direct interviewing, returning of empty chocolate foils and assessment of diet diaries.

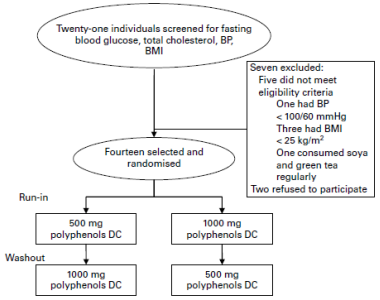

Fig. 1. Diagram showing random allocation of subjects into the different dietary interventions. BP, blood pressure; DC, dark chocolate.

Table 1. Nutritional composition of 20 g of 500 and 1000 mg polyphenol dark chocolate (DC)

Component	500 mg DC	1000 mg DC
Polyphenols (mg)	500	1000
Epicatechin and catechin (mg)	18·99	37·98
Energy (kJ)	425·8	425·8
Fat (g)	7·34	7·34
Protein (g)	1·34	1·34
Carbohydrate (g)	7·44	7·44
Mg (mg)	33·42	33·42
Na (mg)	1·4	1·4
K (mg)	168·42	168·42

Measurements

To measure fasting blood glucose and total cholesterol, 12-h fasting capillary whole blood samples were obtained and analysed using a calibrated Accutrend GC system (Roche diagnostics, Mannheim, Germany). Participants were instructed to consume the last DC dose 12 h before analyses[34], avoid heavy physical activity and alcohol intake 24 h before testing and consume the same diet the day before each test[30]. Waist circumference, hip circumference and BMI were measured. Data on waist and hip circumference were used to calculate waist-to-hip ratio, where waist-to-hip ratio >1·0 in men and >0·85 in women indicate abdominal obesity[35]. Both waist circumference and BMI serve as good indicators of the degree of insulin resistance in overweight and obese individuals[35] while waist-to-hip ratio serves as a predictor of hypertension[36] and hypothalamic-pituitary–adrenal axis hyperactivity, characterised by high baseline plasma cortisol and low 24-h urinary cortisol excretion in obese women[11]. An automated A&D Medical UA-767 BP monitor (A&D Medical, San Jose, CA, USA) was used to measure BP according to Grassi *et al.*[5]. This monitor was previously validated and was shown to achieve grade A for both systolic and diastolic BP according to the British Hypertension Society standard[37].

Urine samples were obtained for estimating 24-h urinary Mg excretion. Urinary Na and K excretion were also

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monitored since they serve as direct measures of Na and K intake, which could act as confounding factors in relation to BP. The 24-h urine collections were validated by measuring creatinine excretion[38,39]. Analyses of urine Na, K and Mg concentrations were conducted using an automated platform (Olympus, Essex, UK) at the Clinical Biochemistry Laboratory, Royal Infirmary of Edinburgh, Scotland, UK. Urinary cortisol and cortisone levels were analysed in duplicates using ELISA according to the method described by Al-Dujaili & Bryant[40] and Al-Dujaili[41]. The data were then used to calculate urinary cortisol-to-cortisone ratio. This ratio serves as a measure of renal 11β-hydroxysteroid dehydrogenase type 2 activity[42]. Monitoring the activity of this enzyme helps detect changes in peripheral metabolism of cortisol[42]. All tests were carried out at baseline, before and after each intervention.

Statistical methods

All data are expressed as means and standard deviations. Mixed between–within subjects ANOVA or split-plot ANOVA was performed for multiple comparison, where time (baseline, week 1, week 2) was the within-group variable and intervention group (500 mg, 1000 mg DC) was the between-group variable and the continuous variable were FG, SBP, DBP BMI, weight, waist circumference, hip circumference and waist-to-hip circumference. A P-value $\leq 0{\cdot}05$ was considered statistically significant. Within each intervention group (500, 1000 mg DC), changes in fasting blood glucose levels, SBP and DBP were analysed using repeated measures ANOVA with Bonferroni *post hoc* tests. A separate split-plot ANOVA was also performed to detect any carry-over effects between the two interventions and to ensure changes in FG, SBP and DBP following each treatment were not affected by the sequence of DC administration (1000 mg followed by 500 mg *v.* 500 mg followed by 1000 mg). Two-tailed paired sample t tests were used to assess changes between baseline and post-intervention total cholesterol, urinary free cortisol or cortisone, urinary cortisol-to-cortisone ratio and mineral excretion. Similarly, differences in response to both DC doses among the various ethnic groups were assessed using one-way between-groups ANOVA with FG, SBP and DBP as the dependent variables and ethnicity as the factor. An independent sample t test was also used to compare the response to DC polyphenols between the abdominally obese individual and the peripherally obese individuals. The relationship between fasting blood glucose levels, total cholesterol, SBP, DBP, BMI, weight, waist circumference, hip circumference, waist-to-hip circumference, urinary Mg, Na and K levels, 24-h urinary free cortisol, cortisone and cortisol-to-cortisone ratio were assessed using Pearson product-moment correlation coefficient, r. The coefficient of determination was estimated by obtaining r^2. All statistical analyses were performed using SPSS for Windows, version 16.0.0 (SPSS Inc., Chicago, IL, USA). The sample size was calculated using G-power software version 3.0.8 (Heinrich Heine University, Dusseldorf, Germany) to detect 0·3 mmol/l reduction in FG with baseline SD = 0·5 mmol/l and post-DC SD = 0·04 mmol/l, which is similar to the reduction reported by Grassi *et al.* [6].

Results

The study included fourteen healthy volunteers (eight males (five Caucasians, two Asians, one African) and six females (five Caucasians and one Hispanic)), 21–50 years old, mean age 26·4 (SD 11·5) years) with a BMI of 27·7 (SD 2·5) kg/m^2. Of these participants, thirteen were peripherally obese and one was abdominally obese (African).

Mixed between–within subjects ANOVA revealed a significant reduction in fasting capillary blood glucose concentrations (P=0·002), SBP ($P<0{\cdot}0001$) and DBP ($P<0{\cdot}0001$) following DC consumption. These effects were independent of the sequence of DC administration and no significant interaction between time, intervention group and sequence of DC administration was observed (FG $F(2,11) = 1{\cdot}057$, P=0·380; SBP $F(2,11) = 0{\cdot}431$, P=0·660; DBP $F(2,11) = 0{\cdot}653$, P=0·539; Figs. 2 and 3). No significant differences between the effect of 1000 and 500 mg polyphenols DC on fasting capillary blood glucose ($P>0{\cdot}05$) and BP ($P>0{\cdot}05$) were observed indicating that both doses have a similar efficacy.

To explore the results further, a one-way repeated measures ANOVA was conducted to compare fasting blood glucose levels, SBP and DBP at baseline, week 1 and week 2 for each of the two dietary interventions. A significant effect of DC on fasting blood glucose levels ($F(2,12) = 4{\cdot}305$, P=0·039), SBP ($F(2,12) = 12{\cdot}330$, P=0·001) and DBP ($F(2,12) = 13{\cdot}937$, P=0·001) was observed after consumption of 1000 mg DC. *Post hoc* comparisons using Bonferroni test indicated that mean fasting blood glucose levels and SBP at week 2 were significantly decreased after chocolate ingestion (FG 3·97 (SD 0·54) *v.* baseline 4·42 (SD 0·70) mmol/l; SBP 112·12 (SD 9·68) *v.* baseline 119·38 (SD 10·51) mmHg). Mean DBP levels were significantly lower at week 1 (74·45 (SD 7·17) mmHg) and week 2 (74·57 (SD 7·39) mmHg) compared to baseline (78·62 (SD 7·74) mmHg).

A significant effect of 500 mg DC on FG levels ($F(2,12) = 5{\cdot}026$, P=0·026), SBP ($F(2,12) = 11{\cdot}971$, P=0·001) and DBP ($F(2,12) = 7{\cdot}709$, P=0·007) was also observed. *Post hoc* comparisons indicated that the mean FG levels at week 2 were significantly different from baseline (3·92 (SD 0·86) *v.* 4·42 (SD 0·30) mmol/l). Mean SBP was also reduced at the end of week 1 (114·24 (SD 9·53) mmHg)

Fig. 2. Capillary fasting glucose (FG) levels at baseline (week 0), and at the end of 1and 2 weeks of each of the polyphenols doses. Group 1 received 1000 mg polyphenols dark chocolate (weeks 1–2) followed by 500 mg polyphenols dark chocolate (weeks 4–5). Group 2 received 500 mg polyphenols dark chocolate (weeks 1–2) followed by 1000 mg polyphenols dark chocolate (weeks 4–5). Changes in FG were independent of the sequences of chocolate administration ($P>0{\cdot}05$). Values are means with their standard errors represented by vertical bars.

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Fig. 3. Systolic blood pressure (SBP) (a) and diastolic blood pressure (DBP) (b) at baseline (week 0), and at the end of 1 and 2 weeks of each of the polyphenols doses. Group 1 received 1000 mg polyphenols dark chocolate (weeks 1–2) followed by 500 mg polyphenols dark chocolate (weeks 4–5). Group 2 received 500 mg polyphenols dark chocolate (weeks 1–2) followed by 1000 mg polyphenols dark chocolate (weeks 4–5). Changes in SBP and DBP were independent of the sequences of chocolate administration ($P>0{\cdot}05$). Values are means with their standard errors represented by vertical bars.

and week 2 (112·40 (SD 9·51) mmHg) as compared to baseline (119·38 (SD 10·51) mmHg). Similar findings were observed with DBP (Week 1 = 74·62 (SD 4·27) and Week 2 = 73·00 (SD 5·06) *v.* baseline 78·62 (SD 7·74) mmHg).

Total cholesterol did not change significantly after 1000 mg (P=0·191) or 500 mg polyphenols DC (P=0·246). There was a trend towards a reduction in 24-h urinary free cortisone levels in both the 1000 and 500 mg DC groups, although this reduction did not reach statistical significance even after adjustment for weight. No changes in anthropometrical data (Table 2), 24-h urinary free cortisol, cortisol-to-cortisone ratio, 24-h urinary Mg, Na, K were observed (Table 3). Likewise, one-way ANOVA revealed no significant effect of ethnicity on changes in FG, SBP, DBP in both DC groups, with the exception of one female subject (Hispanic), who experienced a greater reduction in SBP following both polyphenol doses as compared to Caucasians (1000 mg, P=0·009; 500 mg, $P<0{\cdot}0001$), Asians (1000 mg, P=0·014; 500 mg P=0·003) and African (1000 mg, P=0·0001; 500 mg, $P<0{\cdot}009$). The subject with abdominal obesity showed an increase in urinary free cortisol following 1000 mg polyphenols (+52·44 nmol/d) compared to peripherally obese individuals, who demonstrated a reduction (−11·05 nmol/d; P=0·037). This subject also had a greater reduction in DBP following 500 mg DC compared to other individuals (−15·7 mmHg *v.* −3·10 mmHg; P=0·017).

Pearson's product-moment correlations revealed a significant correlation between changes in 24-h urinary free cortisol, cortisone and changes in 24-h Na excretion (Table 4). There were no significant correlations between age and changes in FG, SBP, DBP following both DC doses ($P>0{\cdot}05$). Addition of DC to the diet did not affect Mg intake or excretion

Table 2. Effect of either 500 or 1000 mg polyphenol dark chocolate (DC) on anthropometrical measurements (Mean values and standard deviations)

	1000 mg DC							500 mg DC						
	Baseline		Week 1		Week 2			Baseline		Week 1		Week 2		
	Mean	SD	Mean	SD	Mean	SD	n	P	Mean	SD	Mean	SD	Mean	SD	n	P
Weight (kg)	81·64	13·40	81·66	12·84	81·36	12·80	14	0·447	81·29	13·99	80·85	13·69	81·13	13·35	14	0·789
BMI (kg/m^2)	27·73	2·53	27·75	2·38	27·66	2·44	14	0·517	27·58	2·70	27·46	2·75	27·56	2·56	14	0·796
Waist circumference (cm)	90·82	11·85	89·86	11·16	89·93	11·412	14	0·175	90·82	11·85	90·04	11·29	90·00	11·31	14	0·326
Hip circumference (cm)	105·61	5·99	105·64	6·21	105·57	6·30	14	0·089	105·61	5·99	105·11	6·19	105·11	6·96	14	0·810
Waist-to-hip ratio	0·86	0·09357	0·85	0·09	0·85	0·10	14	0·165	0·86	0·09	0·86	0·11	0·86	0·11	14	0·992

Table 3. Results for 24-h urine collections
(Mean values and standard deviations)

	1000 mg DC							500 mg DC						
	Baseline		Week 2					Baseline		Week 2				
	Mean	SD	Mean	SD	*n*	*df*	*P*	Mean	SD	Mean	SD	*n*	*df*	*P*
Free cortisol (nmol/d)	77·33	27·09	71·16	38·90	13	12	0·477	86·83	44·05	78·58	47·28	14	13	0·620
Free cortisone (nmol/d)	54·34	26·90	45·82	17·33	13	12	0·159	59·64	32·56	45·80	20·34	14	13	0·139
Cortisol-to-cortisone ratio	1·6812	0·75	1·60	·63	13	12	0·664	1·68	0·72	1·77	0·62	14	13	0·682
Cortisone-to-cortisol ratio	0·71	0·32	0·75	0·37	13	12	0·744	0·71	0·31	0·63	0·21	14	13	0·455
Free cortisol (nmol/kg per d)	0·98	0·37	0·89	0·44	13	12	0·400	1·0529	0·43	0·98	0·64	14	13	0·731
Free cortisone (nmol/kg per d)	0·69	0·36	0·57	0·17	13	12	0·162	0·73	0·38	0·55	0·23	14	13	0·180
Creatinine (mmol/l)	12·74	5·35	11·78	4·40	13	12	0·219	12·83	5·15	19·29	27·37	14	13	0·387
Mg (mmol/l)	3·26	1·23	3·00	1·55	13	12	0·497	3·29	1·19	3·26	1·34	14	13	0·928
Na (mmol/l)	122·07	57·90	114·63	48·71	13	12	0·802	124·35	56·28	132·69	54·44	14	13	0·644
K (mmol/l)	58·20	22·12	56·14	24·08	13	12	0·770	61·19	24·03	67·58	22·42	14	13	0·377

DC, dark chocolate.

Table 4. Pearson product-moment correlations between changes in urinary glucocorticoid levels and changes in selected parameters

Correlation pair	*n*	*r*	*P*
Δ Urinary free cortisol (nmol/kg per 24 h)			
Δ Urinary free cortisone excretion (nmol/kg per 24 h)	27	0·599	0·001
Δ 24 h urinary Na (mmol/g creatinine)	26	0·489	0·011
Δ Physical activity (kJ)	26	−0·384	0·053
Δ Urinary free cortisone (nmol/kg per 24 h)			
Δ Cortisol-to-cortisone ratio	27	−0·662	0·000
Δ Cortisone-to-cortisol ratio	27	0·628	0·000
Δ 24 h urinary Na (mmol/g creatinine)	26	0·478	0·014

significantly. Moreover, no significant correlations were found between changes in Mg intake or excretion and the reductions in fasting blood glucose and BP seen following DC consumption. Energy expenditure, energy, macronutrient and mineral intake did not change significantly through the study period (Fig. 4).

Discussion

The present study demonstrates that polyphenol-rich DC reduces fasting blood glucose levels and BP in overweight and obese individuals. These findings are consistent with previous observations that polyphenol-rich DC intake improved insulin resistance, insulin sensitivity, FG levels and BP in healthy individuals[4], hypertensives[5], glucose-intolerant hypertensives[6] and obese subjects[7]. The results are also in agreement with studies on diabetic obese mice, where reductions in blood glucose and fructosamine levels were reported following consumption of cacao liquor procyanidins[43].

Enhanced vascular function is thought to be the main mechanism by which DC polyphenols improve glucose and BP homeostasis[4–7,23,34,44–47], although other mechanisms like decreased and delayed carbohydrate digestion and absorption might also be involved[48,49]. The present study investigated whether polyphenol-rich DC could alter cortisol metabolism and whether improvements in glucose and BP seen in obese individuals following DC consumption are linked to improved cortisol metabolism. The hypothesis was based on that cortisol plays an important role in glucose and BP homeostasis, probably through a mechanism involving increased reactive oxygen species production and decreased NO bioavailability, and that in obesity several alteration in cortisol metabolism are observed, which are, in turn, linked to increased insulin resistance and hypertension. The study demonstrates that both 500 and 1000 mg polyphenol DC decrease 24-h urinary free cortisol and cortisone levels. However, these reductions were not significant and are not associated with reductions in fasting blood glucose or BP. Such findings differ from previous findings, wherein polyphenols increased[50–53] or decreased[54,55] cortisol levels[56]. The lack of significance could be related to a number of factors. For instance, the sample size might have not been sufficiently large to detect a significant change. In this case, using several parameters of cortisol metabolism including its measurement in urine, saliva and blood might have helped detect any such effect. Additionally, the study population consisted mainly of

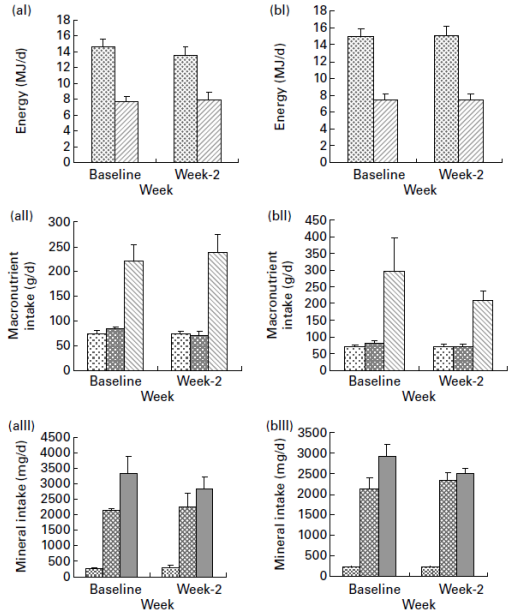

Fig. 4. Energy (l), macronutrient (ll) and mineral intake (lll) at baseline and at the end of each intervention: (a) 20 g dark chocolate with 1000 mg polyphenols and (b) 20 g dark chocolate with 500 mg polyphenols. Values are means with their standard errors represented by vertical bars. (▨), Energy expenditure; (□) energy intake; (▯) fat intake; (▩) protein intake; (▭) carbohydrate intake; (▧) Mg intake; (▨) Na intake; (▩) K intake.

subjects with peripheral obesity rather than those with abdominal obesity, who exhibit more prominent abnormalities in cortisol metabolism as indicated by the association between high waist circumference or waist-to-hip ratio and high urinary cortisol or cortisone-to-cortisol ratio[11,57]. In addition, differences in Na intake were not controlled for and could have acted as confounding factors[58]. This could be observed in the association between changes in urinary free cortisol or cortisone and changes in Na excretion, and the association between changes in Na intake and changes in cortisol-to-cortisone ratio. Dietary factors have been reported to influence cortisol metabolism. High-fat low-carbohydrate diets stimulate cortisol regeneration by 11β-hydroxysteroid dehydrogenase type 1, while reducing cortisol inactivation in liver[59]. Na loading, on the other hand, decreases plasma cortisol levels by enhancing cortisol elimination[60] possibly via a mechanism involving increased hepatic blood flow[61]. The latter may explain the association between increased urinary free cortisol excretion and urinary Na levels. However, subjects did not report significant changes in Na intake during the study, which overall suggest that DC polyphenols influence glucose and BP homeostasis mainly via the NO pathway.

The present study demonstrates that DC with 500 mg polyphenols is as effective in reducing fasting blood glucose levels in overweight and obese individuals as 1000 mg polyphenol DC with a similar macronutrient composition. Furthermore, the results indicate that DC polyphenols reduce blood glucose levels after 2 weeks of commencing a polyphenol-rich DC diet. These findings are important since in relation to glucose metabolism, inconsistencies still exist regarding the treatment duration and dose required to achieve a glucose-lowering effect. For example in their pilot study, Stote *et al.*[62] failed to show any significant improvement in glucose levels, insulin resistance and insulin sensitivity following 5 d of twice daily consumption of procyanidin-rich cocoa beverage containing 22–900 mg procyanidins by insulin-resistant men and women. Similarly, Taubert *et al.*[34] failed to demonstrate any improvement in glucose or insulin levels following 18 weeks of daily ingestion of 6·3 g DC containing 30 mg polyphenols. Conversely, Davison *et al.*[7] showed reduced insulin resistance following consumption of a cocoa beverage containing 902 mg flavanols twice daily for 12 weeks in overweight and obese subjects. Together, these findings suggest that a longer duration and a higher dose of

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polyphenols could be required to achieve a significant reduction in glucose levels. The present study reinforces this hypothesis while demonstrating that increasing polyphenol dose does not necessarily results in further reductions in glucose and BP levels since a saturation effect may occur with increasing DC polyphenol content[63]. It also highlights the need to identify the minimum polyphenol dose at which maximal health benefits could be achieved, since a reduction in the polyphenol content of chocolate implies reduced bitterness[64,65], which could render the chocolate more palatable and acceptable to the general population. In relation to BP, 20 g DC with 500 mg polyphenols reduced SBP and DBP to a similar extent as 20 g DC with 1000 mg polyphenol. Moreover, the reduction in BP observed following the 20 g DC (500 mg polyphenols) was comparable to the reduction reported in a previous study on normotensive subjects (7 and 3 mmHg reduction in SBP and DBP, respectively)[4]. This might suggest that reducing the portion of DC while maintaining a similar total phenol content results in equivalent reductions in BP. This could provide several advantages since reducing the portion of DC would permit delivery of high quantity of polyphenols in a less energy-dense form, which is essential if DC is to be included as part of a healthy balanced diet.

In contrast to Fraga *et al.*[66] and Grassi *et al.*[5], we did not observe any significant changes in total cholesterol. Such results are to be expected since our subjects had normal baseline total blood cholesterol levels as compared to Grassi *et al.*[5] (baseline total cholesterol = 5·4 (SD 0·6) mmol/l). Moreover, Grassi *et al.*[5] suggested that both the catechin and the fat component of DC account for its beneficial effect on total cholesterol. Similar assumptions were made in relation to stearic acid in DC[67]. Since in the present study a lower DC portion was used, the lack of significant change in total blood cholesterol could be related to the lower levels of linoleic, oleic and stearic acids present in this DC. There was also a lack of correlation between the reported energy intake and physical activity, which is similar to the findings of Davison *et al.*[7], who argued that obese individuals might underreport energy intake and overreport physical activity.

In conclusion, the present study confirms previous reports of improved FG levels and BP following DC consumption. It also demonstrates that these effects do not appear to be mediated through changes in cortisol metabolism. Further studies are needed to identify the optimal dose of polyphenols required to improve glucose metabolism and to examine additional parameters that could be influenced by polyphenols.

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Investigating the inhibitory activity of green coffee and cacao bean extracts on pancreatic lipase

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Polyphenols; Green *coffea robusta*; *Theobroma cacao*; pancreatic lipase

Polyphenols and pancreatic lipase

Summary
The present study investigated the effect of green coffee bean extract and *Theobroma cacao* bean extract on pancreatic lipase activity *in vitro*. Green coffee bean extract produced a J-shaped dose-dependent inhibition of pancreatic lipase with the percentage inhibition of pancreatic lipase ranging from 11.8 to 61.5%. Similar concentrations of *Theobroma cacao* failed to produce any effect on pancreatic lipase. Non-linear regression analysis revealed that the concentration of green coffee bean extract required to elicit a 50% inhibition of pancreatic lipase activity (IC50) was ~ 43μM. In conclusion, extracts of green coffee beans but not *Theobroma cacao* possess potent inhibitory activity against pancreatic lipase.

Introduction

It is increasingly recognised that polyphenols can affect the activity of numerous enzymes that regulate glucose, protein and lipid metabolism. Consistent with this, several studies have shown that extracts of *Salacia reticulata* (Yoshikawa *et al.* 2002), grape seed (Moreno *et al*. 2003), berries (McDougall *et al*. 2005, McDougall and Stewart 2005), oolong tea (Nakai *et al.* 2005) and green tea (Koo and Noh 2007) inhibit α-amylase, α-glucosidase, trypsin and pancreatic lipase. Inhibition of pancreatic lipase, in particular, by polyphenolic-rich plant extracts offers a natural dietary approach to preventing obesity (Kulkarni *et al.* 2009). This, if combined with the ability of polyphenols to improve antioxidant status and reduce risk of diabetes, hypertension and coronary heart disease (Fraga 2005; Manach *et al*. 2005; Scalbert *et al*. 2005) will result in additional benefits to obesity, the reduction of obesity-related complications

Obesity is characterised by an imbalance between energy intake and energy expenditure. Strategies aimed at preventing or treating obesity often focus on blocking fat absorption and enhancing thermogenesis, amongst others (Bray and Tartaglia 2000). Both green coffee bean extracts (GCBE) and cocoa reportably affect various pathways involved in lipid metabolism. Shimoda *et al.* (2006) has demonstrated that GCBE reduces hepatic triglycerides and visceral fat accumulation while enhancing the activity of carnitine-palmitoyltransferase. Cocoa, on the other hand, has been shown to reduce serum triglycerides, down-regulate fatty acid synthesis and transport in liver and adipose in addition to stimulating thermogenesis (Matsui *et al.* 2004). The aim of this study was to investigate the effect of GCBE and *Theobroma cacao* bean extract (TCBE) on pancreatic lipase activity *in vitro*.

Methodology
Preparation of extracts
GCBE tablets (Quest Vitamins, Birmingham, UK) were weighed to the nearest 0.0001g, ground to a fine powder using a mortar and pestle, then dissolved in 20mL of acidified water (0.2% v/v formic acid/ water) to give acidified-water extract or in 20ml acetonitrile-water-formic acid solution (50:50:2 v/v/v) to give acetonitrile extract. The resulting solutions were then extracted at room temperature at 200 rpm for 30 min on a Luckham, Model R100/TW rotatest shaker then centrifuged at 4000rpm for 15 min at 4 °C to remove any solid material or soluble polysaccharides. The sample was then filtered through Whatman filter paper under vacuum.

For TCBE, 20g of Peruvian *Theobroma cacao* beans (Creative Nature, Surrey, UK) was dissolved in 200ml of acetonitrile-water-formic acid solution (50:50:2 v/v/v), homogenised 3 times using Ultra-Turrax homogenizer (Rose Sci Ltd,) and then extracted on a rotatest shaker, as described above. Samples were treated with rotary evaporation to remove acetonitrile then loaded onto a 12-ml bed volume C18-E Giga solid phase extraction unit (Strata, Phenomenex, UK) previously conditioned with acetonitrile and equilibrated with acidified water respectively. Samples were loaded onto the unit then washed with acidified water to elute the unbound material. The bound fraction, rich in proanthocyanidins, was eluted with 2x 12ml acetonitrile whereas lipid-like material remained bound to the unit.

Estimation of phenolic content
The phenolic content of the diluted extracts was estimated using the Folin Ciocalteu method. Briefly, 250 μl of 1% diluted sample or water (blank) was transferred into a cuvette and 250μl half strength Folin Ciocalteu reagent was added. After 3min, 500μl of saturated sodium carbonate solution (130g/L) was added to each cuvette and the samples were incubated in the dark for an hour. Absorbance was read at 750nm and phenol content assessed against a standard curve of gallic acid (Singleton and Rossi 1965).

Lipase assay

Pancreatic lipase activity was measured according to the method described by Gilham *et al*. (2003) and Lin *et al.* (1996), with some modifications. Briefly, 50µl of varying concentration of GCBE or TCBE (0, 5, 10, 25 and 50µM gallic acid equivalents; GAE) were added to an assay mixture containing 350µl 100 mM Tris buffer (pH 8.2) and 150µl of porcine lipase solution (10mg/ml). The reaction was started by adding 450 µl of the substrate solution (8 mg/ml *p*-nitrophenyl laurate in 5 mM sodium acetate pH 5.0 containing 1% Triton X-100). The samples were then incubated for 2h at 37°C. Following incubation, all samples were centrifuged at 16000rpm for 2.5 min, transferred to cuvettes and read at 400 nm in a UV spectrophotometer. Samples were assayed in triplicates and appropriate sample controls were prepared for each sample concentration to account for interference from GCBE or TCBE. The percentage inhibitory activity of each sample was calculated using the following equation:

$$\% \text{ inhibitory activity} = \left[1 - \frac{sample(abs) - samplecontrol(abs)}{control(abs)}\right] \times 100$$

Results

Phenolic content

The phenol content in the acidified water extract of green coffee beans varied widely between the triplicates compared to acetonitrile extract (Table 1). This variation could have been due to interference from polysaccharides in the acidified water extract, which are known to affect phenolic content measurement by as much as 50% (Stratil *et al.* 2007). For the purpose of pancreatic lipase assay, the acetonitrile extract, which yielded a higher phenol content and lacked soluble polysaccharides, was selected for study. With regard to TCBE, the SPE-bound fraction contained the majority of phenolic compounds (2952.2µg GAE per ml bound fraction vs. 296.2µg GAE per ml unbound fraction) and was consequently used for lipase assay. Aliquots of these extracts containing known phenol contents were dried in a Speed-Vac to remove acetonitrile and acids then resuspended in ultra-pure water at the required concentrations.

Lipase assay

Fig. 1 shows the effect of GCBE and TCBE on pancreatic lipase activity. GCBE showed a J-shaped dose-dependent inhibition of pancreatic lipase with the percentage inhibition ranging from 11.8 to 61.5%. Similar concentrations of TCBE, on the other hand, had a negligible effect on lipase activity. Non-linear regression analysis revealed that the concentration of GCBE required to elicit a 50% inhibition of pancreatic lipase activity (IC50) was ~ 43µg.

Discussion

This study investigated the inhibitory potential of GCBE and TCBE against pancreatic lipase activity. The key finding of the present study is that GCBE inhibits pancreatic lipase activity dose-dependently whereas concentrations of TCBE up to 50 µg/ml had negligible effect on activity. These findings carry several implications since lipid hydrolysis by pancreatic lipase controls dietary lipid absorption and inhibition of this process by drugs such as Orlistat has been shown to improve post-prandial concentrations of plasma triglycerides, remnant-like particles, cholesterol and free fatty acids (Tan *et al.* 2002). This could be of particular relevance to obesity, as post-prandial lipaemia is associated with an array of obesity-associated complications including insulin resistance and dyslipdaemia (**Kolovou** *et al.* 2004; Kolovou *et al.* 2005; Reaven 2005).

GCBE is rich in chlorogenic acid (CGA) and its derivatives (Fig. 2; Table 2). CGA has been shown to reach the intestines in high concentrations with <1% of ingested CGA being hydrolysed in the stomach and the intestine (Lafay *et al.* 2006a). The low breakdown has been explained by the lack of an esterase enzyme capable of hydrolysing CGA in the stomach or the intestine (Plump *et al.* 1999). In fact, in an *in-situ* rat model of CGA perfusion onto upper intestinal tract (jejunum and ileum), 90.8 % of perfused CGA was found to reach the caecum intact (Lafay *et al.* 2006b), suggesting that CGA had considerable longevity once in the small intestine. Similarly, Olthof *et al*. (2000) demonstrated that following 1-4h incubation in duodenal fluids, 95-99% of CGA is recovered. This poor absorption enhances the availability of CGA to intestinal mucosa, the site of lipase action, a property exhibited by several known pancreatic lipase inhibitors including epigallocatechin gallate (Koo et al, 2007) and Orlistat (Zhi *et al.* 1995). Thus, CGA presents an ideal candidate, which might account for GCBE inhibitory activity particularly that caffeine, the other major constituent of GCBE, is known to be a poor inhibitor of pancreatic lipase (Nakai *et al.* 2005). Moreover, CGA has other beneficial effects on the gastrointestinal tract including increased nitric oxide bioavailability (Peri *et al.* 2005). Nonetheless, further research is required to identify the active component of GCBE. This could be achieved by fractionating GCBE constituents and assessing them individually for their inhibitory activity against pancreatic lipase. Synergism between the different constituents of GCBE remains a possibility.

It is also important to acknowledge that *in vitro* studies can often overestimate the biological effect of polyphenols since they do not take in account *in vivo* metabolism of polyphenols. As a result, *in vitro* inhibition of pancreatic lipase activity does not necessarily imply an *in vivo* effect. Moreover, even if pancreatic lipase activity is reduced, improvements in systemic lipid metabolism are not always observed (Shepard *et al.* 2000). Nevertheless, it remains plausible that GCBE might exert an inhibitory activity against pancreatic lipase *in vivo* particularly that a recent study in mice has shown that consumption of GCBE reduces hepatic triglycerides and visceral fat accumulation and enhances carnitine palmitoyl transferase activity, a rate-limiting enzyme that catalyses fatty acid transport to mitochondria for β-oxidation (Shimoda *et al.* 2006). Subsequently, further research is required to examine whether consumption of GCBE could improve post-prandial lipid metabolism or alter the activity of lipoprotein lipase and hepatic triglyceride lipase especially in obesity.

As for the lack of inhibitory activity on lipase shown by TCBE, it is consistent with the poor inhibitory potential of nonesterified flavan-3-ols like (-)-epicatechin and catechin (Nakai *et al.* 2005) which are likely to account for the major fraction of polyphenols in TCBE. However, TC polyphenols have inhibitory actions against other digestive enzymes such as α-amylase (Queseda *et al.* 1996). Subsequently, their role in glucose metabolism might be more important than their role in lipid metabolism.

In conclusion, this study demonstrates that GCBE, but not TCBE, is an effective inhibitor of pancreatic lipase activity. Further research is warranted to examine whether similar effects could be achieved *in-vivo*.

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Table 1 Phenolic content of GCBE expressed as μg GAE/ml extract. Data presented as the mean (standard deviation) of triplicates.

	Total phenolic content (μg/ml)	
Acidified water extract	1180.4	(111.53)
Acetonitrile extract	1456.7	(61.9)

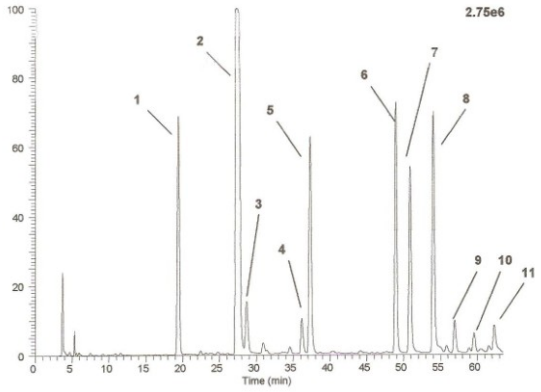

Figure 2 LC-MS profile of green coffee bean extract. Peaks refer to Table 2. Profile is obtained at 280 nm. Assignments supported by previous work (Clifford *et al*. 2003).

Table 2 Retention times of the different LC-MS peaks

Peak No.	**Retention Time**	**PDA**	**M/Z [M-H]**	**MS^2**	**Putative ID**
1	19.42	325	**353.2**, 191.3	191.2, 179.2	3-*O*-caffeolylquinic acid
2	27.33	325	**353.2**, 191.3	**191.2**, 179.2	5-*O*-caffeolylquinic acid
3	28.68	275	**195.3**$^+$	ND	Caffeine
4	36.18	325	**367.2**, 191.2	191.2	3-*O*-feruloylquinic acid
5	37.27	320	**367.2**, 191.2	**191.2**	5-*O*-feruloylquinic acid
6	48.91	325	**515.3**, 353.3	**353.1**, 335.2	3, 4-*O*-dicaffeoyl quinic acid
7	50.84	325	**515.3**, 353.3	353.1	3, 5-*O*-dicaffeoyl quinic acid
8	54.02	325	**515.3**, 353.3	**353.1**, 179.3	4, 5-*O*-dicaffeoyl quinic acid
9	56.92	325	**529.3**, 551.2	**367.1**, 335.2, 193.2	4, 5-*O*-feruloylcaffeoyl quinic acid
10	59.58	325	**529.3**, 551.2	**367.1**, 335.2, 193.2	3, 5-*O*-feruloylcaffeoyl quinic acid
11	62.39	325	**529.3**, 551.2	**367.1**, 335.2, 193.2	3, 4-*O*-feruloylcaffeoyl quinic acid

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Polyphenol-rich dark chocolate: effect on fasting capillary glucose, total cholesterol, blood pressure and glucocorticoids in healthy overweight and obese subjects. By S. Almoosawi, E.A.S. Al-Dujaili and L. Fyfe, *Department of Dietetics, Nutrition and Biological Sciences, Queen Margaret University, Queen Margaret Drive, Musselburgh EH21 6UU, UK*

Excess cortisol is associated with various variables of the metabolic syndrome including hypertension, insulin resistance and dyslipidaemia[1]. This relationship is likely to be mediated via cortisol's ability to regulate NO bioavailability[2,3]. Since increased NO bioavailability is considered the main mechanism by which polyphenols improve glucose, blood pressure and lipid homeostasis, the present study aimed to investigate the effect of two doses of polyphenol-rich dark chocolate on fasting capillary glucose (FG), total cholesterol (TC), systolic (SBP) and diastolic blood pressure (DBP) and urinary free cortisol (F), cortisone (E) and cortisone:cortisol (E:F) in a group of overweight and obese subjects.

The study used a single-blind randomised cross-over design wherein fourteen subjects (eight males, six females; age 36 (SD 11) years; BMI 28 (SD 2.5) kg/m^2) consumed 20 g dark chocolate containing 500 mg or 1000 mg polyphenols for 2 weeks, separated by a 1-week washout period. This 20g portion corresponds to half a portion of habitual chocolate intake and provides similar concentrations of polyphenols as used in previous studies on healthy and hypertensive volunteers that have reported significant reductions in blood pressure following consumption of dark chocolate [4,5].

Capillary FG and TC levels were measured using a calibrated Accutrend GC system (Roche Diagnostics, Burgess Hill, West Sussex, UK). Blood pressure was measured using an automated A&D Medical UA-767 BP monitor (A&D Medical, San Jose, CA, USA). Subjects also completed three 3 d diet and physical activity diaries at baseline and during each intervention. Compliance was measured by direct interviewing, returning of empty chocolate foils and assessment of diet diaries.

Repeated-measures ANOVA revealed a significant reduction in capillary FG levels, SBP and DBP following both treatments. No significant differences were observed between dark chocolate containing 500 mg and 1000 mg polyphenols, suggesting that both doses were equally effective in improving these variables (P>0.05). No changes in anthropometrical measurements, capillary TC and urinary free glucocorticoids levels were observed.

In conclusion, the present study confirms previous findings of a reduction in blood pressure and fasting

	1000 mg polyphenols						500 mg polyphenols					
	Baseline		Week 1		Week 2		Baseline		Week 1		Week 2	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
FG (mmol/l)	4.42	0.70	4.21	0.82	3.97*	0.54	4.42	0.70	3.94	0.72	3.92*	0.86
TC (mmol/l)	5.02	1.01	–	–	4.98	0.90	5.02	1.01	–	–	5.03	0.77
SBP (mmHg)	119	10.5	114	12.0	112**	9.68	119	10.5	114	9.53	112**	9.51
DBP (mmHg)	78.6	7.74	74.5	7.17	74.6**	7.39	78.6	7.74	74.6	4.27	73.0**	5.06
F (nmol/d)	77.3	27.1	–	–	71.2	38.9	86.8	44.1	–	–	78.6	47.3
E (nmol/d)	54.3	26.9	–	–	45.8	17.3	59.6	32.6	–	–	45.8	20.3
E:F ratio	0.71	0.32	–	–	0.75	0.37	0.71	0.31	–	–	0.63	0.21

Mean values were significantly different from those at baseline: *P<0.05, **P<0.01.

glucose following consumption of polyphenol-rich dark chocolate. This effect seems unlikely to be mediated through the glucocorticoid pathway, although involvement of the renin–angiotensin–aldosterone system cannot be excluded. Furthermore, it appears that increasing the polyphenol dose does not result in further improvement in the assessed variables, suggesting that a saturation effect may occur with increasing doses.

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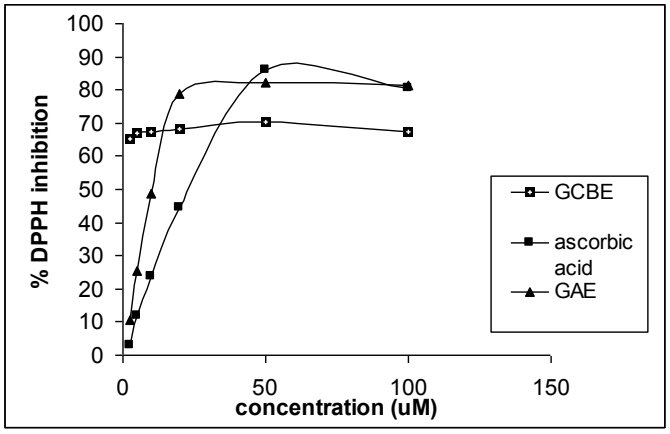
The effect of green-coffee-bean extract rich in chlorogenic acid on antioxidant status of healthy human volunteers. By S. Almoosawi, C. Tsang, I. Davidson, L. Fyfe and E.A.S Al-Dujaili, *Department of Dietetics, Nutrition and Biological Sciences, Queen Margaret University, Queen Margaret Drive, Musselburgh EH21 6UU, UK*

Several studies have linked consumption of green-coffee-bean extract (GCBE) rich in chlorogenic acid (CGA) with reduced blood pressure[1–3]. It is hypothesised that increased antioxidant activity could be one of the underlying mechanisms by which GCBE reduces blood pressure. To test this hypothesis a GCBE preparation rich in CGA was assessed by three extraction methods for antioxidant activity. *In vivo* antioxidant activity was also determined in a group of healthy volunteers.

The phenolic content of GCBE, as determined by the Folin-Ciocalteu method, varied greatly between the ethanolic acidified water (0.2 % (v/v) formic acid) and acetonitrile extract (acetonitrile–water containing 0.2 % (v/v) formic acid; 50:50, v/v): 123 (SD 0.23), 131 (SD 0.66) and 211 (SD 0.51) mg gallic acid equivalents (GAE)/g extract respectively. Similar findings were observed with the Fe^{3+}-reducing ability of plasma (FRAP) assay, wherein the acetonitrile extract exhibited a stronger Fe^{3+}-reducing ability than the ethanolic extract (0.067 mmol/g extract *v*. 0.048 mmol/g extract). The 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) radical-scavenging activity of the ethanolic extract of GCBE was 70.4 % at 50 μM as compared with ascorbic acid (86.1 % inhibition at 50 μM) and GAE (82.2 % at 50 μM).



To examine *in vivo* antioxidant properties of GCBE thirteen healthy volunteers (age 36 (SD 11) years, BMI 28 (SD 2.5) kg/m^2) consumed 200 mg GCBE containing 90 mg CGA twice daily for 2 weeks. *In vivo* antioxidant activity was determined using the Folin-Ciocalteu method and FRAP. There was a significant correlation between urinary polyphenols excretion as determined by the Folin-Ciocalteu method and FRAP (0.664, $P<0.0001$). However no significant increase in urinary antioxidant activity was observed (total phenolics: 173.2 (SD 137.8) mg GAE/ g creatinine *v*. 175.20 (SD 115.7) mg GAE/ g creatinine, $P>0.05$; FRAP: 2.07 (SD 0.9) mmol Fe^{2+}/ g creatinine *v*. 1.56 (SD 0.7) mmol Fe^{2+}/ g creatinine, $P>0.05$). Systolic blood pressure decreased from 119 (SD 10.5) to 114 (SD 9.1) mmHg ($P=0.05$) following the 2-week treatment.

In conclusion, green coffee bean extract has a high antioxidant activity. However, no changes in antioxidant activity are observed in urine. This finding is consistent with previous findings of poor antioxidant activity of hippuric acid, the main urinary metabolite of chlorogenic acid[4]. Further research is required to identify the mechanism(s) of reduction in blood pressure. The antioxidant activity of plasma should also be determined.

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4. Olthof MR, Hollman PC, Buijsman MN *et al*. (2003) *J Nutr* **133**, 1806–1814.

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Effect of green coffee bean extract and chlorogenic acid consumption on 11ßHSD activity in humans and mice

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Increased 11ß hydroxysteroid dehydrogenase type 1 (11ßHSD1) activity is implicated in the development of the metabolic syndrome. Identifying natural compounds that influence 11ßHSD1 activity could lead to novel methods of treating obesity, cardiovascular disease and diabetes. In the present study, we tested the effect of green coffee bean extract (GCBE), rich in chlorogenic acid (CGA), in human volunteers and of CGA in mice on blood pressure (BP), lipid and glucose metabolism. Our hypothesis was that CGA would improve these parameters, by blocking the uptake of microsomal glucose-6-phosphate which in turn would limit the production of co-factor for 11ßHSD1 reductase activity. With local Ethics Committee approval, 13 healthy overweight subjects were given GCBE containing 90 mg CGA twice daily for 2 weeks. Urinary 24 h free cortisol was reduced from 1.0523±0.45 to 0.763±0.40 nmol/kg (P=0.07). Free cortisone excretion was reduced from 0.712±0.38 to 0.432±0.24 nmol/kg (P=0.007). Systolic BP decreased from 119.4±10.5 to 113.8±9.1 mmHg (P=0.05). Fasting plasma glucose (P=0.101), diastolic BP (P=0.114), free cortisol:cortisone ratio (P=0.216) and anthropometrical measurement were not affected. *In vitro*, 11ßHSD1 activity (conversion of added cortisone to cortisol) in isolated mouse microsomes was inhibited dose-dependently by CGA. The effects of feeding diet containing 0.15% CGA for 17 days was tested in male C57BL6 mice. Adiposity was unaffected but liver (27.7±4.9 vs 15.5±2.2 mg/g, P<0.04) and plasma (1.24±0.18 vs 0.86±0.08 mg/ml, P<0.08) triglycerides tended to be reduced. Urinary 24 h cortisol excretion following IP injection of 20 mg/kg cortisone was 30.1±4.1 vs 24.2±5.3 nmol/kg (P<0.4) for control and CGA-treated mice. Peak plasma glucose levels in tolerance tests were earlier with CGA treatment although, over a 2 h period, glucose clearance was not affected.

In conclusion, GCBE decreased urinary cortisol and cortisone excretion in overweight subjects but CGA did not significantly inhibit 11ßHSD1 activity in mice *in vivo*. Treatments lowered blood pressure and triglyceride levels. Further research into the mechanism(s) of these beneficial effects is required.

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International conference on polyphenols, 2009, Harrogate. (Unpublished) P367
Scottish Cardiovascular forum, 2010, University of Glasgow (Unpublished)

Effect of polyphenol-rich dark chocolate on cardiovascular risk factors and glucocorticoids in healthy overweight and obese subjects Suzana Almoosawi; Catherine Tsang; Lorna Fyfe; Emad EAS Al-Dujaili and Davidson I. *Department of Dietetics, Nutrition and Biological Sciences, Queen Margaret University, Edinburgh, UK*

The association between excess cortisol and various parameters of the metabolic syndrome including hypertension, insulin resistance and dyslipidemia is increasingly recognised. The present single-blind randomised placebo-controlled cross-over study investigated the effect of polyphenol-rich dark chocolate (DC) on salivary free cortisol (F) and biomarkers of cardiovascular disease including glucose metabolism, lipid profile and blood pressure (BP) in 21 females with BMI≥25 kg/m^2 and a control group of 23 females with BMI<25kg/m^2 (*n*=42). Volunteers consumed 20g of DC containing 500mg polyphenols or placebo for 4 weeks, separated by a 2-week washout period.

Paired sample t-tests revealed that the reductions in systolic BP (SBP) (-4.17±6.62mmHg from baseline) and diastolic BP (DBP) (-2.29±4.72mmHg from baseline) following polyphenol-rich DC were significantly greater (SBP: *P*=0.007; DBP*: P*=0.003) than the changes seen following placebo. Interestingly, the placebo raised fasting insulin, HOMA-IR and salivary F from baseline by 1.36±4.29mU/L (*P*=0.008), 0.37±1.05 (*P*=0.001) and 13.02±38.38nmol/d (*P*=0.055), respectively, an effect that was significantly different from polyphenol-rich DC which had a negligible effect on fasting insulin (-0.56±2.62mU/L from baseline), HOMA-IR (-0.28±0.60 from baseline) and salivary F (+1.23±6.82nmol/d from baseline). Overweight and obese females, in particular, were found to respond less favourably to placebo than the control group and consequently had higher fasting insulin (*P*=0.006) and HOMA-IR (*P*=0.004) and lower QUICKI (*P*=0.003) after ingestion of placebo compared to polyphenol-rich DC.

In conclusion, this study provides evidence for the metabolic benefits of consuming polyphenol-rich dark chocolate by the overweight and obese group while demonstrating the possibility of adverse effects occurring with polyphenol-poor placebo. The apparent susceptibility of overweight and obese females to insulin resistance following placebo could carry major public health implications and emphasises the need to improve our understating of the relation between polyphenols and health.