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Varsha Rajashekar

School of Life Sciences

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**NOVEL NUTRITIONAL
INTERVENTIONS IN THE
MANIPULATION OF ENERGY
EXPENDITURE AND BLOOD
GLUCOSE REGULATION IN HUMANS**

A thesis submitted in partial fulfilment of the requirements of the
University of Westminster for the degree of Doctor of Philosophy

Varsha Rajashekar

Department of Human and Health Sciences

University of Westminster

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Abstract:

The purpose of the studies described in this thesis was to study the purported metabolic effects of two dietary agents, L-Histidine and Epigallocatechingallate (EGCG). Supplementation of both L-Histidine and pure EGCG in animal models has previously shown effects on components of energy balance and blood glucose regulation. Studies of their supplementation among humans are none (in case of L-Histidine) or very limited (in case of EGCG supplementation). The studies presented herein are novel as there have been no previous studies conducted to measure their effects on thermogenesis in humans and the present studies are the first to do. Three studies were performed to investigate metabolic effects of L-Histidine and two studies were performed to investigate metabolic effects of EGCG among healthy men. Duration of supplementation and varying doses were considered in different studies which led to the variation in study designs and protocols.

Effects of L-Histidine: The metabolic effects of the essential amino acid L-Histidine (25 mg/kg) on healthy and overweight men were investigated in a series of studies. A pilot study was conducted to develop a protocol for measurement of resting metabolic rate (RMR), mean skin (MST), core temperature (CT) simultaneously. The protocol implemented was successful and was applied in all further studies. This study also examined the effect of L-Histidine supplementation (25 mg/kg) on RMR, MST, CT over a short period (3.25 hours). No conclusive results were obtained from this study. A single blind, placebo controlled cross-over study was then conducted to examine the effects of 10-day supplementation of L-Histidine on RMR, MST, CT and fasting blood glucose (FBG) in healthy male subjects (n = 9). Following L-Histidine ingestion, mean body weight (p = 0.008) and FBG (p = 0.04) were significantly reduced compared to baseline measurements. A randomised, parallel, single blinded and placebo controlled study was conducted following on from this to examine the effects of long-term (8 week, 25 mg/kg/day) intake of L-Histidine on RMR, MST, CT, lipid profile, FBG, insulin, leptin, non-esterified fatty acid (NEFA) and body fat composition in overweight and obese but otherwise healthy men (n = 18 (9 participants each in placebo and L-Histidine group)). Results of this study showed significantly decreased weight after 8 weeks (p = 0.007) compared to baseline, irrespective of the type of supplementation. Oral supplementation of L-Histidine improved FBG concentrations and reduced body weight after 10 days' ingestion, however, no effects of long-term supplementation was established.

Effects of EGCG: The second part of this thesis presents studies performed to investigate metabolic effects of pure EGCG. The first study was randomised, cross-over, placebo controlled and single-blinded in design. The effects of an oral intake of 150 mg pure EGCG over an acute period (2.5 hours), on key metabolic parameters and following an oral glucose challenge was measured (n = 8, healthy men). Results of this study were inconclusive on RMR, MST, CT plasma insulin, leptin and NEFA concentrations and appetite regulation. The second study tested the effects of varying doses (75, 150 and 300 mg/day) of pure EGCG on afore mentioned key metabolic parameters when supplemented over a period of 7 days each (n = 8, male). No significant changes in measured parameters were found. The results of these two studies indicate the need for further studies to investigate effects of EGCG on weight loss / weight maintenance possibly in combination with caffeine and/or other green tea flavanols.

Both L-Histidine and EGCG supplementations have previously shown to influence regulators of energy balance, blood glucose and cardiovascular risk factors via activation of the sympathetic nervous system in animal models. The present studies however did not provide any definitive conclusions following EGCG ingestion in healthy humans. L-Histidine supplementation brought about significant decrease in FBG and body weight after 10 days' ingestion but its effect after 3.25 hours and 8 weeks was inconclusive. The effects following supplementation of both L-Histidine and EGCG ingestion needs to be further studied to clarify any potential metabolic consequences in humans.

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Declaration:

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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List of Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BHF	British heart foundation
BMI	Body mass index
BMR	Basal metabolic rate
BNF	British Nutrition Foundation
CLA	Conjugated Linoleic acid
CNS	Central nervous system
COMT	Catechol O-methyltransferase
CT	Core temperature
CVD	Cardio vascular disease
db/db	An obese mouse model with hypothalamic leptin long-form receptor
DIO	Diet induced obese mice model
DOH	Department of Health
EC	Epicatechin
ECG	epicatechin-3-gallate
EGC	epigallocatechin
EGCG	epigallocatechin-3-gallate
ELISA	Enzyme-Linked ImmunoSorbent Assay
FAO	Food and agriculture organisation
FBS	Fasting blood sugar
FMH	α - fluoro methyl histamine
GI tract	Gastro intestinal tract
H1R-KO	H1 receptor knock out
HCA	Hydroxy citric acid
HDC	Histidine decarboxylase
HDL	High density lipoprotein
HPLC	High-performance liquid chromatography
ICV	intracerebroventricular
ip	Intraperitoneal
JAK	Janus kinases
LDL	Low density lipoprotein
LHA	Lateral hypothalamic
l/min	Litres per minute
mg	Milligram

mg/dl	Milligrams per decilitre
ml	Millilitre
mmol/l	Millimoles per litre
mRNA	Messenger ribonucleic acid
MST	Mean skin temperature
mU/L	Milliunits per litre
NAO	National Audit Office
NEFA	Non esterified fatty acid
nm	Nanometre
PBS	Phosphate buffered solution
ppm	Parts per million
PVN	Para ventricular nucleus
RCT	Randomised controlled trials
RER	Respiratory exchange ratio
RNA	Ribonucleic acid
rpm	Revolutions per minute
RQ	Respiratory quotient
SD	Standard deviation
SEM	Standard error of mean
SNS	Sympathetic nervous system
SPSS	Statistical programme for social sciences
STAT	Signal transducers and activators of transcription
TC	Total cholesterol
t-MH	Tele methyl histamine
UCP	Uncoupling protein
UK	United Kingdom
UKPDS	United Kingdom Prospective Diabetes Study
USA	United States of America
VMH	Ventromedial hypothalamus
WAT	White adipose tissue
WHO	World health organisation
μL	Microlitre

1 Introduction

Human health and well-being is dependent upon multiple factors including those heritable traits that may affect health, environmental influences, diet and lifestyle and psychological profile. Amongst these, there is considerable plasticity within the area of diet and lifestyle and the present text is focused in this arena. It is understood that a lack of control of energy balance and frequently associated problems with blood glucose regulation, represent a significant component of risk as understood at both an epidemiological and physiochemical level. The broad aim of the research presented herein is to examine the application of naturally occurring compounds with the aim of positively impacting on human energy balance and blood glucose control. Particular emphasis will be placed on those physiological and biochemical systems upon which the potentially bioactive agents may impact. The thesis will first outline the nature and general mechanistic control of the relevant parameters and will then describe novel interventions with non-pharmacologic agents.

Energy balance regulation is an extremely complex process encompassing multiple interacting homeostatic and behavioural pathways aimed at maintaining constant energy stores (Niswender and Beech, 2008). Body weight control is achieved through integrated interactions between nutrient selection, organoleptic influences and endocrine responses to diet, in addition to being influenced by genetic and environmental factors (Berthoud, 2006). The brain plays a critical role in maintaining whole body energy balance by regulating energy intake and energy expenditure (Schwartz *et al.*, 2000). From an evolutionary stance, humans eat to satisfy their immediate caloric and nutritional requirements from meal to meal, but also to allow energy and nutrients to be stored in anticipation of high energy demands or food shortages. Thus, control of food intake involves the integration of external environmental triggers with multiple internal physiological signals as well as external social elements and reward factors (Zheng and Berthoud, 2007).

Similarly, blood glucose regulation is also dynamically controlled in the body principally by two pancreatic hormones – insulin and glucagon. Together, these hormones co-ordinate the metabolic fate of endogenous glucose, free fatty acids, amino acids and other substrates to ensure that energy needs are met in the basal state and during activity (Costanzo,

2006a). Optimum regulation of blood glucose is imperative in order to maintain nutrient (glucose) homeostasis, therefore preventing chronic metabolic disorders i.e retinopathy, nephropathy etc (UKPDS, 1998).

In broad terms there appears to be an emerging global problem of factors capable of overriding homeostatic control of energy balance. Discourse in academic and medical literature frequently describes an obesogenic environment and even obesogenic economies driving the problem (Burgoine *et al.*, 2009; Horst *et al.*, 2007). It is possible that given the teleological imperative of calorie acquisition and retention, that the modern era represents a special challenge to metabolism in which 'hardwired' responses to food (drive to eat underpinned via powerful reward systems) are more potent than homeostatic controls of energy balance. Perspectives in behavioural science and neurochemistry do seem to support this possibility (Berthoud, 2006; (Davis *et al.*, 2004). In addition it can be argued that there is little evolutionary adaptation to either hyperalimentation or diseases or syndromes of middle to later life ((beyond peak reproductive age) (Bellisari, 2008)).

Investigations into the so called 'obesity epidemic' have revealed an interesting and sometimes counter-intuitive picture of possible drivers of the problem. While much data does show that there is greater availability and affordability of high calorie food (Bellisari, 2008), there is also much evidence that total calories consumed per person in the UK at least, may not have increased significantly (ONS, 2006). In this case the logical assumption is that energy expenditure must have decreased (Hoare *et al.*, 2004). In this connection studies of changing levels of physical activity are of interest and many do suggest that levels of physical activity and hence energy expenditure have been falling steadily for 20 years (BNF, 2007). Increased use of motor vehicles, increasing automation in the workplace and an increase in television watching and other sedentary leisure activities are all implicated in total reductions in average energy expenditure (ONS, 2006).

While public health initiatives have increased general awareness of diet/lifestyle factors in terms of weight and health (Hillsdon *et al.*, 2004), the numbers of overweight and obese have continued to rise (Foresight, 2007). Pharmacological solutions to the problem are expensive and to date, not without side-effects (Rucker *et al.*, 2007). Interest is therefore ongoing in effective interventions with high compliance rates and low incidences of unwanted effects, to unbalance the energy balance equation in favour of weight

loss/control (Hill, 2009; Swinburn *et al.*, 2004). In this connection the manipulation of diet through changing levels of naturally occurring compounds is an attractive option for researchers in the field of human nutrition. The present text is an example of such an initiative.

1.1 Energy balance

1.1.1 Energy intake

Regulation of food intake is controlled by various external and internal stimuli. External stimuli include taste, smell, and sight of food and possibly even time of the day. Eating is also influenced by age, sex and behavioural influences such as and physical activity levels, social, cultural, emotional and psychological needs. Increased buying power, increased availability of ‘fast foods’, access to energy-dense foods all influence food intake (Dulloo and Schutz, 2005c). Internal stimuli for modulation of feeding are more complex and involve neuroendocrine and gastrointestinal signals (Figure 1.1, Table 1.1). The central neuroendocrine pathways include regulatory mechanisms from various parts of the hypothalamus. The peripheral signals for the maintenance of energy intake are controlled by the gut hormones (these include cholecystokinin (CCK), ghrelin, amylin and the glucagon-like peptides (GLP-1 and GLP-2)) (Williams *et al.*, 2000). Adipose tissue also is a major contributor to energy homeostasis. Fat cells secrete several adipokines including leptin, which influence either the storage or mobilisation of fuel along with combustion and energy homeostasis. The central and peripheral regulation of energy intake is extensively reviewed to date by various researchers (Bloom, 2007; Bloom *et al.*, 2005; Chaudhri *et al.*, 2006; Rolls, 2007; Wynne *et al.*, 2004; Wynne *et al.*, 2005).

Figure 1.1 - Sequence of events in food intake control

Reference: (Dulloo and Schutz, 2005a)

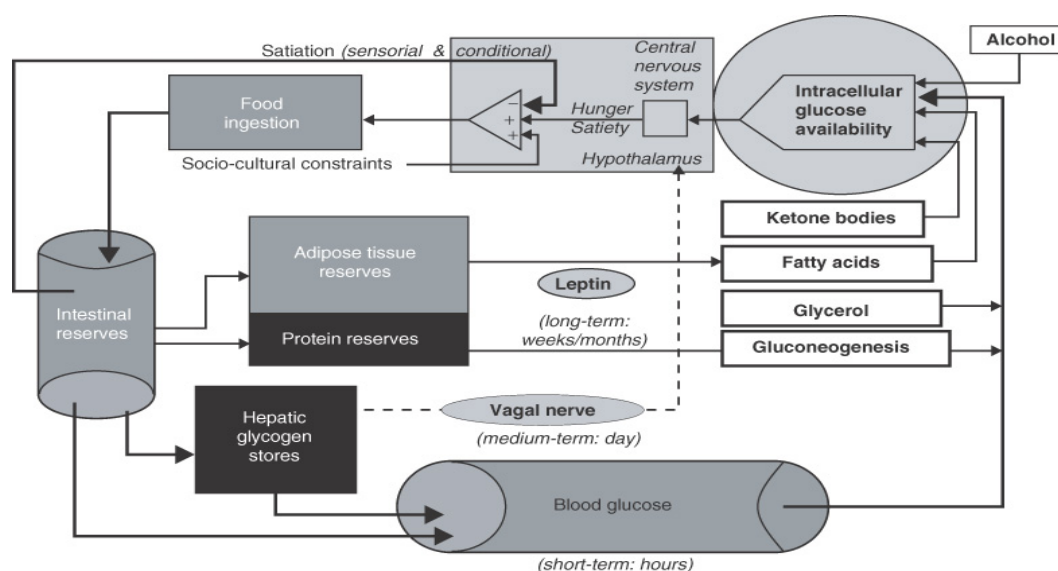


Table 1.1 - Neurotransmitter, hormones, peptides implicated in food intake control

Orexigenic	Anorexigenic
Agouti-related protein (AgRP)	Alpha melanocyte stimulating hormone (α MSH)
Cortisol	Cholecystokinin
Dopamine	Corticotrophin-releasing hormone (CRH)
Endocannabinoids	Glucagon
Glucocorticoids	Glucagon-like peptide 1 and 2 (GLP-1, GLP-2)
Ghrelin	Histamine
Melanin-concentrating hormone (MCH)	Insulin
Neuropeptide Y(NPY)	Leptin
Opioid peptides	Noradrenaline
Orexins	Peptide YY (PYY)
	Serotonin
	Thyrotropin-releasing hormone (TRH)
	Tumor necrosis factor – α

Control of food intake also occurs in a short phase (glucostatic or glycogenostatic), medium term (day-to-day) and over longer terms (lipostatic or adipostatic) (Figure 1.1). The glucostatic theory proposes that the chemoreceptors in the hypothalamic satiety centre are sensitive to the availability and utilisation of glucose (Mayer, 1952). This theory is the basis for the proposition that the control of food intake via the prevention of hypoglycaemia and maintenance of adequate glycogen levels, maintain carbohydrate balance (Flatt, 1995). Long-term control of food intake can be explained by the lipostatic theory (Kennedy, 1950) which proposes that body fat is maintained at a set value and deviations from this value are detected by the hypothalamus via a circulating molecule which is produced in proportion to adipose mass. It is suggested that the hypothalamus then elicits changes in energy intake (and expenditure) to restore the level of body fat stores to its putative set-point. While clearly it is possible to observe both the underweight, and increasingly overweight state in a given population, it is true that for many individuals, long term maintenance of stable weight can be observed in the absence of any conscious effort to maintain such (Schoeller, 1998; Sherwood, 2005).

1.1.2 Energy expenditure

Energy expenditure (EE) consists of different components: namely, basal metabolic rate (BMR), the energy cost of physical activity, the thermic effect of food (post-prandial thermogenesis), and thermoregulation. The role of adipose tissue in thermoregulation is well documented (Frühbeck, 2008). Uncoupling proteins present in the mitochondrial

membrane release energy as heat and thus may play an important role in energy balance (a brief summary of the energy dissipating function of uncoupling proteins is given in the following section). The factors affecting and effecting EE have been extensively reviewed to date (Leibel, 2008; Speakman, 2004; Vermorel *et al.*, 2005; Westerterp, 2008).

A constant expenditure of energy occurs over each 24-hour period in humans. Most of this EE represents the energy required to maintain vital functions (maintaining electrolyte equilibrium across cell membranes, nerve impulse conduction, cellular metabolic processes and respiratory and cardiovascular function). This component of total EE accounts for 60-75% of daily EE and is known as BMR (Frayn, 2003a). BMR is beyond voluntary control and is influenced by are body size (those with greater surface area have higher metabolic rate) (Rolfes *et al.*, 2008) body composition (those with higher fat free mass have higher metabolic rate (compared to weight matched individuals with lower fat free mass, higher fat mass); age (the loss of fat free mass with aging leads to a decline in BMR (Nielsen *et al.*, 2000) ; sex (differences in BMR between men and women is due to difference in body size and composition (Geer and Shen, 2009); hormonal factors (those with endocrine problems i.e hypothyroidism (decreased BMR) and hyperthyroidism (increased BMR), BMR in women fluctuates with the menstrual cycle and is increased in the final stages of pregnancy) (Frary and Johnson, 2004). EE resulting from physical activity can be controlled voluntarily and depends on the type and intensity of physical activity and time spent in different activities (Dulloo and Schutz, 2005).

The human body maintains an average body temperature within the range of 35.5 – 37.7 °C through thermogenesis (Silverthorn, 1998a). EE in response to various thermogenic stimuli has been described as isometric thermogenesis (due to increased muscle tension when no physical work is done), dynamic thermogenesis (heat production of stretched muscle, without any work done), psychological thermogenesis (some psychological states such as anxiety and stress, stimulate adrenaline secretion leading to increased heat production), cold-induced thermogenesis (increased heat production for thermal regulation), diet-induced thermogenesis (heat production following consumption of a meal) and drug-induced thermogenesis (consumption of caffeine, nicotine and alcohol among many others stimulate thermogenesis) (Miller, 1982). Regulation of thermogenesis in the human body can be both voluntary (decreased food intake or decreased physical activity

leads to decreased heat production) and involuntary (increased heat production due to shivering).

1.1.3 Energy regulation and adipocytes

Research presented in later sections will outline interventions with compounds that have possible effects on adipocyte metabolism; therefore the following section is a brief review of the role of adipocytes in the energy balance equation.

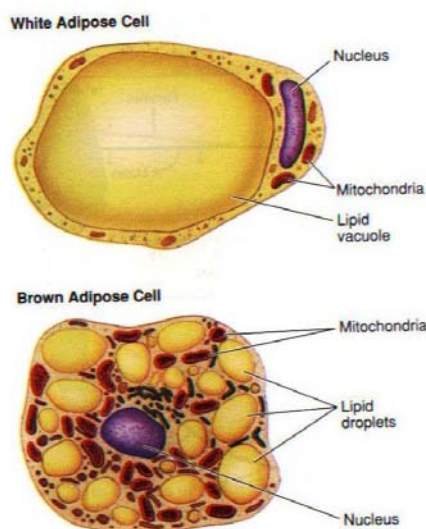
Thermogenesis is an important component of EE and is mediated by the sympathetic nervous system (SNS). Thermoregulation can occur by two mechanisms. Cold-induced thermoregulation or shivering thermogenesis occurs during periods of cold exposure. During this process, heat loss is prevented by vasoconstriction of the cutaneous vasculature and the body uses shivering to generate heat. Non shivering thermogenesis on the other hand is the metabolic production of heat and has been shown as principally occurring in the brown adipose tissue ((BAT) (Himms-Hagen, 1984)).

BAT express uncoupling proteins (UCPs; UCP1 and UCP2) and they function in the generation of heat. The thermogenic effect of BAT is controlled by the SNS and begins with release of noradrenaline from the terminal endings of the sympathetic neurones. Noradrenaline binds with β_3 adrenoceptor and as a consequence fatty acids are mobilised from triglyceride stores and further oxidised following their entry into the mitochondria. The oxidative phosphorylation of fatty acids is then ‘uncoupled’ (ie ATP is not synthesised) by the UCPs present in BAT mitochondrial membrane and the resultant metabolic end product is heat (Trayhurn, 1989). BAT is present abundantly in hibernating animals and to a lesser extent in non-hibernators especially adult humans. Many morphological studies have shown BAT to be present in the perirenal region in humans (Ito *et al.*, 1991). Recent studies have demonstrated the presence of functional BAT in healthy adults in the region extending from the anterior neck to the thorax (Virtanen *et al.*, 2009), which are UCP1-immunopositive (Cypess *et al.*, 2009) and are also activated during cold exposure (Lichtenbelt *et al.*, 2009).

Fat stores in adults are predominantly made up of white adipose tissue (WAT). It is estimated that there is one brown adipocyte for every 200 white adipocytes in humans

(Kozak, 2000). A recent study quantified BAT present in the cervical, supraclavicular depots with combined positron-emission tomography and computed tomography (PET-CT) scanning to be 10g or more in men and women (Cypess *et al.*, 2009). It is of interest however that adipocytes can transdifferentiate from white to brown cell type in response to for example cold ambient temperatures, suggesting that thermic effects at the level of the whole organism may be attainable via BAT (Cancello *et al.*, 1998; Cinti, 2009; Himms-Hagen *et al.*, 2000; Murano *et al.*, 2005). The main distinguishing feature between WAT and BAT are their different metabolic functions. WAT primarily serves as an energy store, whereas BAT's main function is to oxidise substrates to produce heat (Trayhurn, 1989) and this is clearly reflected by the differences in their histological appearances. WAT has one large fat droplet (unilocular structure) and BAT has multiple fat droplets (multilocular structure; Figure 1.2).

Figure 1.2 - Morphology of brown and white adipose cell



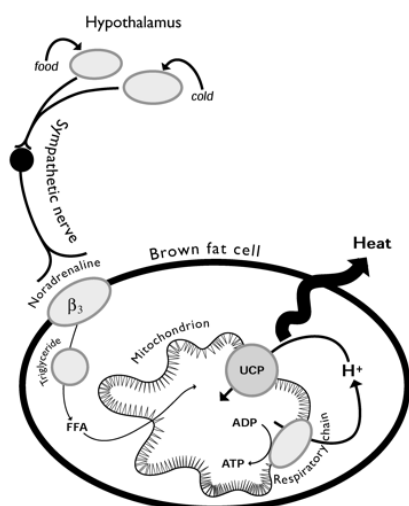
Reference : (Plowman and Smith, 2008)

Further research into the role of BAT and WAT in thermoregulation has led to greater attention towards investigating the role of UCPs in thermogenesis present also in other tissues such as skeletal muscles (Rolfe and Brand, 1996), as they make up 40% of body weight and have been advocated as a major site for adaptive thermogenesis in large mammals, for any expression of UCPs.

Rolfe and others showed that mitochondrial proton leaks exist in tissues other than BAT and contribute as much as 30% of skeletal muscle heat production at rest (Rolfe and Brand, 1996). This led to the discovery of several new members of the UCP family (UCP1, UCP2, UCP3, UCP4, UCP5 and brain mitochondrial carrier protein 1 (BMCP1)) (Ricquier and Bouillaud, 2000). UCP1 is expressed exclusively in BAT and is responsible for thermogenesis in hibernating mammals and mammal neonates. UCP2 is expressed in all tissues so far examined and UCP3 is highly expressed in skeletal muscles and BAT whilst UCP4-5 and BMCP1 are expressed only in the brain (Erlanson-Albertsson, 2003).

Following these breakthrough discoveries, UCP2 and UCP3 have been successfully cloned (Dulloo and Samec 2000, (Ricquier and Bouillaud, 2000)). Transgenic mice generated with skeletal muscle over-expression of UCP were found to have enhanced insulin action and were resistant to weight gain and insulin resistance induced by a high fat diet (Li *et al.*, 2000). It is possible that uncoupling in skeletal muscle by stimulating expression of UCP2 and UCP3 will result in an increase in EE which could be a potential target to treat obesity its complications. Figure 1.3 shows the action of UCPs to release heat. The reader is guided to review articles for further reading about the metabolic actions of UCPs (Costford *et al.*, 2007a; Costford *et al.*, 2007b; Fukuda *et al.*, 2007; Gamber and Ricquier, 2007).

Figure 1.3 - Action of Uncoupling proteins



Reproduced with permission from Dulloo AG and Samec S. Uncoupling proteins: Do they have a role in body weight regulation? *Obesity Matters*, 1999, 2(1), 5-8, (Mediscript, London, UK) (Dulloo and Samec, 2000).

Considering the potential to ‘dissipate’ heat via uncoupling, it is therefore of interest to know if the rate or magnitude of such dissipation can be increased without deleterious effects upon the organism. To this end, research to investigate a specific approach to achieving increased heat production through the induction of uncoupling is detailed later in this thesis.

1.2 Physiological control of blood glucose regulation

Blood glucose is an important fuel and is utilised by muscle, liver, kidney (cortex, medulla), WAT, large intestine, red blood cells and lymphocytes (Dulloo and Schutz, 2005b). It is transported around the body in the blood circulation. The normal levels are maintained between 4 – 7 mmol/l (WHO, 1999), this represent only 3 – 5 g of glucose in circulation at any one time (Benardot, 2006). High levels of circulating blood glucose are damaging as it results in catabolism of muscle proteins and ketosis during acute periods of hyperglycaemia and hyperglycaemia-induced tissue damage, hypertension, dyslipidemia and cardiovascular disease (CVD) over a chronic hyperglycaemic state (Jellinger, 2007). Equally low blood glucose is detrimental because it causes sweating, trembling, anxiety, inability to concentrate, confusion, tiredness, behavioural changes, incoordination, weakness, and drowsiness acutely and if prolonged may lead to seizures, coma and sudden death (Frier, 2002). Therefore the accurate control of the ‘euglycaemic’ state is a physiological set-point which is vigorously defended. Glucose is available from carbohydrates in the diet (post-absorptively) and as glucose released from hepatic glycogen stores. The actual circulating levels reflect a balance between cellular utilisation of glucose, release from the liver and the appearance of glucose in the blood stream following the ingestion of carbohydrates. The hormonal control of the release and uptake of glucose is outlined in the following paragraphs.

1.2.1 Insulin

Blood glucose control is chiefly by the opposing actions of insulin and glucagon. Insulin decreases blood glucose concentrations by increasing glucose transportation into target cells such as muscle and fat tissue via GLUT4 (insulin regulated glucose transporters) into the cell membranes. This pancreatic hormone is synthesised and secreted by the β cells of the islets of Langerhans and has been one of the main adiposity signals to be studied to

date (Schwartz *et al.*, 1992). Factors which influence the secretion of insulin are many and are listed in Table 1.2.

Table 1.2 - Factors influencing the secretion of insulin

Stimulatory factors	Inhibitory factors
Cortisol	Adrenergic agonists
Glucagon	Decreased glucose concentrations
Glucagon-like peptide 1	Exercise
Increased amino acid concentrations	Fasting
Increased fatty acid and ketoacid concentrations	Leptin
Increased glucose concentrations	
Obesity	
Potassium	
Sulfonylurea drugs	

Insulin affects the liver, muscles and adipose tissue by several mechanisms. It inhibits glycogenolysis and thus promotes formation of glycogen in the liver and muscles. Insulin also inhibits gluconeogenesis (Costanzo, 2006). Additionally, insulin decreases fatty acid and ketoacid concentrations in the blood by inhibiting the mobilisation and oxidation of fatty acids in blood and concurrently increasing the storage of fatty acids in adipose tissue. As insulin inhibits lipolysis, decreased fatty acid degradation will result in reduced availability of acetyl-CoA for formation of ketoacids and therefore reducing ketogenesis. Finally, insulin decreases blood amino acid concentrations by stimulating protein uptake into muscle, increasing protein synthesis and inhibiting protein degeneration (Costanzo, 2006).

Insulin also plays an important role in energy balance along with control of blood glucose concentrations. Plasma insulin concentrations are dependent on peripheral insulin sensitivity, which in turn is related to body fat stores and fat distribution with visceral fat being the main determinant (Porte *et al.*, 2002). There is a substantial amount of evidence that insulin acts as an anorectic factor within the central nervous system (CNS) (McGowan *et al.*, 1990; VanderWeele, 1994; Woods *et al.*, 1984). Intracerebroventricular (ICV) administration of insulin and insulin mimetics in rats altered the expression of

hypothalamic genes (POMC and NPY) known to regulate food intake and resulted in a dose dependant reduction in food intake and body weight (Air *et al.*, 2002). Acute injections of insulin antibodies into the ventromedial hypothalamus (VMH) of rats increased food intake suggesting that endogenous insulin normally suppresses food intake (Strubbe and Mein, 1977). Therefore, optimal control of insulin regulation ensuing in blood glucose and energy balance and metabolism are interrelated and essential for longterm well – being.

1.2.2 Glucagon

Glucagon is synthesised and secreted by the α cells of the islets of Langerhans and its actions are in contrast to insulin as it promotes mobilisation and utilisation of metabolic fuels. The actions of glucagon are coordinated to increase and maintain blood glucose concentration. Other main actions of glucagon are increased glycogenolysis, gluconeogenesis, lipolysis and ketoacid formation (McArdle *et al.*, 2007a). Factors which affect glucagon secretion are given in Table 1.3.

Table 1.3 - Factors affecting glucagon secretion

Stimulatory factors	Inhibitory factors
Acetylcholine	Insulin
β -adrenergic agonists	Increased fatty acid and ketoacid concentration
Cholecystokinin (CCK)	Somatostatin
Decreased glucose concentration	
Exercise	
Fasting	
Increased amino acid concentration (especially arginine)	

The main actions of glucagon are on the liver. The mechanism of its action on target cells begins with glucagon binding to the cell membrane receptor; following which cyclic adenosine monophosphate (cAMP) activate protein kinases that phosphorylated various enzymes, which then mediate the physiologic actions of glucagon.

Other neurohormonal factors influencing blood glucose concentrations include growth hormone, thyroxine, cortisol, adrenaline, somatostatin and ACTH, all of which increases blood glucose concentrations. They are less dominant and marginally less important for glycaemic regulation when compared with insulin and glucagon actions. These hormones facilitate increase in glucose production and limit glucose utilisation, suppress insulin release and may act over a prolonged period of time to both directly and indirectly to regulate blood glucose (Costanzo, 2006).

1.3 Exogenous factors influencing energy balance and blood glucose levels

Positive energy balance leads to the development of overweight and obesity and these if uncontrolled, can lead to elevation in blood glucose concentrations due to development of insulin resistance and subsequently other metabolic disturbances such as hyperlipidaemia and hypertension.

Obesity and related diseases are a major contributor to the cost (NAO, 2001) of healthcare and the overall cost is estimated to be £50 billion per annum by the year 2050 if similar trends persist (Foresight, 2007). Public health initiatives such as ‘Change4life’ (January 2009) (DOH, 2008), ‘Small change big difference’ (since 2007) (DOH, 2007), Foresight project (since 2007) (Foresight, 2007) and ‘5 a day’ (since 2005) (DOH, 2004), (BNF, 1999, 2003; DOH, 2001) are currently directed at extending life and also to ensure that increased years are spent free of chronic diseases such as obesity, diabetes mellitus, CVD and metabolic syndrome (syndrome of a cluster of metabolic abnormalities, which comprise of obesity, insulin resistance, hyperlipidaemia and high blood pressure (Grundy *et al.*, 2004)). Below is a brief discussion of factors which influence energy balance and blood glucose regulation.

1.3.1 Diet composition

Diet composition affecting energy balance:

The composition of dietary intake has been suggested to play an important role in regulation of energy balance. A low carbohydrate and high protein diet may cause anorectic effects but may have negative effects on cardiovascular risk factors (increased lipids), despite the weight loss (Astrup, 2005). The amount of water and fibre intake along

with other macronutrients affects satiety (Slavin, 2005). Increased protein intake has been shown to increase satiation and bring about greater weight loss than variation among other macronutrients (Soenen and Westerterp-Plantenga, 2008). Individual amino acids have been suggested as particularly important in this respect, for example, phenylalanine as a dietary precursor of the satiety hormone, cholecystokinin (Pohle-Krauzza *et al.*, 2008). High fat intake appears to have a weak satiating effect and high fat diets which are low in carbohydrates, may promote quick weight loss by inducing ketosis, and if this imbalance between macronutrients continues ketosis will lead to muscle breakdown and may result in certain minerals and vitamins deficiencies (Astrup *et al.*, 2004). In obese individuals, it has been shown that macronutrient composition of the diet has little effect on the rate or magnitude of weight loss unless nutrient composition influences caloric intake (Rolls, 2009; Sacks *et al.*, 2009).

Diet composition affecting blood glucose regulation:

Dietary changes are aimed towards optimisation of blood glucose control in persons with blood glucose dysregulation. Dietary fibre has been shown to modulate the glycaemic response to carbohydrate intake (Ulmius *et al.*, 2009). As glycaemic response to carbohydrate rich foods can be influenced by other factors such as physical state of foods and type of starch present, the *in-vivo* response to varying glycaemic index of foods has been extensively investigated. It has been shown that a diet consisting of low glycaemic index foods results in a reduction in post prandial hyperglycaemia (Fontvieille *et al.*, 1992; Rovner *et al.*, 2009). Recent studies have shown that variation in the type of monomeric and polymeric sugars may affect postprandial metabolism. Intake of a meal consisting of rapidly digestible starch causes significantly more rapid, greater changes in postprandial plasma glucose, NEFA and serum insulin concentrations in comparison with a meal composed of slowly digestible starch (Ells *et al.*, 2005). Dietary fructose seems to produce a smaller postprandial rise in plasma glucose and serum insulin than other common carbohydrates (Bantle, 2009). Variation in macronutrient composition for glycaemic control has also been extensively studied. A low carbohydrate diet (40% carbohydrate) intake may be beneficial over a short period of time to improve insulin sensitivity and blood glucose levels, but dietary adherence to such a dietary change has been found to be poor (Boden *et al.*, 2005; Brinkworth *et al.*, 2004). Low energy, high protein dietary (30% proteins) intake although may facilitate weight reduction and therefore reduction in

circulating glucose concentration, does not affect on blood glucose and insulin concentrations over prolonged periods of time (Nuttall *et al.*, 2003; Sargrad *et al.*, 2005).

1.3.2 Medical and pharmacological influences on energy balance

As compliance with lifestyle modifications of increased physical activity and low fat/calorie eating habits tend to decline over time (Castellani *et al.*, 2003; Shay, 2008), various other means to help minimise the incidence of obesity are being explored. These measures include invasive surgical techniques designed to alter the size of elements (usually the stomach) of the GI tract, or its mechanism of action. These surgical options usually involve either a gastric bypass or gastric banding and are not without potentially serious adverse effects (Pories, 2008; Woodward, 2003). Non-invasive interventions include pharmacological means to help reduce energy intake and reduce nutrient uptake. This is achieved either via suppression of appetite or the induction of malabsorption of nutrients, especially fats. Drug treatments for alleviating obesity (as outlined in Table 1.4) are costly and not without side effects (Rucker *et al.*, 2007; Zieba, 2007).

Table 1.4 - Summary of anti-obesity drugs[†] currently used in healthcare in the UK

Brand name	Active ingredient	Mechanism of action	Side-effects	Dose
Xenical*	Orlistat	Reduces absorption of dietary fat	Oily leakage from rectum, faecal urgency, liquid or oily stools, abdominal distension (gastrointestinal effects minimised by reduced fat intake)	120 mg tds with meals
Reductil **	Sibutramine hydrochloride monohydrate	Inhibits re-uptake of noradrenaline and serotonin	Constipation, dry mouth, hypertension, taste disturbances, depression, anxiety	10 mg od

[†] These drugs are available on prescription only and careful observation of their side-effects are always indicated; * Acts on the gastrointestinal tract; ** Centrally acting appetite suppressant; tds - three times daily; od – once a day; Reference: British National Formulary (BNF, 2008)

1.3.3 Nutritional interventions to control energy balance and blood glucose regulation

Along with public health policies promoting changes in lifestyle, the effectiveness of non-pharmacological measures to address causes and consequences of energy imbalance and associated co-morbidities are being explored (Kumanyika *et al.*, 2008). A breakthrough in identifying such an approach might prove more economical along with conservative methods of treatment in the longer run. There is therefore ongoing interest in developing non-pharmacologic dietary interventions (Norris *et al.*, 2004; Norris *et al.*, 2005) which may have the potential to regulate parameters of energy balance and blood glucose levels, which additionally may have low associated costs to healthcare providers.

Dietary agents may facilitate weight loss by increasing BMR or reducing food intake. Over-the-counter (purported) weight loss supplements (Appendix 1) are widely available and include chromium, guar gum, ephedra, caffeine, chitosan, conjugated linoleic acid, ginseng, glucomannan, green tea, hydroxycitric acid, L-carnitine, psyllium and pyruvate (Lenz and Hamilton, 2004; Saper *et al.*, 2004). The purported mechanism of action of these dietary supplements are suggested to include: increasing EE, modulation of carbohydrate metabolism, increased satiety, increased fat oxidation or reduced fat synthesis, blocking dietary fat absorption, increased water elimination or enhanced mood (DeBusk, 2001). Common dietary and herbal supplements promoted for weight loss frequently lack sufficient supporting efficacy and safety data. More research with larger and better-controlled trials is needed to draw firmer conclusions regarding their effects on energy balance. Table 1.5 gives a brief summary of dietary agents used to regulate energy intake and/or energy expenditure.

Table 1.5 - Dietary agents purported to regulate energy intake and/or energy expenditure

Dietary agent	Purported metabolic effects	Mechanism of action	Adverse effects
Caffeine	Increase energy expenditure	Inhibit phosphodiesterase, resulting in increased cAMP concentrations in cells and prolonged noradrenaline release (Dulloo <i>et al.</i> , 2000; Westerterp-Plantenga <i>et al.</i> , 2006).	↑ Blood pressure, heart rate and cortisol

Dietary agent	Purported metabolic effects	Mechanism of action	Adverse effects
Chitosan	Reduction of body weight	Prevents fat absorption by binding negatively charged fat molecules within the intestinal lumen (Kanauchi <i>et al.</i> , 1995).	Gastrointestinal symptoms such as constipation and flatulence (Mhurchu <i>et al.</i> , 2005).
Chromium	Reduction of body weight and increased insulin sensitivity	Considered to play a role in carbohydrate and lipid metabolism thereby possibly influencing body composition and weight (Anderson, 1998)	Short-term intervention trials have not shown any adverse effects (Anderson <i>et al.</i> , 1997) but long-term intake of chromium has been shown to cause free radical damage as assessed from cell culture and <i>in vivo</i> rat studies (Vincent, 2003)
Conjugated linoleic acids	Reduction in body fat and body weight	Increased fat oxidation and decreased triglyceride uptake in the adipose tissue (DeLany <i>et al.</i> , 1999)	CLA isomer trans-10, cis-12 may produce liver hypertrophy and insulin resistance (Larsen <i>et al.</i> , 2003)
Ephedra	Thermogenic, lipolytic and anorectic effects	Sympathetic activation of the CNS (Astrup <i>et al.</i> , 1992)	Strokes, hypertension, palpitations and tachycardia (Haller <i>et al.</i> , 2000), and may also predispose people to ischemic and hemorrhagic strokes (Chen <i>et al.</i> , 2004)
Glucomanan	Reduction of body weight, blood lipids and blood glucose	Absorbs water within the gut, causing increased satiety and lower caloric intake	None reported
Green tea	Increased thermogenesis and reduction in body weight	Inhibit catechol o-methyl transferase (COMT), thereby increasing noradrenaline effects on thermogenesis (Dulloo <i>et al.</i> , 1999)	None reported
Guar gum	Reduction in weight loss	Soluble fibre in guar gum absorbs water within the gut, causing increased satiety and lower caloric intake (Saper <i>et al.</i> , 2004).	abdominal pain, flatulence, diarrhoea and cramps
Hydroxycitric acid	Increase fat oxidation or reduce fat synthesis	Inhibiting mitochondrial citratelase thereby leading to decreased acetyl coA production and decreased fatty acid synthesis (Lowenstein, 1971)	None reported

Similarly, numerous dietary agents are being investigated for their role in blood glucose regulation. These include ivy gourd (*Coccinia indica*), ginseng species, garlic (*Allium sativum*), fenugreek (*Trigonella foenum graecum*), fig leaf (*Ficus carica*), nopal (*Opuntia streptacantha*), *Gymnema sylvestre*, bitter melon (*Momordica charantia*), *Aloe vera*, chromium, magnesium, vitamin E, L-carnitine, vanadium, alpha lipoic acid. Their purported mechanisms of hypoglycaemic action are suggested to be facilitation of peripheral glucose uptake, decreased rate of carbohydrate absorption and so on (Table 1.6 shows summary of dietary agents along with their purported mechanism). Further evidence is needed from controlled trials using these supplements as current supportive evidence is few and long-term effects of these supplements need to be further examined (Yeh *et al.*, 2003).

Table 1.6 - Dietary agents purported to regulate blood glucose

Dietary agent	Purported metabolic effects	Mechanism of action	Adverse effects
Alpha lipoic acid	Increased glucose uptake, improve insulin sensitivity	Enhanced glucose uptake in muscle and prevents glucose-induced protein modifications (Jacob <i>et al.</i> , 1999; Konrad <i>et al.</i> , 1999)	None reported
<i>Aloe vera</i>	Decrease FBG	Aloe gel contains glucomannan, fibre which accounts for hypoglycaemic effects (Kim <i>et al.</i> , 2009)	None reported
Bitter melon (<i>Momordica charantia</i>)	Decrease FBG, PPG	Increased insulin secretion, tissue glucose uptake, liver muscle glycogen synthesis, glucose oxidation (Akhtar, 1982; Singh <i>et al.</i> , 2008)	None reported
Chromium	Decrease FBG, PPG	Increase number of insulin receptors to enhance receptor binding and to potentiate insulin action (Althuis <i>et al.</i> , 2002; Anderson <i>et al.</i> , 1997)	None reported
Fenugreek (<i>Trigonella foenum graecum</i>)	Decrease FBG, PPG, urine glucose	Delayed gastric emptying, slow CHO absorption, inhibition of glucose transport from the fibre content, modulation of peripheral glucose utilisation (Sharma <i>et al.</i> , 1990; Vijayakumar and Bhat, 2008)	None reported
Fig leaf (<i>Ficus carica</i>)	Decrease PPG	Facilitation of glucose uptake peripherally (Fрати <i>et al.</i> , 1990; Serracarla <i>et al.</i> , 1998)	None reported
Garlic (<i>Allium sativum</i>)	Decrease FBG	Increased secretion or slowed degradation of insulin, increased glutathione peroxidase activity and improved liver glycogen storage (Bailey and Day, 1989; Shane-McWhorter, 2001)	None reported

Dietary agent	Purported metabolic effects	Mechanism of action	Adverse effects
Ginseng species	Decrease FBG, HbA1C	Decreased rate of CHO absorption into the portal hepatic circulation, increased glucose transport and uptake mediated by nitric oxide, increased glycogen storage and modulation of insulin secretion (Vuksan <i>et al.</i> , 2001; Vuksan <i>et al.</i> , 2000; Vuksan <i>et al.</i> , 2008)	None reported
<i>Gymnema sylvestre</i>	Decrease FBG, HbA1C	Increase in glucose uptake and utilisation, increase in insulin release through cell permeability and stimulation of β cell function (Liu <i>et al.</i> , 2009; Persaud <i>et al.</i> , 1999)	None reported
Ivy gourd (<i>coccinia indica</i>)	Decrease FBG and PPG	Due to insulin-mimetic properties (Kamble <i>et al.</i> , 1996; Kamble <i>et al.</i> , 1998; Kuriyan <i>et al.</i> , 2008)	None reported
L-Carnitine	Increase glucose uptake, glucose storage, glucose oxidation	Possibly effect insulin sensitivity and enhance glucose uptake and storage (Giancaterini <i>et al.</i> , 2000; Mingrone <i>et al.</i> , 1999)	None reported
Magnesium	Decrease FBG, improve insulin sensitivity	A cofactor in various enzyme pathways involved in glucose oxidation, it modulates glucose transport across cell membranes, may increase insulin secretion and/or improve insulin sensitivity and peripheral glucose uptake (Paolisso <i>et al.</i> , 1992; Rumawas <i>et al.</i> , 2006)	None reported
Nopal (<i>opuntia streptacantha</i>)	Decrease FBG, insulin	Contains high soluble fibre and pectin content, which may affect intestinal glucose uptake (Shapiro and Wong, 2002)	None reported
Vanadium	Decrease FBG, increase insulin sensitivity	Enhanced glucose oxidation and synthesis, modulate glucose output (Cusi <i>et al.</i> , 2001; Goldwaser <i>et al.</i> , 2000)	Transient gastrointestinal discomfort
Vitamin E	Decrease FBG, PPG	Attributed to its lipophilic anti-oxidant activity, with possible influences on protein glycation, lipid oxidation, insulin sensitivity and secretion (Mooradian <i>et al.</i> , 1994; O'Connell, 2001)	None reported

FBG- Fasting blood glucose; PPG- Post prandial glucose, HbA1C- Glycosylated haemoglobin, CHO- carbohydrate

1.4 Choice of two natural substances with potential to affect energy expenditure and blood glucose control

The remit for the present research was to evaluate, via a review of the literature, likely candidates for novel nutritional interventions affecting key metabolic parameters. Within this the broad intention was to investigate possible mechanisms for increasing EE and stabilising/reducing blood glucose. This is in accord with a generic interest in nutritional

science which seeks to find qualitative perspectives by which health benefits may be elicited from the diet. The concept of functionality of food components is well established and has proved highly efficacious (eg iodisation of salt (UNICEF, 2008), fortification of commonly eaten foodstuffs with vitamins (FAO, 1996), use of plant esters to sequester cholesterol from bile (Plat and Mensink, 2005) etc). In keeping with the search for functional benefit derived from specific manipulation of dietary components, the amino acid L-Histidine and the tea-derived catechin, Epigallocatechingallate (EGCG) were identified as novel agents for testing. The criteria by which they were deemed suitable were that there should be sufficient data and mechanistic information regarding mode of action in the extant literature to suggest that appropriate research could demonstrate a beneficial effect of these compounds. The review of the literature (described in the following study introductions) revealed much interest and relevant preliminary work with L-Histidine and EGCG with respect to energy balance and blood glucose regulation. The dimension of efficacy in free living humans was however found to be incompletely represented for both compounds, suggesting the need for further investigation via controlled interventions.

1.5 Overarching hypothesis for the thesis

Supplementation with L-Histidine or EGCG in healthy subjects (age range: 18 – 55 years) will increase resting metabolic rate, decrease fasting blood glucose and lipid concentrations and affect other parameters of energy balance (reduction in dietary intake and increased lipolysis).

1.6 Aim of the research

The aim of this research was to investigate in humans, the potential exergonic and other metabolic properties of two dietary supplements: an essential amino acid, L-Histidine, and the main catechin present in green tea, EGCG. A review of literature of human trials investigating the metabolic effects of both these dietary agents shows a lack of evidence with very few controlled human studies having been conducted to date. However, many intervention studies have been conducted in animal models to examine the effects of L-Histidine and EGCG and the results from these studies show positive effects of these interventions with respect to increased EE, reductions in body fat, plasma glucose

concentrations and circulating lipids. There is a need therefore to examine these effects in humans and further explore the practicalities of oral ingestion, such as dose and period of supplementation, with respect to potential positive changes in body composition and energy balance.

The metabolic effects of L-Histidine and EGCG were analysed by measurement of RMR, body composition, mean skin and core temperatures, fasting blood glucose and lipids, insulin, leptin and changes in dietary intake.

The aims will be addressed by the following objectives:

- To conduct a pilot study in normal weight volunteers into the effect of L-Histidine supplementation (over 3.25 hours) on parameters of energy balance.
- Investigate the effect of 10 days L-Histidine supplementation on parameters of energy balance and metabolism in normal weight volunteers.
- Investigate the effect of L-Histidine supplementation among overweight and obese subjects on parameters of energy balance and metabolism over an 8 week period.
- Examine the effects on healthy men of acute (2.5 hours) EGCG supplementation on energy balance and metabolism.
- Examine the effect of varying doses of EGCG supplementation on healthy men over a period of 7 days on energy balance metabolism.

1.7 Structure of thesis

The general methods employed to measure outcomes in all studies are detailed in Chapter 2. Chapter 3 is divided into five sections beginning with the review of literature (section 3.1) of metabolic effects of L-Histidine/histamine and sections 3.2, 3.3 and 3.4 serially describing the outcomes of L-Histidine ingestion over an acute, 10-day and 8 weeks period of supplementation respectively. Findings from these studies and further directions for future studies investigating L-Histidine ingestion are discussed in Section 3.5. Chapter 4 describes the studies conducted with EGCG, beginning with the review of literature of EGCG's metabolic effects (section 4.1). Sections 4.2 and 4.3 explore the effects of EGCG ingestion following acute and chronic periods of supplementation among healthy men respectively. Findings from these studies and further directions for forthcoming studies are

discussed in section 4.4. Chapter 5 provides a final conclusion, to allow a clearer understanding of the implications of the studies' results.

2 General Methodology

In the following section, the methods employed for collection of data and the justification for the selection of these methods will be described in detail. In the subsequent chapters of this thesis the reader will be referred to this chapter to find details of methods. Table 2.1 provides a concise summary of the measures performed in each individual study.

2.1 Ethical approval and informed consent:

Experimental plans were prepared for the proposed studies to test the effects of L-Histidine and EGCG ingestion in healthy humans. These proposed plans were used to apply for ethical approval. All studies were approved by the University of Westminster Ethics Committee (Application numbers 04/05-62, 06/07/27; Appendices 2 and 8, respectively). At the time of the initial ethics application, investigations on the rate of lipid oxidation following varying durations of resistance exercise and changes in lipid oxidation following alterations in fat intake in response to ingestion of cordeceps sinesis supplementation in humans was envisaged. Following further review of literature, the experiments on the topic of lipid oxidation following resistance exercises were not conducted as the author chose to investigate effects of dietary agents only, without any changes in physical activity. Continual review of literature on the effects of Cordeceps sinesis on EE and blood glucose concentrations revealed a lack of *in vitro* data to support the proposed *in vivo* mechanisms of its actions among animal models. Therefore, due to the paucity of data regarding effects of cordeceps sinesis among humans the author decided to further study the literature for other dietary agents capable of influencing neurochemical activity *in vivo* and therefore bring about changes in EE and blood glucose concentrations. EGCG has been studied extensively in the recent years and considerably more data regarding its *in vivo* and *in vitro* activities among animals and humans are emerging in the literature. This dietary agent was therefore chosen to be studied and a further ethics application detailing the proposed studies supplementing EGCG among humans was submitted to the University of Westminster Ethics Committee (Appendix 8).

Prior to the commencement of the first test session, participants were provided with a written research study information sheet which detailed the test protocol, safety measures taken and any potential risks associated with the tests (Appendices 5 – 7 and 10 - 11).

Informed consent (Appendices 3 and 9) was obtained from all participants at the start of the study.

2.2 Sample recruitment:

Participants for all studies were recruited by advertising around central London campuses of the University of Westminster, London, England, by means of fliers and posters as well as advertising in local newspapers (Metro and Evening Standard). Lean, healthy men and women along with overweight and obese male participants were recruited according to the following criteria-

Inclusion criteria: Male, (females only for the pilot study Chapter 3.2), BMI < 35, healthy, between the age group of 18 - 55 years, not diagnosed with any chronic medical condition such as cardiac problems, hypertension or diabetes mellitus, not following any stringent training schedule and not supplementing their diets with dietary agents (any amino-acid or green tea ingestion) were included into the studies.

Exclusion criteria: Participants were screened for any health related issues which made them unsuitable as test subjects (including chest pains, respiratory difficulties, heart disease, thyroid disease and orthopaedic problems). Participants were excluded if they were on medications expected to alter the results of the study; including medications that affect metabolism or weight loss; (such as, psychiatric medications, appetite suppressants (Sibutramine), fat absorption inhibitors (Orlistat) and thyroid medications) or suffering from hay fever (taking anti-histamines). Furthermore, subjects who were obese due to a confounding diagnosed disease (for example, Cushing's syndrome) were not recruited.

Apart from the pilot study (Chapter 3.2) which involved investigating the acute effects of oral ingestion of L-Histidine, all other studies were conducted on male participants only as the variations in body temperature during different stages of the menstrual cycle in women would interfere with the mean skin and core body temperature measurements (Kuhl, 2002). All participants recruited for the studies were Caucasians (white). This selection was unintentional as only Caucasian (white) male responded to the adverts.

Table 2.1 - Summary of methods used in studies conducted

Parameter measured	Chapter 3.2 (Pilot study)	Chapter 3.3 (30 day cross-over L-Histidine study)	Chapter 3.4 (8 week parallel L-Histidine study)	Chapter 4.2 (Acute EGCG study)	Chapter 4.3 (Chronic EGCG study)
Height	✓	✓	✓	✓	✓
Weight	✓	✓	✓	✓	✓
Skinfold measurements		✓	✓		✓
BMR	✓	✓	✓	✓	✓
RER	✓	✓	✓	✓	✓
Skin temperature	✓	✓	✓	✓	✓
Core temperature	✓	✓	✓	✓	✓
Fasting blood glucose concentrations	Accutrend GC	✓	✓		
	HemoCue			✓	✓
Fasting total cholesterol concentrations	Accutrend GC	✓			
	Miniphotometer LP20		✓		✓
HDL			✓		✓
LDL			✓		✓
Heart rate				✓	✓
Blood pressure			✓	✓	✓
Dietary analysis		✓	✓	✓	✓
VAS				✓	
Plasma NEFA concentrations			✓	✓	✓
Plasma insulin concentrations			✓	✓	✓
Plasma leptin concentrations			✓	✓	✓

2.3 Participant handling at test sessions:

The handling of all participants prior to the start and during the test session was standardised as follows:

- All participants were instructed to consume their last meal prior to the test session by 8 pm or earlier, allowing an overnight fasting period of 12 hours prior to the test session.
- In order to keep the physical activity to a minimum in the morning of the test session, participants came to the laboratory by public transport.
- On the test day, participants arrived at the laboratory between 7 am – 10:30 pm. The arrival time was kept constant for each participant.

- The participants changed into light indoor clothes, voided urine and were asked to rest for 10 minutes. At the first test session, this time was used to discuss the study information sheet and procedures to be carried out, and informed consent was obtained. At following test sessions, participants used this time to settle in and relax.
- Anthropometric measurements (height, weight) and skinfold measurements were then recorded (details in 2.5 and 2.6). Participants inserted the rectal probe in privacy and further measurements according to each individual study were carried out. These are outlined below.
- Resting metabolic rate (RMR); each test period was 45 minutes. For this period, participants lay on the bed in a comfortable position. Noise was kept to a minimum to avoid arousal stimuli and participants were instructed to remain awake and motionless. Participants listened to meditation music (Sleep, Nukarma) (details in 2.7).
- At the same time, mean skin and core temperatures were recorded continuously as participants were connected to the Squirrel Data Logger with thermistors firmly attached at four sites (described in detail in 2.9) along with having previously inserted the rectal probe.
- At the end of RMR monitoring, participant's blood pressure was measured thrice. The skin thermistors were detached from the participants and the rectal probe removed by the participants in privacy.
- Finger prick blood samples for measurement of fasting blood glucose and total cholesterol were obtained. For later studies (Chapters 3.4, 4.2, 4.3), whole blood samples by venepuncture were obtained following the finger prick blood sample.
- At the end of each session, completed food diaries were collected. Following the test session, subjects were told to continue with the relevant next phase of the study (wash out period or taking capsules and maintaining food diaries).

2.4 Dose of supplementation:

L-Histidine:

Excess L-Histidine ingestion (from a 5% increase in diet to up to 50 g/kg of diet) in rodents causes hypercholesterolemia, hepatomegaly, lipid dysregulation, copper and zinc

loss (Hitomi-Ohmura *et al.*, 1992; Ohmura *et al.*, 1986; Aoyama *et al.*, 1999; Harvey *et al.*, 1981; Soloman and Geison, 1978). Investigations researching the effect of excess dietary histidine in humans are few. Single oral administration of 64.8 g/day in men caused anorexia, taste and smell dysfunction and cerebellar dysfunction with significant decreases in serum zinc concentrations and significant increases in urinary zinc excretion (Henkin *et al.*, 1975). Conversely, 4 g/day oral ingestion of L-Histidine in gelatine capsules among 8 volunteers for a period of 2 weeks, found no significant changes in appetite, taste and smell perceptions (Schechter and Prakash, 1979). The recommended daily requirement of L-Histidine is 12 mg/kg/day (840 mg/day for a 70 kg adult) (FNB, 2002). Reviewing the above literature regarding dose responses to histidine and taking into consideration the WHO/FAO (Kriengsinyos *et al.*, 2002) guidelines on estimated daily requirement of histidine in adults, the test dosage was doubled and chosen to be 25 mg L-Histidine per kilogram body weight to supply a supraphysiological dose in order to bring about metabolic changes. Calculating this dose with mean pre-treatment body weight of study participants results in an average dose of 2.1 g of L-Histidine (1.8 – 3.5 g/day), which was much lower than previously studied doses and was considered safe and non-toxic. Analysis of dietary intake pre-treatment for the L-Histidine supplementation studies showed that the average histidine intake for all study subjects (n = 27) was 2.15 g/day. The orally supplemented L-Histidine was an additional 98% (average) (84 – 163%) over and above the dietary intake and it was therefore presumed to result in metabolic changes.

EGCG:

The United States Department of Agriculture (USDA) database for flavonoid content of foods specify the mean content of EGCG in 100 ml of brewed green tea as 26.00 - 82.89 mg depending on the addition of caffeine or other flavours (USDA, 2003b). There is no statistical data on consumption of green tea among population in the UK; hence for the purpose of the study, the EGCG dose was chosen to be based on an intake of 2 cups/day (250 ml/cup) of green tea. The lower range of EGCG content in brewed green tea (USDA, 2003b) was used to calculate the single oral dose of EGCG to be used in the acute ingestion study (EGCG for 100 ml = 26 mg; for 1 cup (250 ml) = 65 mg; for 2 cups = 130 mg). This dose was rounded up to 150 mg and was used to investigate a single oral dose over a period of 2.5 hours (Chapter 4.2).

Single oral doses of purified EGCG ranging from 50 mg to 1600 mg have been well tolerated among healthy humans (Ullmann *et al.*, 2003). In the chronic ingestion of EGCG study (Chapter 4.3), oral doses of up to 300 mg/day (3 doses, 75, 150 and 300 mg/ day) (as this represents an approximate intake of 1 – 4.5 cups (1 cup measuring 250 ml) of green tea/day) have been used which is much lower than the doses administered in the safety studies, therefore no adverse effect of EGCG administration was envisaged.

2.5 Measurement of height and weight:

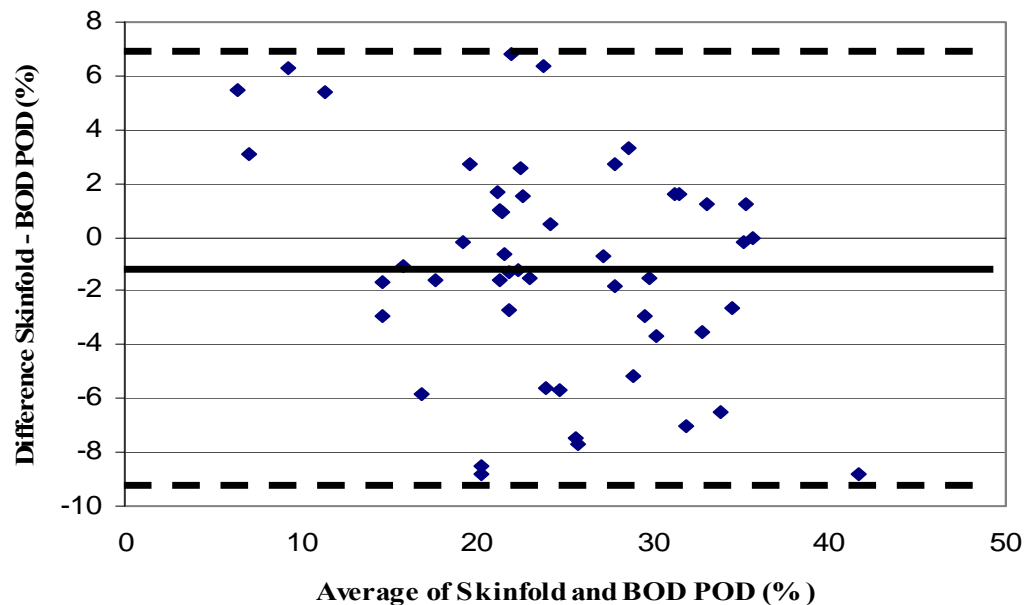
The full standing height of all subjects was measured using a wall mounted stadiometer (Holtain Ltd, Britain) at the beginning of the first test session. The subjects were standing straight, looking ahead with their feet flat and heels almost together. They had their arms at the sides with shoulder blades, buttocks and heels touching the stadiometer (Gibson, 2005). Body weight was measured at the start of each test session, with subjects wearing light indoor clothes and no shoes on a digital balance (Salter 461, UK) and recorded to the nearest 100 g.

2.6 Measurement of body fat percentage by skin-fold measurements:

Estimation of body fat was by measurement of skinfold thickness in all studies presented in this thesis. This method was chosen over the use of BODPOD system (version 4.1, Body Composition System; Life Measurement Instruments, Concord, CA) which measures body volume by using air displacement plethysmography as it was unavailable to use due to technical malfunction at the beginning of the project. Skinfold thickness measurements provide an estimate of the size of subcutaneous fat depots, which in turn, provides an estimate of body fat. In a previous study conducted in the Department of Human and Health Sciences, University of Westminster, to measure bias, if any, in the different methods of measuring percentage body fat, it was found that both BODPOD and skinfold measurements by the use of Harpenden callipers can be used interchangeably as the results obtained from both techniques were similar and reliable (CV of 3.5%) (Stiegler *et al.*, 2005). There were no statistically significant differences when comparing the BODPOD and skinfold measurement by analysis of variance. A Bland-Altman plot (Figure 2.1) for body fat determined by the two techniques revealed a wide scatter of data points without an apparent trend ($r = -0.16$, not-significant). The limits of agreement of the difference

between the two techniques indicate that 95% of the results for percentage body fat determined by skinfold measures were between 6.9% higher and 9.1% lower than results from the BODPOD measurement (Figure 2.1).

Figure 2.1- Agreement between BODPOD and skinfold measurements, showing bias (-1.1%) and limits of agreements (-9.1%, +6.9%)



For the purpose of studies presented herein, skinfold measurements were employed to measure body fat composition as this method has been shown to detect changes in body fat reliably and accurately over time (Demura and Sato, 2007; Orphanidou *et al.*, 1994). Care was taken to perform these measurements by a single, trained anthropometrist so as to avoid interrater differences and maintain accuracy (Jebb and Elia, 1993; Kispert and Merrifield, 1987) (investigator is a trained level one anthropometrist, International Society for the Advancement of Kinanthropometry).

Skinfolds were measured by the use of Harpenden callipers (British Indicators, England) and a Harpenden anthropometer (Holtain, UK), all on the right side of the body and at four sites: namely, subscapular, biceps, triceps and supraspinale (ISAK, 2001) (see Appendix 23 for sites). Skin folds were measured three times at each site to the nearest 0.1 mm and

the mean of the closest two measurements was used for the calculation of percentage body fat. The percentage body fat was calculated first by determining body density:

$$\text{BD (Body Density)} = C - [M (\log_{10} \text{ of all four skin folds})]$$

where, C and M are constants according to gender and age (Durnin and Womersley, 1974).

The result from the calculation of BD is substituted into the Siri equation to estimate percentage body fat (Siri, 1993).

$$\% \text{ BF (Body Fat)} = ([4.95 \div \text{BD}] - 4.5) \times 100$$

2.7 Measurement of resting metabolic rate (RMR):

Many energy releasing actions in the body depend on oxygen use. Measuring a person's oxygen consumption either during rest or physical activity, provides an indirect and highly accurate estimate of EE (McArdle *et al.*, 2007b).

RMR for all studies was measured with the use of Deltatrac II (Datex-Ohmeda, Finland) which employs indirect calorimetry using a continuous, open circuit, ventilated canopy system. Numerous studies have been conducted to validate and test the reliability of Datex Deltatrac to accurately measure gas exchange. The measurements of VO_2 and VCO_2 has been found to be accurate under a variety of simulated clinical conditions and when compared with other models (Makita *et al.*, 1990; Phang *et al.*, 1990; Takala *et al.*, 1989; Weissman *et al.*, 1990).

In the following studies described in this thesis, the measurement unit was adjusted for ambient pressure and calibrated using a reference gas mixture of 95% oxygen and 5% carbon dioxide before the beginning of each test session. Participants were required to lie on the bed for 45 minutes with a perspex hood over their head. They were encouraged to be in a complete state of rest but not asleep. To help them achieve this, they were supplied with a set of headphones with calming music playing (Sleep, Nukarma), to block out any distractions. For calculation of RMR, the initial 15 minutes' data were not used as this time was given for acclimatisation under the hood.

Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were calculated from continuous measurements of oxygen and CO_2 concentrations in inspired and expired air.

The estimate of RMR was calculated using the formula:

RMR (kilocalorie) = (3.9 * litres O_2 used) + (1.11 * litres CO_2 produced) (Weir, 1949).

2.8 Measurement of respiratory exchange ratio (RER):

RER reflects the pulmonary exchange of carbon dioxide and oxygen under differing physiological and metabolic conditions (McArdle *et al.*, 2007b). This measurement is an indicator of relative percentages of carbohydrate and fat utilisation by the whole body. It was calculated by using the formula:

$$RER = VCO_2 \text{ (litres/minute)} \div VO_2 \text{ (litres/minute)}$$

where VCO_2 and VO_2 data were obtained from Deltatrac during RMR measurement.

2.9 Measurement of skin and core temperature:

Mean skin (MST) and core temperatures (CT) were measured in all studies presented in this thesis to examine the effects of L-Histidine and EGCG supplementations on thermogenesis. This interest in the measurement of possible changes in MST and CT was to observe variation in adaptive thermogenesis, thereby contributing to an increase in overall EE. Both L-Histidine and EGCG supplementations have been shown to increase expressions of UCP 1 and 2 in BAT and UCP 3 in WAT among animal studies (Kao *et al.*, 2006; Masaki *et al.*, 2004; Takahashi *et al.*, 2002) and UCPs produce heat instead of ATP. Therefore MST and CT were measured to detect any increase in body temperature, and as a consequence, increase in EE.

The measurements of skin and core temperatures were taken using a Squirrel data logger (Grant Instruments Ltd, 2020 series, Cambridge, UK). Measurements of skin temperature involved placing thermistors on four different skin sites on the body (namely the sternum, forearm, calf and thigh) and securing them with hypoallergenic tape (TransporeTM, 3M, USA). Core temperature was measured by a rectal probe inserted to a depth of 8cm beyond the external anal sphincter (Reilly and Waterhouse, 2005). The participants inserted the probe themselves, in privacy, under sterile conditions. The probe and skin thermistors were

left in position for 45 minutes (period of time when participants were under the perspex hood during the measurement of RMR). There are different methods (invasive and non-invasive) for the measurement of CT; such as, measuring temperature orally, in the axilla or the tympanic membrane. Invasive measurement techniques include measurements taken in the oesophagus, pulmonary artery or the urinary bladder or the use of a radio-sensitive pill. The use of these measurement sites have been found to be complicated and rectal temperature is considered the gold standard for CT measurement (Moran and Mendal, 2002; Reilly and Waterhouse, 2005). The Squirrel data logger was calibrated to record data every minute. The 45 minute data thus obtained were averaged and used to calculate the final skin and core temperatures.

Mean skin temperature (MST) was calculated by using the equation:

$$\text{MST } (^{\circ}\text{C}) = 0.3 * t_{\text{chest}} + 0.3 * t_{\text{arm}} + 0.2 * t_{\text{thigh}} + 0.2 * t_{\text{leg}} \text{ (Ramanathan, 1963)}$$

where t_{chest} , t_{arm} , t_{thigh} and t_{leg} are the temperatures recorded by the chest, arm, thigh and leg thermistors, respectively.

2.10 Blood metabolites:

Blood glucose and lipid concentrations reflect the individual's well-being. Reduction in any elevated concentrations reveals positive changes towards health. Measurement of their markers such as fasting blood glucose, high density lipoprotein, and total cholesterol indicate any predisposition to diabetes or hypercholesterolemia. Hormones such as insulin and leptin are implicated in various metabolic processes such as blood glucose regulation, lipolysis, amino acid uptake, glycogen synthesis (McArdle *et al.*, 2007a, c) and appetite regulation. Fatty acids are delivered to the tissues in the form of non-esterified fatty acid (NEFA). NEFA is released by lipolysis of adipose tissue triacylglycerol (Yaqoob *et al.*, 2005). Measurement of NEFA therefore gives an indication towards rate of lipolysis and overall indication of metabolic changes. These blood metabolites are measured in the following studies to assess the metabolic pathways of action of the two supplements.

2.11 Measurement of fasting blood glucose:

An Accutrend GC monitor (Roche, Germany) was used to measure fasting blood glucose (FBG) for all L-Histidine studies. The process involved obtaining a drop of capillary blood

sample with the use of an Accucheck soft clix pro lancet device (Roche, Germany) and placing the drop of blood on a BM-Accutest test strip (Roche, Germany). A reading (in mmol/l) from the glucometer was obtained after 30 seconds. The technique used during measurement was as instructed by the manufacturer. A reliability study previously conducted to assess the Accutrend meter showed good correlation coefficients ($r = 0.988$) with those obtained by the laboratory method (Devreese and Leroux-Roels, 1993).

The system automatically performs a control check before each measurement by reading the bar code on the test strip and by comparing the code-strip data stored before the blood sample is applied. In addition, the control solution Accutrend® Control G (glucose) was used for performance checks. The intra-assay co-efficient of variation (CV) performed for studies described in Chapters 3.3 and 3.4 was 5.5% at 3.7 mmol/l (the hypoglycaemic range) and 4.8% at 9.6 mmol/l (the hyperglycaemic range).

For all the EGCG studies, a HemoCue® Glucose 201 analyser (HemoCue AB, Sweden) was used for measurement of FBG. This change in apparatus was due to availability of more funds and thus procurement of HemoCue® Glucose 201 analyser for all further studies in the Department of Human and Health Sciences, University of Westminster, London. The method involved obtaining a capillary blood sample using the Accucheck soft clix pro lancet device (Roche, Germany). By capillary action 5µL of blood were drawn into the cavity of a HemoCue® Glucose 201 microcuvette which was then placed into the analyser for measurement. All techniques used in the measurements were as per manufacturer's instructions. The accuracy and precision of HemoCue® has been found previously to be comparable to other laboratory diagnostic methods (Fogh-Anderson, 2004). The HemoCue® Glucose 201 analyser has an internal electronic self-test system. It automatically verifies the performance of the optronic unit of the analyser every time it is switched on. This self-test is also performed at regular intervals if the analyser is on for a period of time. The intra-assay CVs ($n = 10$ for each) using Eurotrol GlucoTrol-NG control solution, by the current investigator during the EGCG studies, were 1.63% at 6 mmol/l and 1.15% at 10 mmol/l.

The accuracy of the type of blood sample used (capillary blood samples) for glucose monitoring have been studied during various conditions such as measurement during gestational diabetes and among patients undergoing haemodialysis, and has been found to

be comparable and highly correlated with plasma blood glucose levels investigated in the laboratory (Bosch and Hyneck, 1984; Meriggi *et al.*, 1988). Correlation of results obtained from capillary blood samples to laboratory assay technique ranged from 0.74 - 0.99 in hospitalised patients with diabetes (Bustamante *et al.*, 1994), during resuscitation (mean difference of 0.01 mmol/l between the capillary sample and lab result) (Kumar *et al.*, 2004) and in general intensive care patients (the mean difference (bias) was 0.12 mmol/l and precision 0.77 mmol/l) (Kulkarni *et al.*, 2005). Capillary blood glucose measurements have been found to be significantly different when compared with venous blood sample (Boyd *et al.*, 2005) and in healthy volunteers (correlation coefficient with venous blood sample was 0.24) (Funk *et al.*, 2001). In all of the studies, all subjects were euglycaemic and only capillary blood samples have been employed for measurement of FBG. Hence consistency has been maintained and accuracy was expected to be high as shown with the CVs.

2.12 Measurement of total cholesterol:

The measurement of total cholesterol for the first L-Histidine study (Chapter 3.3) was done using an Accutrend GC monitor (Roche, Germany) and an Accu-chek soft clix pro lancet device (Roche, Germany) to obtain a capillary blood sample, which was then dropped onto an Accutrend Cholesterol test strip (Roche, Germany). The test strip was completely covered by the blood sample and a result was obtained after 120 seconds when the cholesterol value (in mmol/l) was read on the monitor. Measurement technique was carried out according to manufacturer's instructions. The accuracy and precision of Accutrend GC monitors to measure cholesterol concentrations has been found to be high in previous studies (correlation co-efficient = 0.96 against enzymatic laboratory method and CV of 0.07% and differences of +2.5% to -3.2% with the laboratory technique for total cholesterol assay (Cañizo *et al.*, 1996; Gottschling *et al.*, 1995) respectively.

The system automatically performs a control check before each measurement by reading the bar code on the test strip and by comparing the code-strip data stored before blood is applied. The control solution Accutrend® Control CH1 (cholesterol) was used for performance checks. The intra-assay CV (n = 10) performed by the current investigator was 3.61% at 4.5 mmol/l.

2.13 Blood procurement for plasma and serum samples:

Venous blood samples (8 ml) were obtained by a trained phlebotomist. Following mild occlusion of the upper arm using a tourniquet, a suitable antecubital vein was located, and the skin sterilised using a cotton wool ball containing Hydrex Denaturated Ethanol B 96% (Adams Healthcare, Leeds, UK). The needle (21-gauge blood collection set, Becton, Dickinson and Company, Plymouth, UK) was inserted, the tourniquet was released, and blood was collected in heparinised Vacutainer® tubes (Becton, Dickinson and Company, Plymouth, UK). Then the needle was removed and sufficient pressure was applied until bleeding had stopped. The blood was immediately centrifuged for 11 minutes at 3000 rpm in room temperature (Centaur 2, MSE, Centaur, Leicester, UK). Supernatant plasma was aliquoted into conical tubes (Eppendorf, Hamburg, Germany) and stored at -80°C (Ultralow Freezer, Revco Technologies, Asherville NC, USA) until analysis. To obtain serum samples, blood was collected in serum separator tubes with clot activators (9.5 ml) and stored at room temperature for 1 hour to allow for full coagulation, centrifuged and immediately analysed.

2.14 Measurement of total cholesterol (TC), High density lipoprotein (HDL) and Low density lipoprotein (LDL) by Miniphotometer LP20:

Due to additional University investment during the course of the study, analyses of TC and further components of lipid profile (HDL and LDL) were performed using a spectrophotometrical method (miniphotometer LP20, Dr Lange, Germany) for the studies described in Chapters 3.4 and 4.3.

Venepuncture was used to collect 9.5 ml of whole blood using the vacutainer system (Becton and Dickinson, USA) and drawn into a serum separator tube (Becton and Dickinson, USA). This was thoroughly mixed and left standing at room temperature for 1 hour, before being centrifuged for 12 minutes at 3500 rpm. This resulted in the separation of the blood components and accumulation of serum at the top of the tube. This serum was collected and used to measure total cholesterol, LDL and HDL with the use of miniphotometer LP20 and reagents from Dr. Lange, Germany. The procedures employed for measurements were in accordance with the manufacturer's instructions. The repeatability measure for the miniphotometer was measured by measuring the same serum

sample, 5 times, by the current investigator and using the same procedures. The standard deviation (0.02) was small and well within the probability of 95%.

Measurement of total cholesterol – Serum (20 µl) was pipetted into a cuvette containing pre-pipetted cholesterol buffer solution as following manufacturer's instructions. The starter reagent (in start caps) was added, mixed and left to stand at room temperature for 5-8 minutes. A blank cuvette (containing no blood) was used to calibrate the miniphotometer before measuring the sample cuvette. The results were read at 546 nm. The intra-assay CV (n = 5) for total cholesterol performed by the current investigator was 4.0% at 7.05 mmol/l.

Measurement of LDL – LDL precipitant (100 µl) was added to 200 µl serum and left to stand at room temperature for 15 minutes before being centrifuged at 1500 rpm for 15 minutes. Clear supernatant (50 µl) obtained after centrifugation, was pipetted into a cuvette containing pre-pipetted cholesterol buffer solution and mixed thoroughly with the starter reagent (in start caps). This was then left standing at room temperature for 5-8 minutes. A blank cuvette (containing no blood) was used to calibrate the miniphotometer before measuring the sample cuvette. The results were read at 546 nm. The intra-assay CV (n = 5) for LDL cholesterol was 7.01% at 5.16 mmol/l.

Measurement of HDL – HDL precipitant (50 µl) was added to 500 µl serum and left to stand at room temperature for 10 minutes before being centrifuged at 3000 rpm for 5 minutes. Clear supernatant (50 µl) obtained after centrifugation, was pipetted into a cuvette containing pre-pipetted cholesterol buffer solution and mixed thoroughly with the starter reagent (in start caps). This was then left standing at room temperature for 5-8 minutes. A blank cuvette (containing no blood) was used to zero the miniphotometer before measuring the sample cuvette. The results were read at 546 nm. The intra-assay CV (n = 5) for HDL cholesterol was 1.54% at 1.30 mmol/l.

2.15 Measurement of plasma NEFA:

Plasma NEFA concentrations were measured on an ILAB 600 clinical chemistry analyser at the Hugh Sinclair Nutrition Research Group laboratory at the University of Reading. A WAKO NEFA-C kit (Wako Chemicals, Germany) was used in the enzymatic quantitative colorimetric test. The samples, after treatment with reagents according to manufacturer's

instructions (see Appendix 21 for detailed instructions), were measured at a wavelength of 560 nm. Quality control was done with the use of 3 control sera (control serum I, II and I + II) with the assay. The values of all control sera were within the expected values for the assay. The measuring range of the assay kit is up to 2mmol/l. The lowest detectable level on the ILAB is 10 $\mu\text{mol/L}$ and the range of the assay on the machine is 10-4000 $\mu\text{mol/L}$. A single point calibration was used for the test; the concentration of the calibrator was 1000 $\mu\text{mol/L}$. The intra-assay CV (n = 20) was found to be 1.2%.

2.16 Measurement of plasma insulin:

To measure insulin concentrations in plasma, an Enzyme-Linked ImmunoSorbent Assay (ELISA) technique was employed. The kit was manufactured by Merckodia AB, Sweden. The procedure involved using 25 μl of unknown plasma along with standard solutions with a range of 0 – 200 mU/l. The assay was performed as specified by the manufacturer (see Appendix 19 for details and standard curve) the results were read at 450 nm. The R^2 value of calculated values from the assay was 0.9998. The intra-assay CV for 6 replicates of 4 samples was found to be between 2.8% and 4.0% (data obtained from pack insert).

2.17 Measurement of plasma leptin:

An ELISA technique was employed to measure leptin concentrations in plasma samples. The ELISA kit was obtained from DRG Instruments GmbH, Germany. The leptin ELISA test kit required 15 μl of standard (the range of concentration was 0 – 100 ng/ml), control and unknown plasma. Following the manufacturer's instructions (see Appendix 20 for details and standard curve) the results were read at 450 nm. The R^2 value of calculated values from the assay was 1. The intra-assay variation of 10 replicates of 2 samples was 5.95% and 6.91% (data obtained from pack insert).

2.18 Measurement of heart rate:

Heart rates were measured in all subjects participating in the EGCG studies as analysis of heart rate variability is an useful tool to assess cardiovascular function and stress (Omerbegovic, 2009). Heart rates were measured using a Polar heart rate monitor S610i (Polar Electro, Kempele, Finland). A Polar T61 transmitter belt was firmly attached onto

the participant's chest during the measurement of RMR (45 minutes) in each test session. The belt detected the electrocardiogram (ECG) and sent an electromagnetic signal to the Polar S610i wrist receiver which was worn by the participant at all times during the test session. This recorded the ECG and the recording was downloaded by using Polar IR Interface™, onto a computer for ease of analysis. The Polar IR Interface™ communicates between the Polar heart rate monitor and Polar software. The data thus obtained were used to calculate the mean heart rate for each test session and used for further analyses. The precision and accuracy of portable heart rate monitors have been well documented for subjects both at rest and during different types and intensities of exercise (Durant *et al.*, 1993; Gamelin *et al.*, 2006; Seaward *et al.*, 1990).

2.19 Measurement of blood pressure:

A bosomedicus sphygmomanometer (Bosch + Sohn GmbH, Germany) was used to measure participants' blood pressure. The measurement of blood pressure was performed as per the manufacturer's instructions. Participants were supine and rested during the measurements. All three measurements were recorded after the measurement of RMR, while the participants lay on the bed. The participants were rested and comfortable, and all the measurements were performed at the same point during each test session. An average of 3 recordings was calculated and used for analysis.

2.20 Dietary assessment and analysis:

All participants kept 3-day food diaries during varying periods of the studies. Diet diaries recorded pre-treatment were used as baseline data and those recorded during the course of the study were used to detect changes from baseline intake.

2.20.1 Formulation of diet diary and a guide to keeping the diet diary to record food intake:

In view of the data available regarding the validity and reliability of 3-day dietary recalls, a three-day prospective diet diary was designed for the analysis of food intake during the study (Appendix 15). This was the chosen method as it provides a direct measure of the current diet (Bates *et al.*, 2005). The 3-day dietary intake record has been validated for use

among all age groups and when compared with other methods of dietary intake assessment (Crawford *et al.*, 1994; Lührmann *et al.*, 1999; Schröder *et al.*, 2001). The food diary was prepared based on a model used nationwide by the European Prospective Investigation of Cancer and Nutrition (EPIC) (Frobisher and Maxwell, 2003). Participants were asked to record their dietary intake on 2 weekdays and 1 day of the weekend, before and after the supplementation.

A photographic guide to aid in the keeping of the food diary was prepared based on the ‘photographic atlas of food portion sizes’ and ‘food portion sizes: a user’s guide to the photographic atlas’ formulated by the Food Standards Agency (Nelson *et al.*, 2002). This involves the use of colour pictures of everyday foods in varying quantities where each quantity was assigned a code, to be entered into the food diary (Appendix 16). This was used as a visual aid to assist with quantification of portion sizes and also aid to minimise coding errors. This was further used to input quantities of food intake during analysis.

2.20.2 Analysis of food intake:

Quantification of dietary intakes was carried out using the prospective 3-day diet diary and photographic atlas as described above. The food diaries were coded and data entered into the dietary analysis software, Dietplan 6 (Forestfield Software Ltd, West Sussex, UK). This allowed the calculation of nutrient intakes. The Dietplan 6 database is pre-installed with the full set of UK food tables (from the 5th and 6th Editions of McCance and Widdowson’s *The Composition of foods* plus all the published supplements, including Fatty acids and the *Composition of Foods Integrated Data Set*), Dietary Reference Values (COMA, 1991), Food Portion Sizes (MAFF 2nd Edition, 1993), Food Labelling Data (EC Directive, 1990). A small number of foods were not defined in Dietplan 6, which were then defined individually by the author by inputting each ingredient separately to make up the reported food product. The food database and results are produced in pdf format, with clearly labelled graphs and tabulated list of foods. The guidelines for selecting a dietary analysis system specify choosing the software based on characteristics such as validity of the database, clear and complete documentation, an easy-to-read computer output (Frank and Pelican, 1986; Stumbo, 2008). Dietplan 6 met all of these characteristics as its database was based on National standard food tables and the search and analysis process is efficient with clearly produced reports and was therefore chosen to be used for dietary analyses for all studies presented in this thesis. Averages of all the 3-day diaries were used

during statistical analyses for dietary intakes of all macronutrients (pre and post treatments).

Histidine quantification of reported dietary intakes was performed by using the INTERMAP (International Collaborative Study of Macronutrients, Micronutrients and Blood Pressure) database to control for dietary histidine intake in the analyses following a personal communication (discussion followed by email on 16/6/09) with Dr Claire Robertson, UK Country Nutritionist (Schakel *et al.*, 2003) (permission to use the data from the principal authors was sought via email, their response is awaited). The database constituted of 1448 commonly consumed foods in UK and histidine content expressed as mg/100 g.

To assess the validity of the reported dietary intakes, the Goldberg cut-off method was used on all diet diaries. This was performed to quality control the reported dietary intakes as underreporting of dietary intakes might occur in all dietary assessment methods and among all age groups and in both males and females (Livingstone and Black, 2003). The Goldberg cut-off method is based on the principle that an individual of a given sex, age and body weight needs a minimum energy intake and intakes below this level are considered to be unacceptable representation of habitual intake (Goldberg *et al.*, 1991). This method compares the daily mean reported energy intake (EI_{rep}) to the FAO/WHO/UNU (1985) recommended energy intakes for a sedentary lifestyle of $1.5 \times BMR$. BMR estimated (BMR_{est}) was calculated using the Schofield equation (Schofield, 1985). Underreporting for individuals can be identified by calculating the ratio $EI_{rep} : BMR_{est}$. The ratios were then compared with the cut-offs given in table 5 (page number: 576) of Goldberg *et al.* (1991) for $n = 1$ and number of days of dietary assessment used to calculate the EI_{rep} . A ratio of less than 1.0 was considered as energy intake lower than the minimum intake needed and therefore considered as under-reported.

The challenges in obtaining accurate dietary intake information are manifold (Black *et al.*, 1991; Cook *et al.*, 2000; Samaras *et al.*, 1999; Voss *et al.*, 1998). The present series of studies attempted wherever possible to circumvent issues of subject compliance and recording accuracy through the use of clear and consistent guidelines, and adjuncts to written information in the form of visual cues (shown to enhance recording accuracy) (Turconi *et al.*, 2005). In this way, and based on best practice as described in the extant

literature, the data collection process regarding qualitative and quantitative aspects of intake was made as robust as possible. As mentioned above, macronutrient intake, as it would affect total energy intake was evaluated for validity through the use of the Goldberg Cut-off method. In addition, it should be noted that the designs of the studies in the present series were cross-over in nature with subjects acting as their own controls. Therefore, consistency in the explanations given to subjects regarding their role as self-recorders of nutrient intake was of paramount importance and prioritised by the present author.

2.20.3 Assessment of appetite sensations:

Visual analogue scales (VAS) were used to assess subjective appetite sensations during the first EGCG study (Chapter 4.2). The validity and reproducibility of VAS for measurements of appetite sensations, with and without diet standardisation prior to the test day has been shown previously (Flint *et al.*, 2000). The VAS (Appendix 14) used were 100 mm in length with words anchored at each end, expressing the most positive and the most negative rating. This was used to assess hunger, satiety, fullness and prospective food consumption. The questionnaires were made as small booklets showing one question at a time. Participants marked on the scale according to their appetite sensations at the beginning and end of each test session.

2.21 Analysis of biomarkers:

Biomarkers of L-Histidine metabolism were not analysed in the studies described as existing literature indicates that oral consumption of the doses utilised (25mg/kg BW, > 2 x RDA) in the present studies leads to a significant increase in the levels of blood histidine. L-Histidine is rapidly and extensively absorbed when administered orally or intravenously in humans, with bioavailability of oral doses reaching 80% or higher (Wade and Tucker, 1998). An oral dose of 100 mg/kg L-Histidine (average intake of 7 g for a reference man weighing 70 kg) increased mean peak plasma concentration to 8.8-fold above baseline and an oral dose of 50 mg/kg L-Histidine (average intake of 3.5 g for a reference man weighing 70 kg) has shown to increase mean peak plasma concentration to 4.7-fold above baseline (Sitton *et al.*, 1988). It has been suggested that the relationship of L-Histidine dose (oral or intravenous) and peak plasma concentration is linear up to at least 200 mg/kg (Wade and Tucker, 1998). These elucidations in pharmacokinetics of L-Histidine administration

provide evidence that the doses administered in the studies presented in this thesis (1.9 – 3.5 g/day) will be rapidly absorbed and will increase plasma concentrations and may account for any changes observed in the study parameters. Following the acute phase of L-Histidine metabolism, there is a compartmentalisation of resultant compounds and financial constraints upon the research dictated that it was not feasible to assay possible metabolites or products of further biochemical reactions induced by L-Histidine consumption.

The pharmacokinetics of EGCG in humans is well documented and it has been shown that EGCG concentrations in plasma accounts for 2% of the ingested amount (Nakagawa *et al.*, 1997) and the plasma and urine concentrations reach undetectable levels in 24 hours following ingestion and it has been suggested that most of the ingested EGCG does not get into the bloodstream and the absorbed EGCG is preferentially excreted through the bile to the colon (Lee *et al.*, 2002). With respect to EGCG, there is no normal blood level for this compound and it was determined from the literature that 4 cups of green tea would deliver ~300mg EGCG. It was therefore decided that the test dose would be designed to reflect this level of intake as this would be of broad relevance (ie apply to a large number of tea drinkers) in terms of any effects measured. Following the completion of all EGCG supplementation studies, the analysis of urine following ingestion of EGCG over an acute and chronic period was performed to detect EGCG in the urine. However, the procedure (in collaboration with University of Reading) at the time of submission of this thesis was not optimised and is not presented in this thesis

2.22 Statistical analyses:

2.22.1 Sample size calculation:

The number of subjects required for both the studies was determined by sample size calculations to determine the size of significant differences in EE and metabolic responses that may be observed after ingestion of L-Histidine and EGCG. Sample size calculations were performed taking into account the probability of failing to detect a real difference (type II error) and the significance level, that is the probability of incorrectly rejecting the null hypothesis (type I error) (Armitage and Berry., 1971). The sample size was calculated to incorporate a 5% significance level (two sided), and 80% power (1- β). The standardised

difference was calculated as the physiologically significant difference divided by the standard deviations (SD) of the measures in question: weight loss, EE and fasting blood glucose.

L-Histidine studies:

One of the aims of the L-Histidine studies was to detect a difference in fasting blood glucose concentrations following chronic ingestion of L-Histidine. Prior to commencement of this investigation, review of literature showed that apart from the current study, there were no other studies performed to test efficacy of L-Histidine supplementation in humans. The investigator was required to use the key metabolic parameter (blood glucose) measured in the below mentioned study, as a guide during sample size calculation.

From a previous study (Song *et al.*, 1998), it was identified that after ingestion of bovine prostrate powder (containing cyclo His-Pro, a metabolite of thyrotropin releasing hormone, and a cyclic form of the di-peptide L-Histidine and Proline) men with type 2 diabetes showed significantly reduced fasting blood glucose levels as much as 27 mg/dl. This was used to calculate the sample size for this study. The significance level was set at 5% (α , two-sided) and a power of 80% (corresponding to a 20% chance of a type II error) is required to detect a mean difference between the experimental and control groups.

The following equation was used to determine the size of the sample (Armitage and Berry, 1971):

$$n > 2 \left\{ \frac{(z_{2\alpha} + z_{2\beta})\sigma}{\delta_0} \right\}^2$$

Using the equation with $z_{2\alpha} = 1.96$, $z_{2\beta} = 0.842$, $\sigma = 20$, $\delta_0 = 27$ gives

Therefore, at least nine participants need to be included in the study to measure the effect of the intervention within subjects.

$$n > 2 \left\{ \frac{(1.96+0.842) 20}{27} \right\}^2 = 8.6$$

EGCG study:

Using the same equation for sample size calculation as above, it was determined that 8 participants were required to take part in studies investigating the efficacy of EGCG ingestion.

According to a double-blind study conducted by Nagao *et al* (2005), body weight of healthy men were significantly lower in the group ingesting green tea extract than in the control group. The authors concluded that ingestion of catechins might be useful in the prevention and improvement of lifestyle-related diseases, mainly obesity. As this was the main objective to the study the weight loss data (73.9 ± 1.8 kg before ingestion of green tea extract and 71.5 ± 1.7 kg after 12 weeks of ingestion) was used in sample size calculation as below-

Using the equation with $z_{2\alpha} = 1.96$, $z_{2\beta} = 0.842$, $\sigma = 1.7$, $\delta_0 = 2.4$ gives

$$n > 2 \left\{ \frac{(1.96+0.842) 1.7}{2.4} \right\}^2 = 7.87$$

2.22.2 Statistical tests employed to analyse data:

Data are presented as mean \pm standard error of mean (SEM). Raw data obtained from the study, were entered into the Statistical Programme for Social Sciences (SPSS 14.0 for windows, SPSS Inc, Illinois, USA) for analyses. To test the normal distribution of pre-treatment data, a Kolmogorov-Smirnov test was performed on body weight, body mass index (BMI) and percent body fat as these measures were used to recruit participants for the studies. Baseline data for all studies except the pilot study were analysed for normal distribution. The significance value for all data sets were >0.05 (non-significant) confirming that the distribution of the sample was not significantly different from a normal distribution.

Table 2.2 - Tests of normality performed for body weight, BMI and body fat measures at baseline for subjects who completed the studies supplementing L-Histidine and EGCG

	Kolmogorov-Smirnov	
	df	Significance
Baseline data for Chapter 3.3		
Body weight	9	0.200
Body fat %	9	0.200
BMI	9	0.200
Baseline data for Chapter 3.4 1=Placebo group, 2=Histidine group		
Body weight	1	9
	2	9
BMI	1	9
	2	9
Body fat %	1	9
	2	9
Baseline data for Chapter 4.2		
Weight	8	0.200
BMI	8	0.200
Baseline data for Chapter 4.3		
Weight	8	0.168
BMI	8	0.200
Body fat %	8	0.187

Significance > 0.05, therefore all data are normally distributed.

Repeated measures analysis of variance (ANOVA) was performed to detect statistical significance among variation in data due to treatment and over time. A 0.05 level of significance was used so that the probability of falsely rejecting the null hypothesis (type I error) is only 5%. Sphericity of data (equality of variances of the differences between treatment levels) was examined by observing the 'Mauchly's test of Sphericity' output. If the p value was ≤ 0.05 , it was concluded that there were significant differences between the variances of difference and sphericity was not met. If in any instance this violation of sphericity was noted, the Greenhouse-Geisser correction was used. Bonferroni post-hoc test for pair-wise comparison was performed to observe significant difference between groups, as this method is the most robust, especially in terms of power and control of the type I error rate (Field, 2000).

To detect any linear relationship between variables, Pearson's correlation coefficient tests were performed. A p value of ≤ 0.05 was considered significant. Paired samples t-tests

were performed for the acute EGCG study as only two groups of data were available. As previously, a p value ≤ 0.05 was considered significant. Statistical analyses for the pilot study (Chapter 3.2) were performed as the sample size was small. Analyses via non-parametric tests were not possible as more than three group means needed to be compared. ANOVA is used when comparing means for three or more groups of a single independent variable (Ritchey, 2007). As analysis of variance (ANOVA) compares each group mean to the grand mean (mean for all cases in the sample), it was the most appropriate method to test differences among three or more means and was therefore chosen for the pilot study (Ritchey, 2007). The variations between all data, between treatments, between subjects and between time periods were calculated. Following on from this data, the sum of squares and mean square were calculated. The results for the F test were obtained by using table of values according to the degrees of freedom (Table 2.3).

Table 2.3 - Five percentage points of the F-Distribution

ν_1	Upper 5% points									
	1	2	3	4	5	6	7	8	9	10
ν_2										
1	161.45	199.50	215.71	224.58	230.16	233.99	236.77	238.88	240.54	241.88
2	18.51	19.00	19.16	19.25	19.30	19.33	19.35	19.37	19.38	19.40
3	10.13	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81	8.79
4	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00	5.96
5	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77	4.74
6	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10	4.06
7	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68	3.64
8	5.32	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.39	3.35
9	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18	3.14
10	4.96	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02	2.98
11	4.84	3.98	3.59	3.36	3.20	3.09	3.01	2.95	2.90	2.85
12	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.80	2.75
13	4.67	3.81	3.41	3.18	3.03	2.92	2.83	2.77	2.71	2.67
14	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.65	2.60
15	4.54	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.59	2.54
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.54	2.49
17	4.45	3.59	3.20	2.96	2.81	2.70	2.61	2.55	2.49	2.45
18	4.41	3.55	3.16	2.93	2.77	2.66	2.58	2.51	2.46	2.41
19	4.38	3.52	3.13	2.90	2.74	2.63	2.54	2.48	2.42	2.38
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39	2.35
21	4.32	3.47	3.07	2.84	2.68	2.57	2.49	2.42	2.37	2.32
22	4.30	3.44	3.05	2.82	2.66	2.55	2.46	2.40	2.34	2.30
23	4.28	3.42	3.03	2.80	2.64	2.53	2.44	2.37	2.32	2.27
24	4.26	3.40	3.01	2.78	2.62	2.51	2.42	2.36	2.30	2.25
25	4.24	3.39	2.99	2.76	2.60	2.49	2.40	2.34	2.28	2.24
26	4.23	3.37	2.98	2.74	2.59	2.47	2.39	2.32	2.27	2.22
27	4.21	3.35	2.96	2.73	2.57	2.46	2.37	2.31	2.25	2.20
28	4.20	3.34	2.95	2.71	2.56	2.45	2.36	2.29	2.24	2.19
29	4.18	3.33	2.93	2.70	2.55	2.43	2.35	2.28	2.22	2.18
30	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21	2.16
40	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.12	2.08
60	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	2.04	1.99
120	3.92	3.07	2.68	2.45	2.29	2.18	2.09	2.02	1.96	1.91
∞	3.84	3.00	2.60	2.37	2.21	2.10	2.01	1.94	1.88	1.83

The table consulted for F value. All observed values from ANOVA were lower than the tabulated value, therefore indicating that there were no statistically significant variations between samples; Ref (Gravetter and Wallnau, 2008).

3 Possible histaminergic modulation of energy expenditure and blood glucose regulation in man

This chapter presents the background information and studies conducted with L-Histidine as an oral dietary agent with a potential to affect various components of energy balance. The L-Histidine supplementation studies proposed herein are the first of their kind. The theoretical potential of L-Histidine to increase EE and affect blood glucose via increasing insulin sensitivity appear to be supported by animal studies (Masaki *et al.*, 2001; Yoshimatsu *et al.*, 2002b; Yasuda *et al.*, 2004a; Lee *et al.*, 2005). A review of literature however demonstrates a lack of knowledge regarding the effects of L-Histidine intake in humans. The present study was initiated with a view to examining the metabolic effects of L-Histidine, but also to assess the viability of implementing a complex series of metabolic measurements in healthy human volunteers. These measurements included whole body metabolic measurements alongside skin and core temperature assessments. No previous studies have looked at indirect calorimetric measures being taken at the same time as both peripheral and deep body temperature. The rationale for doing such being that should an intervention bring about increased EE, it may be possible to localise the area of increased metabolism. In the case of the present study this was of particular interest due to the documented effects of L-Histidine on uncoupling reactions in peripheral adipose depots.

3.1 Review of Literature

The metabolic effects of L-Histidine and consequently histamine in animals and humans are discussed in the following section. The literature search showed that histidine/histamine actions have been studied extensively among animal models, and evidence from human studies is few. The review of literature is divided into sections discussing the amino acid's dietary quantification, its bioavailability, mechanism of action (in particular effects on energy balance, blood glucose and blood lipid concentrations) and finally data on possible toxicity are explored.

L-Histidine is an essential amino acid (FAO/WHO/UNU, 1985) as a histidine free diet reduces haemoglobin levels (Kriengsinyos *et al.*, 2002) and results in negative nitrogen balance, along with a marked decrease in both muscle and plasma histidine concentrations,

lower serum albumin concentrations, anaemia and clinical symptoms (such as, scaly and dry skin with erythema) (Kopple and Swendseid, 1975). There is little evidence that a metabolic pathway exists for histidine synthesis in the human body. Histidine stores in the human body are large as it is present in haemoglobin and carnosine (also called β -alanylhistidine, a di-peptide present in the muscle) and the gut flora synthesises an unknown amount of histidine which may be absorbed and used (Visek, 1984). L-Histidine is a precursor of the neurotransmitter histamine which has important regulatory roles on lipolysis, EE, food intake and blood glucose regulation within the body (Panula, 2007).

3.1.1 Histamine biosynthesis and action

Histamine is synthesised in the brain by the enzymatic decarboxylation of L-Histidine catabolised by histidine decarboxylase (HDC, a histamine synthesising hormone) (Panula, 2007). It has been previously seen that slices of rat hypothalamus when incubated in the presence of ^3H -L-Histidine, rapidly absorbed the amino acid and partially converted to ^3H -histamine. Inhibitors of HDC prevented this conversion and the regulation of histamine synthesis was independent of the addition of exogenous histamine (Verdiere *et al.*, 1975). Dietary L-Histidine intake influences concentrations of tissue histamine, HDC, and histamine methyltransferase (HMT, a degradative enzyme) activity. Feeding rats diets supplying inadequate, adequate, or excess amounts of histidine for 14 days, showed that as the levels of dietary histidine increased, tissue concentrations of free-histidine and of histamine increased in all the tissues analysed (Lee *et al.*, 1981). The increase of histamine was greatest in brain and stomach (5- and 4-fold, respectively), HDC activity was not detected in muscle, but doubled from the lowest to the highest histidine intake in brain and increased almost 6-fold between the lowest and the highest histidine levels in stomach. The increases in tissue histamine concentrations observed in the tissues analysed generally reflected the changes and magnitudes of enzyme activities for HMT and HDC. Similarly, research into the properties and localisation of HDC enzyme in adult human brain shows that a major portion of the enzyme is localised in a sub-cellular fraction containing nerve terminals and it showed an uneven regional distribution (Nowak and Zelazowska, 1987). Considering all of the above, it is apparent that L-Histidine metabolises histamine with the help of HDC.

Histamine transfers signals through its interaction with its receptors. Histamine receptors (a class of G-protein coupled receptors with histamine as their endogenous ligand), of which there are four known subtypes (H₁, H₂, H₃ and H₄), are widely distributed in most tissues such as:

H₁ smooth muscle, endothelium, adrenal medulla, heart and CNS

H₂ gastric parietal cells, vascular smooth muscles, neutrophils, CNS, heart and uterus

H₃ CNS, heart, lung, GI tract, endothelium (Homaidan *et al.*, 2001)

H₄ various cells of the immune system, mast cells, lymphocyte T cells, dendritic cells and basophils (Parsons and Ganellin, 2006).

The functions of histamine include its roles during allergic reactions, vasodilation, vasoconstriction, gastric acid secretion and neurotransmission. Of special interest to the current study is its action in the CNS as a neurotransmitter. Histaminergic neurons project from the tuberomammillary nucleus of the posterior hypothalamus to all regions of the brain (Mercer, 1997). The activation of the histaminergic system is responsible for a collection of physiologic effects; including, increased activity of the hypothalamic-pituitary-adrenal axis, decreased blood pressure, decreased pain perception, wakefulness, hyperactivity, hyper secretion of corticotropin releasing hormone, hypercortisolemia, temperature regulation, thirst, food intake (satiety), breathing and cardiovascular function (Costanzo, 2006b).

3.1.2 Dietary sources of histamine

Histamine is readily available from foods such as marine products, cheeses, fermented soy products and other fermented foods, alcoholic beverages and vinegars. Some plant sources such as aubergine, spinach, and tomato also contain high levels of histamine naturally (Table 3.1). In addition, fish meat, eggs, food additives such as azo dyes and preservatives mediate the release of histamine within the body (Table 3.2) (Sellers *et al.*, 2005). Recommended daily intake of histidine for adults is 12 mg/kg body weight (FAO/WHO/UNU, 1985).

Table 3.1 - Foods rich in histamine

Food categories	Histamine content	
	mg/kg	mg/l
<u>Fish (frozen/smoked or salted/canned)</u>		
Mackerel	ND - 1788*	
Herring	1 - 479*	
Sardine	ND - 2000*	
Tuna	ND - 402*	
<u>Cheese</u>		
Gouda	10 - 900	
Camembert	0 - 1000	
Cheddar	0 - 2100	
Emmental	5 - 2500	
Swiss	4 - 2500	
Parmesan	10 - 581	
<u>Meat</u>		
Fermented sausage	ND - 650	
Salami	1 - 654	
Fermented ham	38 - 271	
<u>Vegetables</u>		
Sauerkraut	0 - 229	
Spinach	30 - 60	
Eggplant	26	
Tomato ketchup	22	
<u>Red wine vinegar</u>	4	
<u>Alcohol</u>		
White wine		ND - 10
Red wine		ND - 30
Top-fermented beer		ND - 14
Bottom-fermented beer		ND - 17
Champagne		670

ND = Not detected; Reference: Adapted from (Maintz and Novak, 2007) *Original authors have collated data from (Beutling, 1996; Izquierdo-Pulido, 1996; Jarisch, 2004; Sarkadi, 2004).

The formation histamine in foods requires the availability of free amino acids, the presence of decarboxylase-positive microorganisms and conditions allowing bacterial growth and decarboxylase activity. Changes in the presence of any of these factors account for the variation in histamine content in foods. Numerous bacterias and some yeast display high HDC activity and thus have the capacity to form histamine. Therefore, high concentrations of histamine are found mainly in products of microbial fermentation (aged cheese, sauerkraut, wine) or in microbially spoiled food (Pechanek *et al.*, 1983). It has been suggested that some foods have the capacity to influence the release of histamine directly from tissue mast cells in the body (TNO, 1996) (Table 3.2). Histamine release following

ingestion of these foods is triggered in an Ig-E independent manner (by direct interaction of molecules with cell membrane-associated G proteins or transmembrane lipid mediators) and is regulated by several factors other than Ig-E, such as cytokines, prostaglandins and neuropeptides (Steinhoff *et al.*, 2004b).

Table 3.2 - Foods with suggested histamine releasing capacity

Plant-derived	Animal-derived	Other
Citrus fruit	Fish	Additives
Papaya	Crustaceans	Liquorice
Strawberries	Pork	Spices
Pineapple	Egg white	Azo dyes
Tomatoes		
Spinach		
Nuts		
Peanuts		
Chocolate		

Reference: ((Steinhoff et al., 2004a); Sellers et al., 2005)

3.1.3 Metabolic action of L-Histidine and histamine *in-vivo*

There have been numerous studies conducted on animal models, demonstrating the actions of L-Histidine and/ or histamine on lipolysis, EE, food intake and blood glucose regulation.

Effects on parameters of energy balance: Both peripheral (intraperitoneal (i.p)); 0.35mmol/kg) and central (intraventricular; 1nmol/rat) administration of L-Histidine and histamine results in an elevation in plasma glycerol and free fatty (FFA) concentrations in rats, indicating lipolysis in progress (Yasuda *et al.*, 2004a; Yoshimatsu *et al.*, 2002b). This was due to increased activity of sympathetic nerves innervating WAT following peripheral administration of histidine mediated by neuronal histamine. Investigations on the effect of peripheral administration of L-Histidine on BAT sympathetic nerve activity as a marker for the central regulation of EE has shown that a bolus i.p injection of L-Histidine (0.3 mmol/rat) induced a significant increase in nerve activity in BAT as a consequence of an excitatory effect of histamine on the SNS (Yasuda *et al.*, 2004b). Histamine infusion for 7 days (ICV; (0.05 μ mol/g)) in mice reduced body fat, weight, *ob* gene expression and serum leptin concentration to a greater extent than in pair-fed controls due to increased EE via BAT and WAT (Masaki *et al.*, 2001). One study (the only human study looking at changes in dietary intake) examining the effects of 4 g/day oral ingestion of L-Histidine in gelatine

capsules among 8 human volunteers for a period of 2 weeks, found no significant changes in appetite, taste and smell perceptions, food intake and body weight (Schechter and Prakash, 1979).

Similar to the lipolytic effects observed following peripheral and central administration of L-Histidine and histamine, dietary supplementation of L-Histidine and central infusion of histamine have both demonstrated effects on upregulation of UCPs in BAT and WAT indicating influence on energy regulation. Increased dietary L-Histidine supplementation (50 g/kg diet) in rats has shown an increase in UCP1 mRNA in BAT (Kasoaka *et al.*, 2004) and ICV infusion of histamine up-regulated mRNA expression of BAT UCP1 and WAT UCP3 in diet induced obese and leptin resistant mice (Masaki *et al.*, 2001). The role of histamine in upregulation of UCPs was further confirmed by the development of H₃ receptor knockout mice (H₃^{-/-}) which were obese and their expression of UCP1 and UCP3 in BAT, UCP3 in WAT and in skeletal muscles was reduced, with decreased EE and increased body weight (Takahashi *et al.*, 2002). Similarly, H₁ receptor knockout mice (H₁KO), gradually develops maturity-onset obesity, which is accompanied by hyperphagia and decreased expression of UCP1 mRNA thus suggesting that H₁ receptors are also crucial for feeding behaviour and development of obesity (Masaki *et al.*, 2004). Furthermore, targeted disruption of the HDC gene were characterised by visceral adiposity, increased amount of BAT, impaired glucose tolerance, hyperinsulinemia, and hyperleptinemia (Fulop *et al.*, 2003). These metabolic changes are attributed to the impaired regulatory loop between leptin and hypothalamic histamine and the attenuated ability to induce UCP1 mRNA in the BAT. Impairment of either the key enzyme needed in the conversion of L-Histidine to histamine or the receptors through which histamine exerts its actions can bring about changes in parameters affecting energy balance and also confirms the essential role of L-Histidine and histamine in regulation of food intake and upregulation of UCPs in both BAT and WAT.

Hypoglycaemic effect: Increased insulin sensitivity, reduction in body fat content, reduced leptin, insulin and glucose concentrations may bring about overall positive change in energy metabolism and help alleviate insulin resistance and obesity associated comorbidities. Evidence is emerging suggesting that activation of histamine signalling in the hypothalamus may have anti-diabetic actions, particularly in leptin resistant states (Masaki *et al.*, 2001). It was observed that serum concentrations of glucose and insulin were

reduced and tests for glucose and insulin tolerance showed improved insulin sensitivity in those mice infused with histamine compared with pair-fed controls. Targeted disruption of HDC in mice resulted in impaired glucose tolerance and hyperinsulinemia (Fulop *et al.*, 2003). Intake of histidine and carnosine (β -alanylhistidine) (each – 1 g/l of water) decreased glucose and fibronectin levels (therefore decreased risk of glaucoma caused by high blood glucose levels) and increased insulin levels in diabetic mice (Lee *et al.*, 2005). Experiments with histidine and carnosine were found to dose-dependently decrease triglyceride and cholesterol levels in the heart and liver. This study also showed that histidine and carnosine effectively protect LDL against glucose induced oxidation and glycation by scavenging free radicals in LDL and retarding oxidation and glycation thus delaying long term complications (retinopathy, neuropathy, nephropathy, heart attacks and strokes) of blood glucose dysregulation. The action of peripheral and central supplementation of L-Histidine and histamine and enzymes involved in histamine metabolism clearly indicates towards their role in blood glucose regulation among animal models but needs further investigation among humans due to lack of data in the literature.

Effects on factors regulating dietary intake: Investigations on the dose-response effect of dietary histidine on food intake and body fat accumulation in male Wistar rats have shown decreased food intake with increasing concentration (0, 1, 2.5 and 5 %) of histidine in the diet (Kasaoka *et al.*, 2004). The authors found a negative, significant correlation between dietary histidine intake and retroperitoneal fat pads after 8 days of supplementation indicating reduction in body fat. A similar study exploring the differences in daily food consumption in male Wistar rats following i.p histidine administration combined with either phosphate buffered solution (PBS) or α - fluoromethylhistidine (FMH; a suicide inhibitor of HDC) found that histidine administration pre-treated with PBS decreased food intake to 64.2% of the baseline value compared to 88.1% of the baseline value after treatment with histidine pre-treated with FMH, thus demonstrating that peripheral administration of L-Histidine suppressed food intake and hypothalamic neuronal histamine suppresses food intake through H₁ receptors in the VMH nucleus and the paraventricular Nucleus (PVN) (Yoshimatsu *et al.*, 2002a). To study the specific areas involved in histamine-involved suppression in feeding, an infusion of chlorpheniramine (an H₁ antagonist) was infused into the VMH, PVN, lateral hypothalamus area (LHA), the dorsomedial nucleus and the preoptic anterior hypothalamus of Wistar King A rats. It was

observed that VMH and PVN, which are the areas with the highest distribution of H₁ receptor and hypothalamic histamine, responded to chlorpheniramine by increasing feeding in rats. There were no observed effects in the remaining regions. This indicates that neuronal histamine transmits signal for suppression of food intake through H₁ receptors in the VMH and PVN (Sakata *et al.*, 1997). The involvement of hypothalamic histaminergic activity with leptin in the central regulation of feeding behaviour has been investigated in animals and it is suggested that histamine is involved in leptin-induced inhibition of food intake (Gotoh *et al.*, 2005; (Toftgaard *et al.*, 2003). Infusion of leptin into the third cerebroventricle of mice has shown increased levels of *t*-MH (*tele*-methylhistamine, a major metabolite of histamine) (Yoshimatsu *et al.*, 1999). Leptin deficient obese mice (*ob/ob*) exhibit lower histamine turnover compared to lean littermates, which has been shown to increase after leptin infusion (Itateyama *et al.*, 2003). Also, leptin injection to wild type mice significantly reduced food intake but had no effect in H₁R-KO (male mutant mice lacking in H₁ receptors) (Morimoto *et al.*, 1999).

The above mentioned animal studies all confirm various effects of histidine or histamine actions *in-vivo*. Histidine and histamine administration increases EE, via increase in thermogenesis and increase in lipolysis thereby reducing body weight as increased sympathetic activation mediated by noradrenaline in BAT and WAT following histidine and histamine administration release brings about mobilisation of energy reserves, through the accelerated breakdown of glycogen in muscle and liver cells and the release of lipids by adipose tissues; altogether facilitating reduction in body weight (Martini, 2006). Histidine intake and histamine infusion increase UCP mRNA expression and this influence upon UCP function might be a pathway by which L-Histidine exerts its thermogenic influence. Leptin is a hormone secreted from the adipose tissue which influences energy homeostasis, neuroendocrine and immune function (McArdle *et al.*, 2006). Plasma leptin concentrations are highly correlated to total body fat and body mass index in humans and animals (Stanley *et al.*, 2005). It is considered a regulatory hormone for EE as lack of circulating leptin leads to hyperphagia, obesity neuroendocrine and immune disturbances which can be normalised by leptin administration in *ob/ob* mouse (Campfield *et al.*, 1995). Histamine affects leptin action in the hypothalamus, thus decreasing food intake and therefore possibly reduce body weight.

3.1.4 Toxicity

A study investigating a 5% increase in dietary histidine resulted in hypercholesterolemia and enlargement of the liver in rats (Hitomi-Ohmura *et al.*, 1992). The authors suggest that the hypercholesterolemia caused by histidine-excess diet (50g/kg diet) appears to be due to the stimulation of cholesterol synthesis in the liver. They attributed this increased cholesterol synthesis to the significantly higher hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in histidine-excess diet rats than in rats fed a basal diet. A similar study investigating the effect of 5% increase in dietary histidine for 3, 6, 14 or 30 days on serum and liver lipids in rat models, found that it caused growth retardation, hepatomegaly and decreased liver lipids throughout the period of the experiment compared to rats fed the basal diet (Ohmura *et al.*, 1986). This effect has been further investigated with rats being fed basal (no histidine + cupric carbonate 6mg copper kg diet), histidine-excess (the addition of 50 g L-Histidine/kg diet + cupric carbonate 6mg copper kg diet) or copper-deficient diets (no cupric carbonate supplemented in the diet) for 0, 7, 21 and 42 days *ad libitum* (Aoyama *et al.*, 1999). It was found that liver triacylglycerol accumulated and the serum triacylglycerol level decreased after feeding of the histidine-excess diet for 21 or 42 days, but not after feeding of the copper-deficient diet. Serum cholesterol level increased (from 5.85 ± 0.25 to 5.90 ± 0.18 ; 7.99 ± 0.36 ; 9.57 ± 0.67 $\mu\text{mol/g}$) in rats fed the histidine-excess diet for 7, 21 and 42 days respectively, but not in rats fed the copper-deficient diet. Copper content in the liver and serum significantly decreased in rats fed the histidine-excess diet. Urinary copper and zinc increased in rats fed the histidine-excess diet, and decreased or showed a decreasing tendency in rats fed the copper-deficient diet. A previously conducted similar study fed rats histidine (50 g/kg of diet) for a period of 7 days and observed that dietary excess histidine lowered total liver lipids and liver phospholipids and serum cholesterol increased (Aoyama *et al.*, 1990). They concluded that excess histidine lowers liver copper, serum copper, and ceruloplasmin (enzyme carrying 90% of the copper in plasma), and increased urinary output of copper, zinc, iron, and magnesium. Other studies have shown that excess dietary histidine induced hepatomegaly, retarded growth, increased plasma cholesterol and significantly reduced plasma copper and zinc and liver copper concentrations in rats (Harvey *et al.*, 1981 ; Soloman and Geison, 1978).

Toxicity studies for histidine ingestion in humans are very few. One study performed on 6 patients with systemic sclerosis found that an initial daily oral administration of 8.1 g

histidine, increased by 8.1 g once every 3 days till a maximum dose of 64.8 g/day was reached, caused anorexia, taste and smell dysfunction and cerebellar dysfunction (Henkin *et al.*, 1975). These changes were associated with significant decreases in serum zinc concentrations and significant increases in urinary zinc excretion but the research article fails to clarify the exact doses at which the above alterations occur. Another study investigating intake of L-Histidine (4g/day) in healthy men observed a significant increase in urine zinc excretions after supplementation for 1 week, but not after 2 weeks (Schechter and Prakash., 1979). The effects of oral ingestion of higher doses of L-Histidine in humans are ambiguous, due to fewer human investigations and needs to be studied further before drawing any definite conclusions.

3.1.5 Rationale for L-Histidine studies

L-Histidine consumption and histamine actions *in vivo* have been associated with increased EE, up-regulation of UCPs, therefore reduction in body weight and loss of body fat and glycaemic control (Costentin *et al.*, 1973; Sakata, 1995; Sakata and Yoshimatsu, 1995). The majority of studies have been conducted on animal models and there is a need to conduct studies of oral administration of L-Histidine on humans to observe its effects on key metabolic functions. Thus, the following studies were designed and carried out to assess the influence of L-Histidine ingestion on: EE, change in mean skin and core body temperature, fasting blood glucose and total cholesterol concentrations, and food intake and substrate oxidation in lean and obese adult humans. It was hypothesised that ingestion of L-Histidine would increase RMR, thermogenesis, lower total body fat and increase lipolysis, resulting in loss of body weight and lower fasting blood glucose and total cholesterol concentrations, some of which may be key to alleviate risk factors for body weight dysregulation and development of comorbidities.

3.2 Experimental pilot study to evaluate effects of L-Histidine on energy expenditure and thermogenesis

As described in the above literature review a strong association of histidine/ histamine with increased sympathetic nerve activation resulting in increased lipolysis and EE has been found (Yoshimatsu *et al.*, 2002b; Yasuda *et al.*, 2004a). It has also been observed that histidine/histamine administration increases insulin sensitivity and reduces circulating blood glucose concentrations (Masaki *et al.*, 2001; Lee *et al.*, 2005). Interestingly, histidine/histamine administration has been positively correlated with increasing expression of UCPs in BAT and WAT (Kasoaka *et al.*, 2004; Masaki *et al.*, 2001), which indicates a dissipation of heat and therefore increased EE. The metabolic effects of L-Histidine and histamine are investigated exclusively among animal models and there is a need to verify the effects of oral ingestion of this essential amino acid among humans. Hence, the current and following studies (Chapters 3.3 and 3.4) were designed to investigate metabolic effects of L-Histidine over varying periods of supplementation and also among participants with varying body composition.

The pilot study was conducted to develop an optimal technique for the real-time simultaneous measurement of RMR, MST and CT (and hence did not employ sample size calculations as the primary aim was methodological/developmental). As mentioned earlier, this set of measurements taken concurrently has not been previously reported in the literature. Due to the dearth of data regarding optimum doses of L-Histidine supplementation and its effects in humans, it was an opportunity to begin working with L-Histidine as an intervention compound in a human study. Due to the lack of previous data from human trials, the author was unaware of the variations to expect in measured parameters.

3.2.1 Hypothesis

Acute ingestion of L-Histidine will increase mean skin and core temperatures in concert with an increase in resting metabolic rate over the test period.

3.2.2 Aims

The aims of the study were:

1. To organise the study protocol and evaluate the practicality and feasibility of measurement techniques used.
2. To study the acute effects of oral ingestion of L-Histidine on energy expenditure and thermogenesis in healthy men and women.

3.2.3 Methods

3.2.3.1 Participant recruitment and profile

Five healthy participants (3 male and 2 female) were recruited by advertisements placed around the university campus (see section 2.2 for further details on recruitment). The study protocol was approved by the University of Westminster Ethics committee (Appendix 2) and informed consent (Appendix 3) was obtained at the start of the test sessions from all participants. The study protocol and measurements to be carried out during the test sessions were explained to all participants with the aid of the participant information sheet (Appendix 5). The mean anthropometric pre-treatment data are presented in Table 3.3.

Table 3.3 - Mean anthropometric data of study participants pre-treatment

	Mean \pm SEM	Range
Age (years)	38 \pm 3.2	33 - 44
Weight (kg)	72.5 \pm 4.0	57 - 79
BMI (kg/m ²)	25.5 \pm 2.4	20.2 - 34.3
<i>n</i>	5	

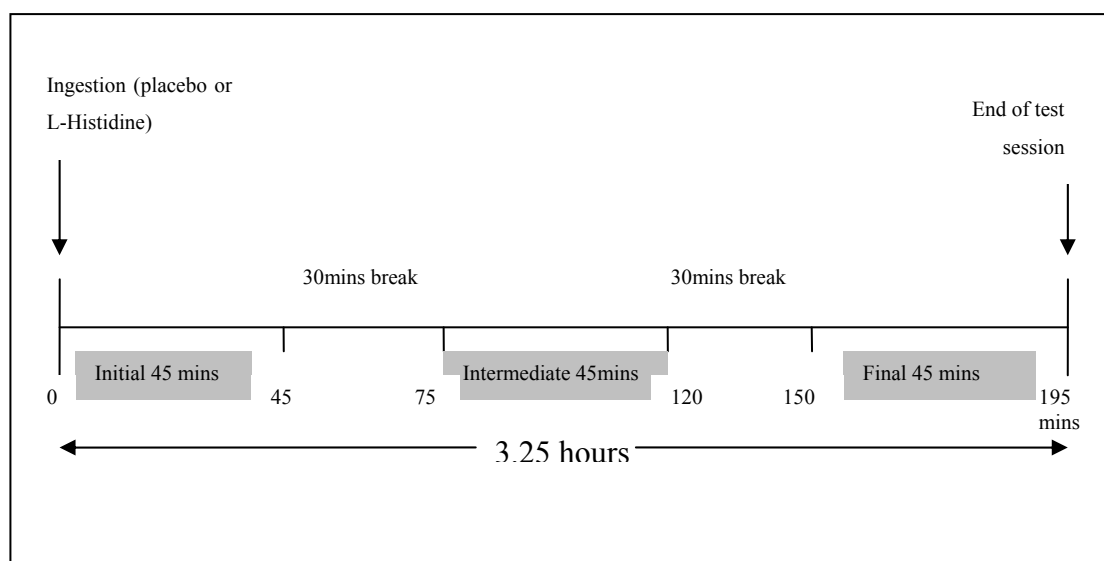
3.2.3.2 Study design

This study was randomised and crossed-over in design. Each recruit acted as their own control. All participants were required to visit the laboratory on two occasions having fasted for a minimum of 12 hours before each visit, although they were permitted to drink water if desired. Participant handling was standardised as detailed in section 2.3. The administration of L-Histidine and placebo was single blind, randomised, with a wash out period of 7 days provided between each test session. A dose of 25 mg/kg L-Histidine (Lamberts Healthcare Ltd, Kent, UK) was used (section 2.4 for dosage calculation). The nitrogen content of this dose was calculated and used to calculate the placebo dose (45

mg/kg) and it was ensured that the placebo and L-Histidine were isonitrogenous (Casilan-90, Complan foods Ltd, UK). The placebo and L-Histidine doses were also isocaloric (Casilan-90 providing 1.82 kcals and L-Histidine providing 2 kcals).

There was a wash out period of 7 days between the two test sessions. This time was necessary because of the possibility that the intervention administered first could affect the outcome variable for some time even after treatment ceases. Analysis of urine for total radioactivity in a study performed to examine the metabolism of 3-methylhistidine (an amino acid, formed by the methylation of peptide-bound histidine in actin and myosin) in men using an intravenous infusion of radioactive ^{14}C -3-methylhistidine showed that 75% of the administered dose was excreted in 24 hours and 95% in 48 hours (Long *et al.*, 1975). Considering the above, a period of seven days was considered sufficient time to ascertain that the first administered intervention was no longer in the system.

Figure 3.1 - Diagrammatical representation of study design



Each recruit acted as their own control. The above timeframe is not to scale, only representative.

Each test session began with participants relaxing for a period of 10 minutes during which time the tests to be carried out were further explained. The test supplement or placebo was mixed with 150 ml of tap water before being given to the subject to ingest in one acute dose. The participant's RMR was measured by the use of a Deltatrac II, for a period of 3.25 hours (section 2.7). Each test session was for 3.25 hours as there were three RMR sampling periods in each test session: 0 – 45, 75 – 120 and 150 - 195 minutes and between

them breaks of 30 minutes were given which totalled to 3.25 hours/test session (Figure 3.1). The skin thermistors were attached (section 2.9) after insertion of the rectal probe by the participants and MST and CT was measured along RMR measurements for a period of 3.25 hours. The same measurements and test protocol were repeated with the other test supplement following the wash out period in the second and final test session.

3.2.4 Statistical analyses

Analysis of variance was performed to analyse the variation between all data, between the two treatments, within subjects and between time periods. There were no significant differences in any of the parameters (RMR, MST, CT and RER) (Appendix 12).

3.2.5 Results

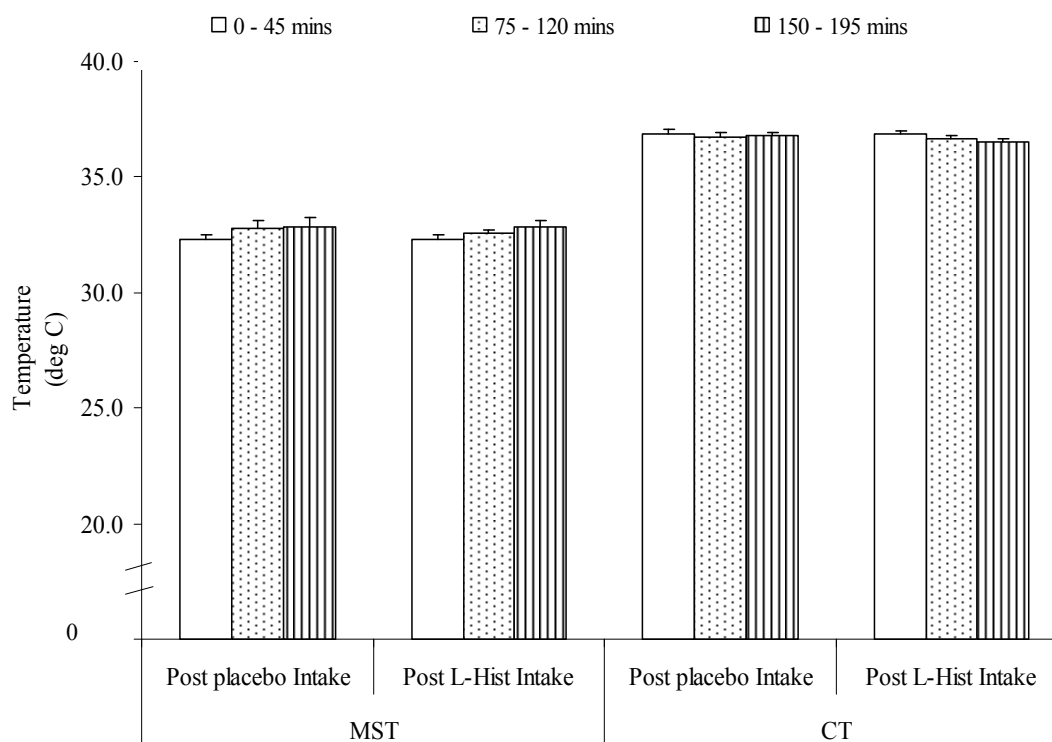
Table 3.4 - Mean RMR (\pm SEM) at 0 - 45 mins, 75 - 120 mins and 150 - 195 mins post placebo and L-Histidine ingestion

	RMR		
	0 - 45 mins	75 - 120 mins	150 - 195 mins
Post placebo intake	1917.5 \pm 45.8	1913.3 \pm 70.2	1938.6 \pm 78.0
Post L-Histidine intake	1853.2 \pm 96.8	1974.5 \pm 43.9	1780.7 \pm 108.9

n = 5

There appeared to be an increase in mean RMR measured between 75 – 120 minutes post L-Histidine ingestion and a decrease when measured between 150 - 195 minutes in comparison to 0 - 45 minutes post L-Histidine ingestion (Table 3.4). This variation was however, non-significant (f values = 0.0023 for treatment and 0.0020 for changes over time). Post placebo intake, mean RMR remained unchanged when measured at either 75 – 120 or 150 – 195 minutes in comparison to RMR measured during 0 - 45 minutes. The average MST and CT during the 3.25 hour period post ingestion of placebo or L-Histidine test sessions were similar when measured at 0 - 45, 75 – 120 and 150 – 195 minutes (Figure 3.2). The AT was kept stable at 23.2 \pm 0.5 °C throughout the test session (3.25 hours) to avoid inducing any shivering thermogenesis. There were no differences in RER post placebo or L-Histidine ingestion over the 3.25 hour period (Table 3.5).

Figure 3.2 - Mean skin (MST; \pm SEM), core temperature (CT; \pm SEM) measured for a 3.25 hour period after ingestion of either placebo or L-Histidine



n = 5; Ambient temperature (AT; \pm SEM) for 0 – 45, 75 – 120 and 150 – 195 mins post placebo intake was 23.5 ± 0.4 , 23.4 ± 0.5 , 23.6 ± 0.5 °C respectively; post L-Histidine ingestion 23.2 ± 0.6 , 23.3 ± 0.7 , 23.8 ± 0.7 °C for 0 – 45, 75 – 120 and 150 – 195 mins respectively.

Table 3.5 - Mean respiratory exchange ratio (RER; \pm SEM) at three intervals (0 - 45 mins, 75 - 120 and 150 - 195 mins) post placebo and L-Histidine ingestion

	RER		
	0 - 45 mins	75 - 120 mins	150 - 195 mins
Post placebo intake	0.85 ± 0.04	0.85 ± 0.02	0.83 ± 0.01
Post L-Histidine intake	0.82 ± 0.05	0.82 ± 0.05	0.80 ± 0.03

n = 5

3.2.6 Discussion

This study was primarily conducted to determine the most practical protocols to measure RMR, MST and CT simultaneously. The techniques and protocols employed for measuring RMR, MST and CT were fully developed via this pilot study, and therefore were suitable for use in further studies. The second objective was to assess the acute effects of placebo and L-Histidine ingestion on RMR, MST, CT and RER. There was no effect of placebo on any of the measured parameters. There was no observed effect of L-Histidine ingestion on RMR, MST, CT and RER.

Practicality of measuring RMR, MST and CT

The measurement of RMR involved participants lying on a bed with a perspex hood over their head. They were instructed to relax and limit movement as much as possible. This was to minimise any increase in EE due to increased activity. Participants were unable to be under the hood for a continuous period of 3.25 hours. To overcome this difficulty, the protocol for RMR testing was designed to be measured in 45 minute intervals with 30 minutes break between sampling periods. Participants needed a minimum of 30 minutes to relax; during which time they sat up to either read or use the computer and avoided any strenuous physical exertion. During the measurements, the initial 15 minutes' data were not used for calculations of RMR, to allow for acclimatisation to being under the enclosed hood. The later 30 minutes data were found to be more consistent and were used for calculations of RMR.

The ambient temperature during all test sessions was maintained at 23.2 ± 0.5 °C. A constant ambient temperature is essential during studies conducted to measure variations in body temperature as high environmental temperature results in vasodilation of cutaneous blood vessels, increased sweating and maximum heat loss as against low environmental temperature which results in vasoconstriction of cutaneous blood vessels and minimised heat loss (Silverthorn, 1998b). Maintaining constant ambient temperature was essential in all the following studies as any change in MST or CT could then be attributed as a result of ingestion of L-Histidine. During the study, participants experienced no discomfort during insertion or removal of the rectal probe, which was used for CT measurement, and were at ease with being attached to the skin thermistors (for MST measurement) over a prolonged

period of time (3.25 hours). The use of Squirrel data logger 2020 was easy and was found to be an uncomplicated technique for temperature measurement.

Effect of L-Histidine ingestion on RMR, MST, CT and RER

There was no effect of placebo ingestion on RMR, MST, CT and RER. There however seemed to be a $6.0 \pm 2.7\%$ rise in RMR measured between 75 – 120 minutes post L-Histidine ingestion when compared to 0 – 45 minutes measurement, which gradually seemed to decline to $10.0 \pm 1.2\%$ below the 0 – 45minutes post L-Histidine ingestion measurement by the end of the test session (3.25 hours). These variations however, were not statistically significant. The effect of histidine in the central regulation of EE has been examined previously in animal models. Predominantly, the effects on lipolysis, appetite suppression and weight loss post histidine intake and histamine infusion have been studied rather than changes in RMR. One such study investigated the effect of peripheral administration (bolus i.p injection) of L-Histidine on BAT sympathetic nerve activity as a marker for the central regulation of EE (Yasuda *et al.*, 2004a) and found significant increases in BAT nerve activity indicating an increase in EE. A similar study (Yoshimatsu *et al.*, 2002b) examined the effect of histidine via i.p injection of histamine on rats and found a significant increase in WAT nerve activity, again showing possible increases in EE. The RMR results obtained in this study were inconclusive and if any previous animal/human studies had measured RMR via indirect calorimetry, further comparisons and conclusions might have been drawn.

There were no changes in MST or CT measurements during each 3.25 hour test session, or between the two test conditions. The main rationale for measuring MST and CT during the present and proposed studies was to establish whether L-Histidine ingestion increases thermogenesis, thereby increasing EE and possibly upregulating expression of UCPs. The proposed studies on oral L-Histidine ingestion are novel and the first of their kind that we are aware of. These studies were designed after reviewing the literature on the expression of UCP mRNA in BAT and WAT and its regulation by hypothalamic neuronal histamine. Studies conducted using rat models have shown an increase in UCP1 mRNA in BAT with increases in dietary histidine (Kasaoka *et al.*, 2004). Also, targeted disruption of the HDC gene has shown to down-regulate UCP1 mRNA in the BAT (Fulop *et al.*, 2003). Several other studies (Masaki *et al.*, 2001; Masaki *et al.*, 2003; (Yasuda *et al.*, 2004b) support these findings of increased expression of UCPs via neuronal histaminergic system, consequently

increasing thermogenesis and EE. This mechanism following L-Histidine ingestion needs to be further investigated among humans.

There was no effect of either placebo or L-Histidine ingestion on RER over the 3.25 hour period. Histidine administration and histamine infusion studies performed in animal models have not measured RER previously as more direct measures of fuel (fat) oxidation such as circulating fatty acid concentrations are measured. Most studies, apart from being conducted on animal models and not directly measuring changes in the current study outcome measures, also predominantly employ infusion techniques of histidine rather than oral ingestion. Central or peripheral infusion of histamine and histidine although a possibility in humans, is not practical to study free-living conditions and most importantly not an aim of the current and proposed studies. Therefore, it is important to further study effects of peripheral ingestion of L-Histidine, which can cross the blood-brain barrier and is taken up by the histaminergic neurons and converted to histamine in one step synthesis by decarboxylation with HDC.

3.2.7 Conclusion

The techniques and protocols employed for measuring RMR, MST, CT and RER were found to be simple, easy and therefore were used in all further studies. Literature regarding acute infusion of histidine and histamine in animals has shown promising effects on energy regulation. Similarly, the present experimental study did reveal a possible trend towards an increase in RMR in a short period of time, which then decreased below the pre-treatment measurement. As no other study has been performed to measure the acute effects of L-Histidine consumption in humans, it is difficult to explain this rise and fall in RMR over a short period of time. It can however be reasoned that acute effects of L-Histidine consumption might not be clearly observable in a short period of time without further work. As the acute effects of L-Histidine ingestion on the current study outcomes was not conclusive, a further study was designed and carried out to investigate the effects of the same dose of L-Histidine ingestion over a longer period of supplementation (10 days) among healthy men. The dose of L-Histidine and the standardised measurement protocols for simultaneous measurements of RMR, MST and CT from the current study were kept unchanged in the following study.

3.3 Chronic ingestion of L-Histidine and its effects on energy expenditure, thermogenesis and blood glucose regulation in healthy humans

The minimum period of L-Histidine supplementation in humans to observe any possible metabolic effects, is unclear. The pilot study (Chapter 3.2) established the protocol for the concurrent measurement of RMR, MST and CT. The results obtained in the pilot study showed no significant effect of an acute period of L-Histidine supplementation on the measured parameters. L-Histidine is a precursor of neuronal histamine (Panula, 2007), which exerts various metabolic effects on factors affecting energy balance and blood glucose regulation via diverse mechanisms (Kasoaka *et al.*, 2004; Masaki *et al.*, 2001; Morimoto *et al.*, 1999; Sakata *et al.*, 1997; Yasuda *et al.*, 2004a; Yoshimatsu *et al.*, 2002b; Yoshimatsu *et al.*, 1999). In spite of the clear actions of L-Histidine/histamine in animal models, its effects have not yet been researched in humans. Given the mechanistic understanding of L-Histidine, there is likely to be some metabolic effect following its ingestion. Therefore a logical step in investigation is to increase supplementation duration. The exact *in-vivo* pathway of L-Histidine partitioning (uptake rate and pathways) in humans is unclear. It is however plausible that relevant receptors need to be exposed to histamine derived from dietary L-Histidine for longer periods of time to be upregulated and consequently affect components of energy balance. For example, acute effects of opiates ingestion inhibits the functional activity of cAMP by inhibiting adenylyl cyclase (enzyme that catalyses the synthesis of cAMP), but with chronic exposure to opiates inhibition of cAMP pathway gradually recovers due to increased tolerance, thus affecting energy balance differently following acute and continued exposure (Nestler and Chao, 2006). This therefore was the rationale for the design of the current study following on from the previous study investigating the effect of L-Histidine ingestion over a short period (3.25 hours). The supplementation period was chosen to be 10 days L-Histidine ingestion. As the study design was cross-over in nature, each participant needed to ingest either placebo L-Histidine (for 10 days each) and also maintain a wash out period (10 days) ie, 31 days for the complete study.

As it is also well established that increasing the length of the study and number of study visits increases the drop out rates among study participants (Stewart *et al.*, 2004), supplementation for 10 days was ideal as the ingestion and wash out periods could be the

same and the visits to the laboratory was only thrice in 31 days. The dose of L-Histidine (25 mg/kg) was well tolerated among study subjects in the pilot study and was maintained in the current study. This study was designed to address this gap in knowledge by investigating in healthy male subjects the effects of oral ingestion of L-Histidine (25 mg/kg) over a period of 10 days. The outcome measures studied were changes in body weight, RMR, MST, CT, body fat composition, fasting blood glucose and total cholesterol concentrations.

3.3.1 Hypothesis

Supplementation with L-Histidine for 10 days will affect RMR, MST, CT and fasting blood glucose concentrations in healthy men.

3.3.2 Aim

The aim of this study was to determine the effects of 10-day oral ingestion of L-Histidine (25 mg/kg/day) on RMR, MST, CT, RER, blood glucose and total cholesterol concentrations in healthy male subjects.

3.3.3 Methods

3.3.3.1 Participant recruitment and profile

Ten lean, healthy male participants were recruited for the study by advertisements placed around the campuses of the University of Westminster, London, UK (section 2.2 for detailed inclusion and exclusion criteria). The sample size calculations (section 2.22.1) were based on changes in fasting blood glucose concentrations. The results of the calculation estimated that at least 9 participants were needed to show any statistical significance post L-Histidine ingestion. By the end of the study, 1 participant had dropped out and 9 completed the study. The mean pre-treatment anthropometric data of participants who completed the study are as shown in Table 3.6.

Table 3.6 – Mean anthropometric data of participants who completed the study at the pre-treatment test session:

	Mean ± SEM	Range
Age (years)	24.8 ± 2.0	19 - 36
Weight (kg)	75.4 ± 1.3	69.3 - 82
Body fat 120	16.0 ± 1.0	11.4 – 20.4
BMI (kg/m ²)	23.8 ± 0.6	21.9 – 24.9
<i>n</i>	9	

3.3.3.2 Study design

The study was approved by the Ethics Committee at the University of Westminster, London, UK (application number 04/05 – 62; Appendix 2). Written informed consent (Appendix 3) was sought from all participants after having explained the research study to them using an information sheet (Appendix 6).

The supplementation of L-Histidine (Lamberts Healthcare Ltd, UK) and placebo (Casilan-90, Complian foods Ltd, UK) followed a randomised, cross over and single blinded experimental protocol. A wash out period of 10 days was given in between the two ingestion periods, to eliminate traces of the previous supplement ingestion as justified previously (Chapter 3.2.3.2, para 2). The placebo and L-Histidine were both prepared as capsules for easy ingestion. Size 0, gelatine capsules (G. Baldwin & Co, London) with a fill capacity of 500 mg, were used. The L-Histidine dosage was 25 mg/kg and the isonitrogenous and isocaloric dose of placebo was determined to be 45 mg/kg. All participants were required to visit the laboratory on 3 separate test occasions, each lasting up to 2 hours, having fasted for a minimum of 12 hours before each visit. In the first laboratory session (day 1), body weight, RMR, percentage body fat, fasting blood glucose (FBG) and total cholesterol (TC) concentrations were measured along with MST and CT (sections 2.5, 2.7, 2.6, 2.11 and 2.12 respectively for detailed methods of measurement). The participants were given a 3-day food diary (Appendix 15) with a photographic food atlas (Appendix 16) to record their prospective food intake over the last 3 days of each 10-day ingestion period (section 2.20 for details of formulation and analysis of diet diary). Every effort was made to ensure maximum reporting accuracy through the use of photographic food atlas and a clear explanation of portion size issues and requirements for written diary entries (details of the analysis software and construction of the food atlas are

given in section 2.20.2; Histidine quantification of reported dietary intakes was performed by using the INTERMAP database (section 2.20.2 for further details). At the end of the first test session, participants were given either placebo or L-Histidine capsules according to the randomisation to be consumed for the next 10 days. The participants were asked to report back to the laboratory on the 11th day for intermediate testing (2nd test session, day 11) where all measurements were repeated as in session 1. Following this test session, participants were asked to maintain a wash-out period of 10 days (days 11-20) after which they were asked to ingest the second set of capsules for the next 10 days (day 21-30) and maintain a food diary for the final 3 days of supplementation and report back to the laboratory on the next day for the final (3rd) test session (day 31). In the final test session, all measurements were repeated as in the first two test sessions to determine the effects of the supplementation.

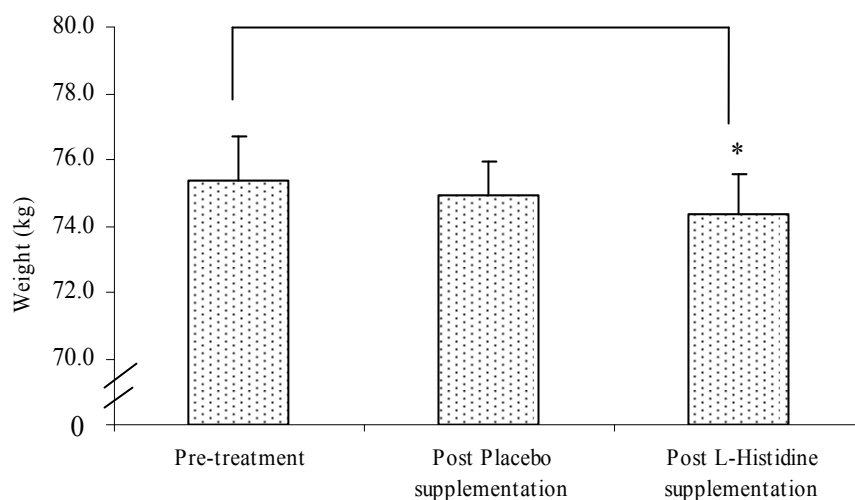
3.3.4 Data analyses

Statistical analyses were performed using the Statistical Programme for Social Sciences (SPSS) 14.0 for Windows (Section 2.22.2). Analyses of data were carried out by following the general linear model of one-way repeated measures analysis of variance (ANOVA) to assess possible differences between the placebo and L-Histidine supplementations in all measured parameters over time. A difference of $p \leq 0.05$ was regarded as statistically significant.

3.3.5 Results

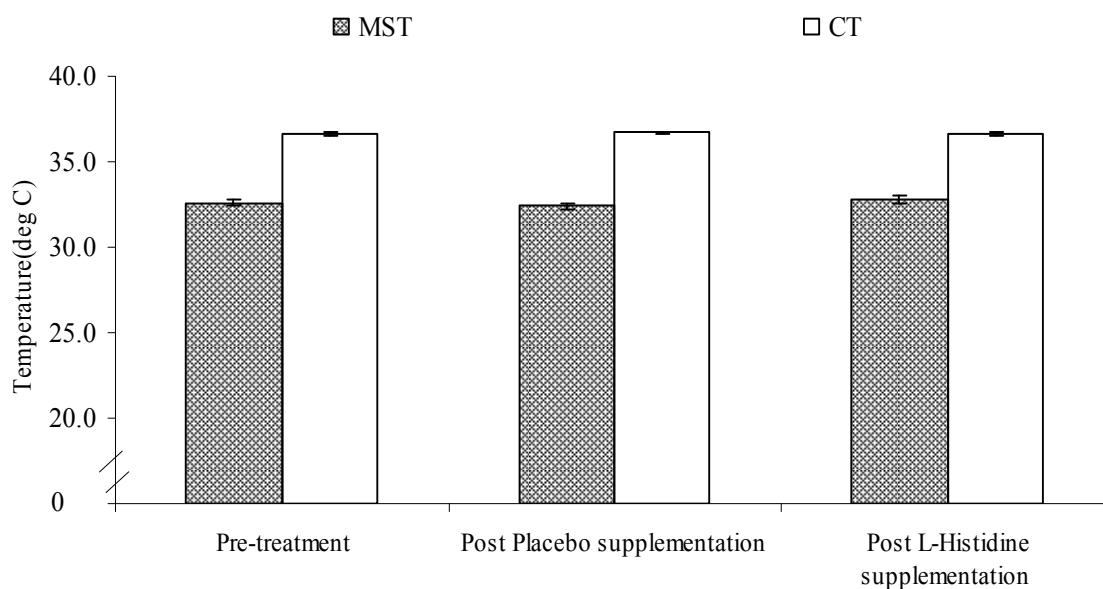
Post L-Histidine ingestion, there was a significant decrease in body weight among participants when compared to body weight measured pre-treatment (pre-treatment: 75.4 ± 1.3 kg compared to post L-Histidine: 74.4 ± 1.2 kg; $p = 0.008$; Figure 3.3). There was no significant change in body weight post placebo intake in comparison with pre-treatment measurements (pre-treatment: 75.4 ± 1.3 kg compared to post placebo: 74.9 ± 0.9 kg; Figure 3.3). There was no effect of order of supplementation due to randomisation.

Figure 3.3 - Mean body weight (\pm SEM) at the end of 10 days supplementation with either placebo or L-Histidine compared to pre-treatment



$n = 9$; * Significantly different from pre-treatment ($p = 0.008$).

Figure 3.4 -Mean of means data for mean skin (MST; \pm SEM), core (CT; \pm SEM) over a 45 minute sampling period pre-treatment, post placebo and post L-Histidine ingestion



$n = 9$; Ambient temperature (AT; \pm SEM) over a 45 minute sampling period pre-treatment, post placebo and post L-Histidine ingestion was 22.8 ± 0.6 , 23.5 ± 0.4 , 22.4 ± 0.6 °C respectively.

MST and CT remained unchanged from pre-treatment readings, after placebo and L-Histidine supplementation (Figure 3.4). Ambient temperature was maintained at $22.9 \pm 0.5^{\circ}\text{C}$ during all test sessions (Figure 3.4). There was no effect of L-Histidine ingestion on RMR (Table 3.7). There were no changes in RER following placebo and L-Histidine supplementations compared to pre-treatment values (Table 3.7). No change was measured in total body fat percentage post placebo and post L-Histidine supplementation in comparison to pre-treatment values (Table 3.7). Consumption of total energy and macronutrients remained unchanged during both periods of supplementation (Table 3.8). Dietary histidine intake did not vary significantly during the last 3 days of either L-Histidine or placebo supplementation (Table 3.9).

Table 3.7 - Mean resting metabolic rate (RMR; \pm SEM), respiratory exchange ratio (RER; \pm SEM) and percentage body fat (\pm SEM) pre-treatment, post placebo and post L-Histidine supplementation.

Parameter	Pre-treatment	Post placebo supplementation	Post L-Histidine supplementation
RMR (kcal/ day)	1666.4 ± 44.7	1751.8 ± 61.4	1762.1 ± 92.0
RER	0.83 ± 0.01	0.82 ± 0.01	0.83 ± 0.01
Body Fat (%)	16.0 ± 1.0	15.6 ± 1.1	15.5 ± 1.0

n = 9; There were no significant differences.

Table 3.8 - Mean daily dietary intake (\pm SEM) and nutrient intake expressed as a percentage of total energy intake (%TEI) during the final three days of placebo supplementation compared to mean dietary intake in the final three days of L-Histidine supplementation

	Total Energy Intake (kcal)	Protein %TEI	Fat %TEI	Carbohydrate %TEI
Placebo	1970.7 ± 218.9	18.1 ± 1.5	30.1 ± 2.1	50.3 ± 2.4
L-Histidine	2086.3 ± 266.6	16.5 ± 1.6	36.2 ± 2.5	45.8 ± 3.3

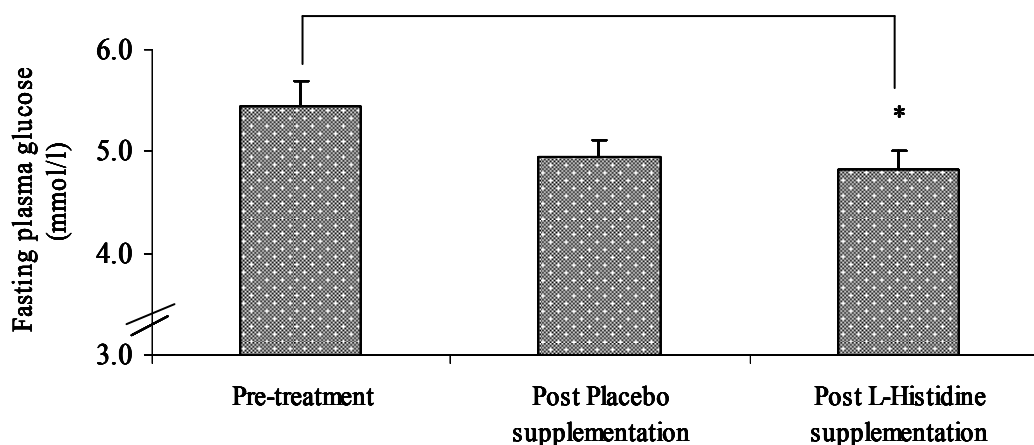
n = 9; There were no significant differences; † Likely validity of reported kcal intake discussed in appendix 13.

Table 3.9 - Mean dietary histidine intake during the final three days of placebo supplementation compared to mean dietary histidine intake in the final three days of L-Histidine supplementation

Supplementation	Histidine intake (mg/day)
Placebo	2149.8 ± 173.4
L-Histidine	2484.3 ± 641.0

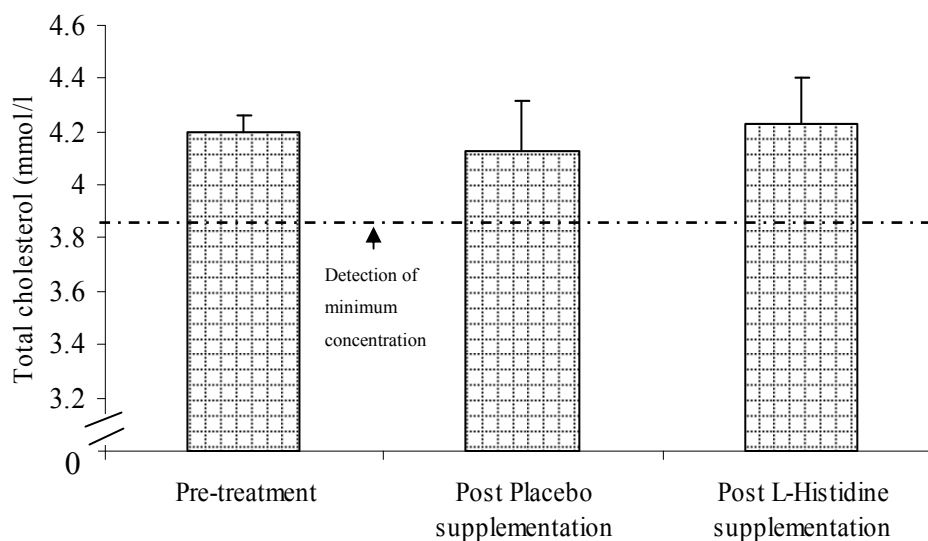
n = 9; There were no significant differences.

Figure 3.5 - Mean fasting blood glucose concentrations (± SEM) following placebo and L-Histidine supplementation compared with pre-treatment



*n = 9; * Significantly different from pre-treatment (p = 0.04).*

Figure 3.6 - Mean total cholesterol concentrations (\pm SEM) measured pre-treatment, post placebo and post L-Histidine supplementation



Complete data set (n = 3), all other samples were below the level of detection at either of the test sessions

Post L-Histidine supplementation, there was a significant decrease in mean fasting blood glucose concentrations from pre-treatment values (pre-treatment: 5.4 ± 0.3 mmol/l compared to post L-Histidine: 4.8 ± 0.2 mmol/l; $p = 0.04$; Figure 3.5). There was no effect of placebo on fasting blood glucose concentrations. There were no significant differences in blood glucose concentrations between post placebo and L-Histidine supplementation. Meaningful interpretation of total cholesterol concentrations could not be made due to missing values during statistical analysis (Figure 3.6).

3.3.6 Discussion

This study was conducted to assess the chronic effects of L-Histidine supplementation for 10 days on metabolic indices of energy balance such as EE, thermogenesis and blood glucose regulation. There was a significant decrease in body weight and fasting blood glucose concentrations post L-Histidine ingestion when compared with pre-treatment. There were no significant effects of L-Histidine on RMR, MST, CT, RER and total cholesterol concentrations. There were no effects on any of the parameters measured following supplementation with placebo. There was no effect of the order of supplementation for any parameter measured.

Effects of L-Histidine on metabolic indices of energy expenditure and thermogenesis (body weight, RMR, MST, CT, % body fat and RER)

There were no effects of placebo supplementation on body weight. There was a significant reduction in body weight following L-Histidine ingestion when compared to pre-treatment measurement (pre-treatment: 75.4 ± 1.3 kg compared to post L-Histidine: 74.4 ± 1.2 kg; $p = 0.008$; Figure 3.3). Normally, a reduction in body weight is associated with either an increase in RMR, decrease of total body fat composition or a decrease in dietary intake. However, there were no significant differences in either RMR, total body fat percentage or the rate of fat oxidation (which would indicate an increase in lipolysis) in the current study. There were also no differences observed in nutrient and calorie intake between two periods of supplementation. The change in body weight over 10 days is small and it maybe that changes in RMR, RER and dietary intake are too small to detect statistically but sufficient to have a biological effect over time. As there was no change in obvious markers of energy balance, the change in body weight measured in the current study needs to be considered with care. Perhaps the change in body weight is due to the participants taking part in the study and bringing about lifestyle changes and by staying motivated. Further work, such as longer periods of supplementation, is required to demonstrate the chronic effects of L-Histidine supplementation.

Even though in this study there appeared to be no effect on RMR, fat oxidation and dietary intake, histamine has been shown to affect parameters of energy balance. Several studies in rodents have shown that histamine stimulates EE and lipolysis in adipose tissue by

activation of the SNS (Yoshimatsu *et al.*, 2002b) suppression in food intake is mediated via H1 receptors in the hypothalamus (Fulop *et al.*, 2003). The majority of studies performed in animal models clearly showing that histaminergic activity is associated with regulation of food intake (Gotoh *et al.*, 2005; Morimoto *et al.*, 1999), administer either histamine via intracerebral infusion (Masaki *et al.*, 2001; Yoshimatsu *et al.*, 1999) or histidine via intraperitoneally injections (Yoshimatsu *et al.*, 2002a). There are very few human studies examining the anorexic effect of oral histidine ingestion. One such human study found that oral intake of very high doses (64.8 g/day) of histidine caused anorexia, taste and smell dysfunction along with cerebellar dysfunction and significant decreases in serum zinc concentrations (Henkin *et al.*, 1975). However, data obtained from the current study is partly in accordance with findings from a study by Schechter and Prakash (1979) who found no change in appetite, taste and smell perception, food intake or body weight after oral intake of L-Histidine 4 g/day, for a period of 2 weeks by eight, healthy volunteers.

Dietary histidine intake quantified with the aid of the INTERMAP database (personal communication (discussion followed by email on 16/6/09) with Dr Claire Robertson), showed no difference in histidine intake during either placebo or L-Histidine supplementation period and therefore no confounding effect of dietary histidine is envisaged. It should be noted however, that although histidine is generally accepted as an essential amino acid, few available databases (i.e. those incorporated in dietary analysis software systems) include histidine. It was therefore of considerable benefit to access quantitative information regarding histidine levels in a wide range of foods (1448) via the compilation as provided by INTERMAP study (personal communication (discussion followed by email on 16/6/09) with Dr Claire Robertson, UK Country Nutritionist; Schakel *et al* (2003)).

There were no differences in MST and CT from pre-treatment measurements after ingestion with either placebo or L-Histidine. The present study attempted to measure changes, if any, in MST and CT post L-Histidine ingestion to observe changes in thermogenesis. Also, as there is no valid marker to depict upregulation of UCP expression apart from mRNA analysis and as this technique was beyond the scope of this study, these measurements attempted to infer that a possible increase in MST and/or CT might indicate upregulation of UCPs. UCP2 and UCP3, are abundant in WAT, skeletal muscle, the spleen and pancreatic β cells and are of particular interest because of their potential role in EE

(Thompson and Kim, 2004). They are found in the inner mitochondrial membrane and under control of noradrenaline released from the SNS, are able to uncouple the oxidation of fuels via the electron transport chain from ATP synthesis, thus dissipating energy as heat and potentially affecting metabolic efficiency (Dulloo and Samec, 2000). Previous studies have shown that infusion of histamine into the lateral cerebroventricle of mice upregulated gene expression for UCP3 in WAT (Masaki *et al.*, 2001) and H1 and H3 receptor knock out mice have decreased expression of UCP1 and UCP3 (Masaki *et al.*, 2004; Takahashi *et al.*, 2002). It is unclear whether the activation/upregulation of UCPs could be detected by body temperature measurements by means of skin thermistors and rectal temperature measurement by rectal probe. Other methods of UCP analysis via muscle biopsy and northern blotting to quantify the amounts of RNA species for the UCP genes might provide accurate results for any increase in BAT or WAT UCP mRNA expression (Masaki *et al.*, 2001). These measurement techniques were not possible to conduct in the present and following studies as there was neither ethical approval nor the medical expertise to conduct muscle biopsies and genetic analysis. These methods need to be considered by others conducting any future studies of this nature.

There was no difference in fat oxidation post placebo or L-Histidine supplementation from pre-treatment. Further sensitive measures of lipolysis and techniques of measurement of EE might clarify the observed weight loss in study participants and clarify the cause and mechanism of weight loss observed. Techniques have been developed to quantify substrate turnover in abdominal subcutaneous adipose tissue *in vivo* after cannulation of the left superficial epigastric vein to allow sampling adipose tissue venous blood which might show more accurate and significant results (Frayn *et al.*, 1993). The characteristics of the adipose tissue venous blood are quite distinct from those of the superficial (mainly skin) or the deep (mainly muscle) tissues of the forearm and appears to be typical of adipose tissue as a whole in terms of non-esterified fatty acid release. This method is difficult and time consuming and was beyond the practicalities of this study. Circulating glycerol and fatty acids could be good markers to indicate whole body substrate oxidation and are easily assayed. If these assay techniques were employed in the current study it might have shown significant differences.

Effect of L-Histidine ingestion on glucose regulation

There was no effect of placebo on fasting blood glucose concentrations while there was a significant decrease in fasting blood glucose concentrations after L-Histidine ingestion when compared to pre-treatment measurements (pre-treatment: 5.4 ± 0.3 mmol/l compared to post L-Histidine: 4.8 ± 0.2 mmol/l; $p = 0.04$; Figure 3.5). This could be attributed to the fact that L-Histidine may indirectly enhance insulin's action. L-Histidine is a zinc-chelating agent and helps in intestinal zinc absorption (Horn *et al.*, 1995). Zinc is involved in the regulation of insulin receptor-initiated signal transduction mechanisms (Song *et al.*, 1998). The role of zinc in insulin metabolism is well established and studies have demonstrated that the mineral increases insulin binding to receptors (Arquilla *et al.*, 1978). It has been shown that zinc supplementation in genetically obese mice resulted in a reduction in plasma insulin and glucose concentrations and also attenuated the glycaemic response after the glucose load and this effect was due to enhanced insulin activity by zinc (Chen *et al.*, 1998). Another hypoglycaemic mechanism of histidine has been shown by a study which observed that treatment of diet induced obese (DIO) and leptin resistant (*db/db*) mice with histamine lowered serum glucose and insulin concentrations as well as improving glucose tolerance (Masaki *et al.*, 2001). This was a result of the decrease in circulating free fatty acids which is a major determinant of improving insulin sensitivity as it increases hepatic glucose output and decreases glucose disposal in the muscle. In the current study, neither zinc nor free fatty acid concentrations were measured as it was beyond the scope of the study. Future studies need to incorporate these measurements in order to fully determine the hypoglycaemic mechanism of L-Histidine.

Effect of L-Histidine on total cholesterol concentration

There were no statistical advantage to report for changes in the total cholesterol concentrations following both placebo and L-Histidine supplementations. Six participants' blood cholesterol were recorded as 'low' during one of the three test sessions as the Accutrend GC meter employed to test blood cholesterol did not read values below 3.88 mmol/l. These participants' missing data were excluded during statistical analysis by SPSS. The number of full data sets included in the statistical analysis was only 3 and this sample size is small and it is understood that the reliability of such a data set is low and conclusions cannot be drawn from their analysis. However, the observation of no effect of histaminergic action on total cholesterol concentrations has previously been shown by Takahashi and colleagues (2002) in mice with targeted disruption of the H_3 receptor ($H_3^{-/-}$).

These $H_3^{-/-}$ mice presented with elevated concentrations of leptin and insulin (i.e. were leptin and insulin resistant), but no changes in cholesterol and triglyceride concentrations were observed. No human studies have examined this effect of L-Histidine on total cholesterol concentration and this study is the first to do so. Unfortunately, due to small dataset, conclusions could not be drawn.

3.3.7 Conclusion

Oral administration of L-Histidine for 10 days influenced body weight and fasting blood glucose concentrations. It would be expected that a change in body weight might be due to change in BMR, increased fat oxidation or decrease in dietary intake, but these changes were not observed. The change in body weight and fasting blood glucose concentrations were subtle though statistically significant. If calorie deficit were ongoing at the same rate, it stands to reason that if L-Histidine was supplemented for a longer period greater weight loss may be observed. While it would be of interest to measure changes in plasma insulin, leptin and free fatty acids which might show the mechanism of L-Histidine action, weight loss without compositional changes, suggests equal losses of fat and non-adipose body tissue/water. To further investigate this question it is likely that longer-term (than the present study) ingestion of L-Histidine could clarify its metabolic and consequential physical effects in humans (described in Chapter 3.4).

3.4 Metabolic effects of long term (8 weeks) oral ingestion of L-Histidine in overweight and obese men

It has been previously shown that histamine infusion reduces fat accumulation and upregulates UCPs in leptin resistant obese mice (Masaki *et al.*, 2001) and that leptin affects feeding behaviour through activation of the central histaminergic system via histamine receptors (Masaki *et al.*, 2003; Morimoto *et al.*, 1999; Sakata *et al.*, 1997; Yoshimatsu *et al.*, 1999) in obese mice. All of these studies demonstrating interaction between leptin and histamine pathways have been conducted on animal models and there is a need to assess the implication of these findings for humans. The following study was designed to study the metabolic effects of oral ingestion of L-Histidine for 8 weeks in overweight and obese men, who are most likely to be leptin resistant due to increased adipose tissue mass (Jequier and Tappy, 1999; Stanley *et al.*, 2005; Trayhurn *et al.*, 1999) when compared with healthy (normal weight) humans from the previous study.

In the previous study (Chapter 3.3), body weight and fasting blood glucose concentrations decreased significantly post L-Histidine ingestion among healthy subjects. To investigate these effects further, the following study was designed to explore the effects of longer-term ingestion of L-Histidine on factors affecting energy balance and glycaemia in overweight and obese sample populations. Variations in physiology have been shown to alter nutrient utilisation and metabolism (e.g. during pregnancy, there is increased maternal plasma volume which alters the concentrations of nutrients such as zinc, folic acid etc in the blood (Campbell, 1988)). As there are alterations in the amount of fat mass in the study sample population due to overweight and obesity, the effects previously seen (chapter 3.3) may or may not be apparent in the current study population. Also, as the effects of L-Histidine on RMR, lipolysis were inconclusive from the findings of the previous study, it was supposed that data from further long-term (> 2 weeks) study investigating the metabolic effects of L-Histidine supplementation would elucidate these metabolic effects noticeably. It was supposed that exposure to L-Histidine over a prolonged period might alter the uptake and transport of L-Histidine and may perhaps modify histamine interaction with the receptors resulting in augmented upregulation of histamine receptors and thereby affecting the study measures. The dose of L-Histidine was maintained at 25 mg/kg/day and was unchanged as

this dose is already twice the RDA nevertheless non-toxic and it is unclear whether any further increments in doses might cause deleterious effects.

3.4.1 Hypothesis

Long-term supplementation (8 weeks) of L-Histidine will increase components of energy expenditure (RMR, MST, CT) and reduce fasting blood glucose concentrations in overweight and obese but otherwise healthy men.

3.4.2 Aim

The aim of this study was to investigate the effects on RMR, MST, CT, fasting blood glucose (FBG), blood lipid, plasma insulin, leptin and non-esterified fatty acid (NEFA) concentrations of 8 weeks ingestion of L-Histidine (25 mg/kg/day) in overweight and obese men.

3.4.3 Methods

3.4.3.1 Participant recruitment and profile

Twenty two male participants between the ages of 20-55 years were recruited by advertisements placed in the Metro and Evening Standard newspapers, London, UK. Inclusion criteria were a BMI of ≥ 25 and not suffering from any chronic disorder (such as, diabetes mellitus or hypertension) or ingesting any dietary or herbal supplements or medications (section 2.2 for further details).

The sample size calculations (section 2.22.1) used changes in blood glucose concentrations, and resulted in nine participants to be recruited in each supplementation group. It was noted that as this calculation was based on just one parameter, it may not necessarily show significant effects on other metabolic parameters. As the study was randomised and parallel in design eighteen participants needed to be recruited, nine in the placebo group and nine in the L-Histidine group. Of the 22 participants initially recruited, 18 participants completed the study while 4 participants dropped out during various stages of the study. All 4 of the participants who dropped out reported being unable to spare time for laboratory test sessions due to work commitments. Two participants from each group dropped out of the study and this was a random occurrence as participants dropped out following varying test sessions (1 participant dropped out after the 1st test session whereas the remaining three participants dropped out following the 2nd test session).

Table 3.10 – Mean anthropometric data of the participants following random assignment to either placebo or L-Histidine supplementation prior to the supplementation period

	Placebo Group		L-Histidine group		Statistical difference
	Mean \pm SEM	Range	Mean \pm SEM	Range	
Age (years)	39.56 \pm 2.76	29 - 48	42.89 \pm 2.50	30 - 50	NS
Weight (kg)	99.67 \pm 6.44	75.7 – 141.1	90.48 \pm 6.10	68.5 – 115.4	NS
Body fat (%)	27.24 \pm 1.50	18.12 – 31.6	25.92 \pm 2.06	21 – 34.6	NS
BMI (kg/m ²)	31.08 \pm 1.82	25.2 – 43.8	28.41 \pm 1.72	25 – 37.4	NS
<i>n</i>	9		9		

NS = not significant; only participants who completed the study are included in this data set.

Statistical analysis via paired samples t-tests (tested at 5% level of significance; SPSS 14.0) of the difference in initial weight of participants (due to randomisation) found no difference between the groups ($p = 0.130$).

3.4.3.2 Study design

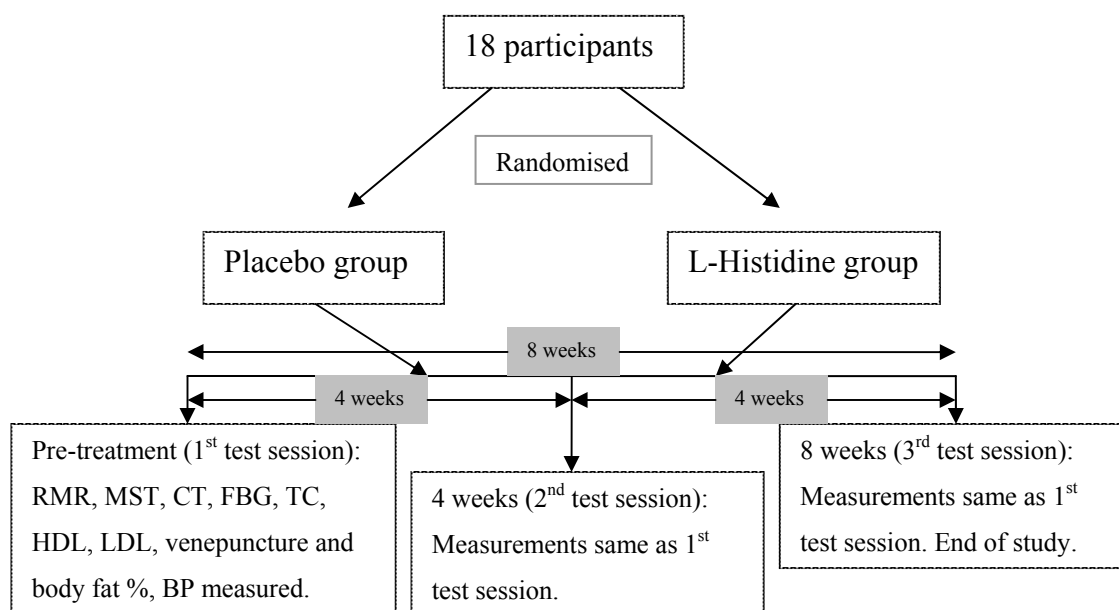
Participants were required to visit the laboratory on 3 separate occasions, over the test period of 8 weeks, each visit lasting up to 2 hours in a fasting state (Figure 3.7). The study was single blinded and each participant was randomised into either the placebo or L-Histidine group for the duration of the study. Simple randomisation of participants was accomplished by choosing a folded note with the group specified on it among many similar unspecified pieces of folded notes.

Measurements including RMR, MST, CT, TC, HDL, LDL, FBG and % body fat and blood pressure (BP) were recorded during the first laboratory session (pre-treatment) and repeated after 4 (second test session) and 8 weeks (third test session), as described in sections 2.7, 2.9, 2.14, 2.11, 2.19 respectively. The analysis of food intake was carried out with the use of a prospective 3-day food diary (section 2.20) at pre-treatment and at the end of 8 weeks to detect changes in nutrient intake. The doses of placebo and L-Histidine were kept isonitrogenous and isocaloric. Participants in the placebo group ingested Casilan-90 (Complan foods Ltd, UK) 45 mg/kg/day and the participants in the L-Histidine group ingested 25 mg/kg/day L-Histidine (Lamberts Healthcare Ltd, UK) for the duration of the study (8 weeks). The placebo and L-Histidine were both prepared as capsules for easy

ingestion. Size 0, gelatine capsules (G. Baldwin & Co, London) with a fill capacity of 500 mg, were used.

In addition to measuring the previously studied metabolic parameters as outlined in Chapter 3.3, NEFA analysis, insulin and leptin assays were performed to provide an insight into the possible metabolic processes of oral ingestion of L-Histidine (sections 2.15, 2.16, 2.17 and Appendices 19-21 for details on the methods used to assay NEFA, insulin and leptin). Whole blood was obtained by venepuncture at the beginning of each test session and collected in heparinised vacutainers. The vacutainers were centrifuged for 12 minutes at 3500 rpm and the plasma removed. The plasma was stored immediately at -80°C until required for further analyses of insulin, NEFA and leptin (section 2.13).

Figure 3.7 - Diagrammatic representation of study design



3.4.4 Data analyses

Data are presented as mean \pm standard error of mean (SEM). Repeated measures ANOVA using SPSS 14.0 for Windows was performed with post hoc tests to determine statistical significance in variations observed in the measured parameters. A p value ≤ 0.05 was considered statistically significant. Pearson's correlation coefficient analysis was performed to analyse the correlation between study variables (e.g. correlation between

plasma insulin and blood glucose). This analysis was also performed with the use of SPSS 14.

3.4.5 Results

There were no significant differences in body weight between the two groups of participants pre-treatment ($p = 0.315$; independent samples T-test; Table 3.11). Overall, both groups had lost weight at the end of the 8 week period ($p = 0.011$). There was however no effect of L-Histidine ingestion on weight loss ($p > 0.05$; Table 3.11).

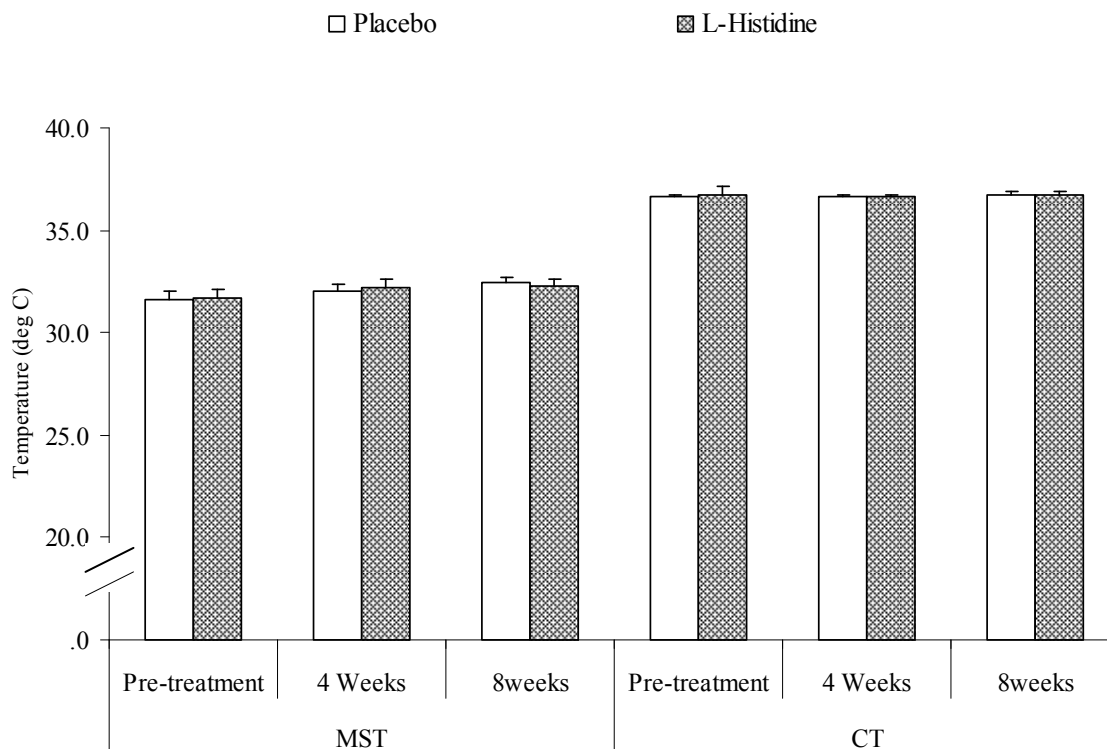
Table 3.11 - Mean body weight (\pm SEM) following placebo and L-Histidine ingestion for 4 and 8 weeks compared to pre-treatment

Body weight (kg)	Placebo	L-Histidine	Mean of both groups
Pre-treatment	99.67 \pm 6.44	90.48 \pm 6.10	95.07 \pm 6.27
4 Week	98.83 \pm 6.47	89.96 \pm 6.34	94.39 \pm 6.41
8 Week	98.54 \pm 6.33	89.70 \pm 6.37	94.12 \pm 6.35 *

*n = 18 (9 in placebo group, 9 in L-Histidine group); There was a significant difference between mean body weight pre-treatment and post L-Histidine ingestion; *Significantly different from pre-treatment ($p = 0.011$).*

Data collected at 4 and 8 weeks post placebo and L-Histidine ingestion showed no changes in MST and CT when compared with pre-treatment data (Figure 3.8). The ambient temperature was maintained at a range of $22.7 \pm 0.4^\circ\text{C}$ during all test sessions to avoid cold-induced thermogenesis (Figure 3.8). There were no significant differences in RMR between the two groups (Table 3.11). Mean overall RER increased after 4 weeks when compared to pre-treatment RER (pre-treatment: 0.82 ± 0.01 compared to 4 weeks: 0.87 ± 0.02 ; $p = 0.006$; Table 3.13). However, there was no significant difference between RER measured at 4 and 8 weeks. There was no difference between the groups therefore no effect of L-Histidine ingestion was detected (Table 3.13). There were no changes observed in body fat composition after 4 and 8 weeks ingestion with either placebo or L-Histidine when compared with pre-treatment measurements (Table 3.14).

Figure 3.8 - Mean of means of skin (MST; \pm SEM) and core (CT; \pm SEM) pre-treatment and at 4 and 8 weeks following placebo and L-Histidine ingestion measured over a 45 minute sampling period



n = 18 (9 in placebo group, 9 in L-Histidine group); Ambient temperature (AT; \pm SEM) pre-treatment and at 4 and 8 weeks following placebo and L-Histidine ingestion monitored over a 45 minute sampling period was 22.6 ± 0.2 , 22.3 ± 0.3 , 23.0 ± 0.2 and 23.7 ± 0.8 , 22.3 ± 0.4 , 22.5 ± 0.2 °C respectively; There were no significant differences.

Table 3.12 - Mean resting metabolic rate (RMR; \pm SEM) pre-treatment and following placebo and L-Histidine ingestion for 4 and 8 weeks

RMR (Kcal/day)	Placebo	L-Histidine
Pre-treatment	2017.81 \pm 143.71	1809.37 \pm 78.63
4 Week	1982.71 \pm 125.48	1797.08 \pm 88.67
8 Week	2012.17 \pm 117.76	1842.33 \pm 91.13

n = 18 (9 in placebo group, 9 in L-Histidine group); There were no significant differences.

Table 3.13 - Mean respiratory exchange ratio (RER; \pm SEM) pre-treatment, 4 weeks and 8 weeks of ingestion with either placebo or L-Histidine

RER	Placebo	L-Histidine	Mean of both groups
Pre-treatment	0.81 \pm 0.01	0.82 \pm 0.01	0.81 \pm 0.01
4 Week	0.86 \pm 0.03	0.88 \pm 0.02	0.87 \pm 0.02*
8 Week	0.83 \pm 0.02	0.89 \pm 0.02	0.86 \pm 0.02

n = 18 (9 in placebo group, 9 in L-Histidine group); *Significant difference in overall mean RER measured at 4 weeks in comparison with overall RER pre-treatment (*p* = 0.006).

Table 3.14 - Percentage body fat (\pm SEM) measured by skin-fold measurements pre-treatment and after 4 and 8 weeks ingestion throughout with either placebo or L-Histidine

Body Fat (%)	Placebo	L-Histidine
Pre-treatment	28.38 \pm 1.11	25.14 \pm 2.11
4 Week	28.04 \pm 1.22	24.44 \pm 2.10
8 Week	28.63 \pm 1.19	24.89 \pm 2.45

n = 18 (9 in placebo group, 9 in L-Histidine group); There were no significant differences.

Table 3.15 - Mean daily dietary intake (\pm SEM) and nutrient intake expressed as a percentage of total energy intake (%TEI)[†] pre-treatment and at the end of 8 weeks following either placebo or L-Histidine ingestion

Group	Time	Energy (kcal/day)	Protein (%TEI)	Fat (%TEI)	Carbohydrate (%TEI)
Placebo	Pre-treatment	2895.43 \pm 236.20	14.01 \pm 0.96	29.05 \pm 3.84	48.50 \pm 4.60
	Post 8 weeks	3023.86 \pm 282.63	13.80 \pm 0.75	30.73 \pm 2.72	47.22 \pm 3.15
L-Histidine	Pre-treatment	2007.00 \pm 231.88	18.70 \pm 2.23	32.53 \pm 3.15	45.53 \pm 2.71
	Post 8 weeks	2230.29 \pm 305.26	16.89 \pm 1.68	32.35 \pm 2.91	44.02 \pm 3.37

n = 18 (9 in placebo group, 9 in L-Histidine group); There were no significant differences in any of the nutrient intakes; [†] Likely validity of reported kcal intake discussed in appendix 13.

Table 3.16 - Mean dietary histidine intake pre-treatment and at the end of 8 weeks following either placebo or L-Histidine ingestion

Group	Time	Histidine intake (mg/day)
Placebo	Pre-treatment	2073.85 ± 446.99
	Post 8 weeks	1923.82 ± 295.70
L-Histidine	Pre-treatment	1913.49 ± 221.54
	Post 8 weeks	2093.99 ± 258.63

n = 18 (9 in placebo group, 9 in L-Histidine group); There were no significant differences

Diet diaries were maintained for 3 days prior to the start of either supplementation and again for the last 3 days prior to the final test session (Table 3.15). There were no differences in the average total calories and percentage of nutrient intakes (carbohydrates, proteins, fat) consumed between the two supplementation groups or between the mean overall dietary intakes during the two sampling periods. Dietary histidine intake from pre-treatment to post 8 weeks did not change over time or between groups (Table 3.16).

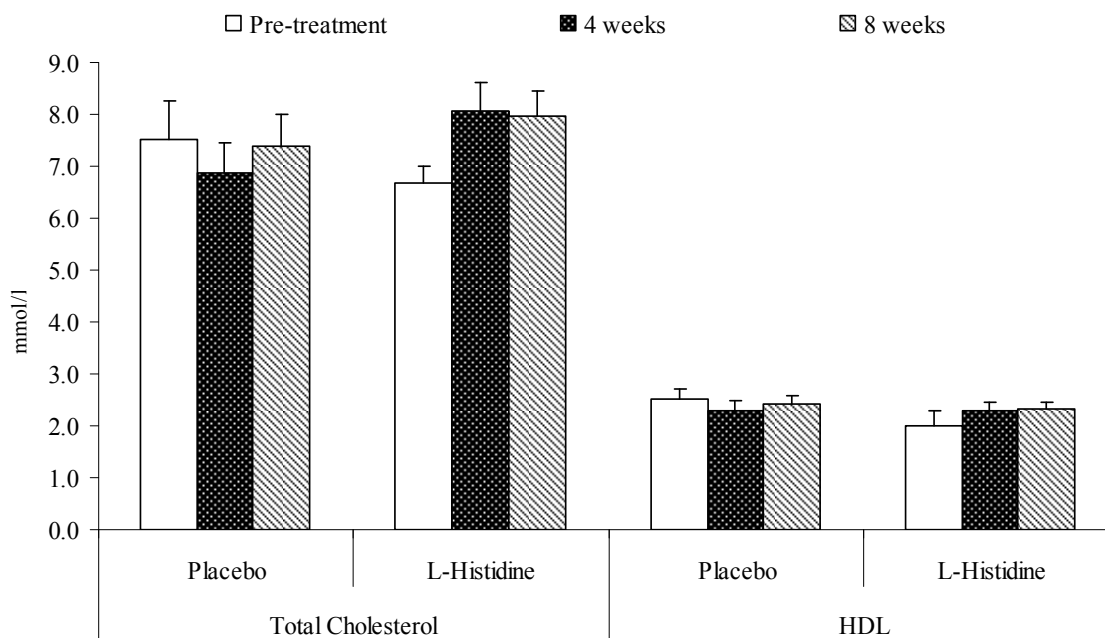
There were no differences in fasting blood glucose or insulin concentrations between the placebo and L-Histidine groups (Table 3.17). There were no correlations between insulin and blood glucose concentrations due to supplementation or time (Pearson's correlation coefficient). There were no differences in plasma leptin and NEFA concentrations between pre-treatment, 4 and 8 weeks and between placebo and L-Histidine (Table 3.17).

Table 3.17 - Mean fasting glucose (FBG; \pm SEM), insulin (\pm SEM), leptin (\pm SEM) and non-esterified fatty acid (NEFA; \pm SEM) concentrations in circulation measured pre-treatment and after 4 and 8 weeks of either placebo or L-Histidine supplementation

Parameter	Time	Placebo	n	L-Histidine	n
FBG (mmol/l)	Pre-treatment	4.91 \pm 0.15	9	5.33 \pm 0.34	9
	4 Week	4.93 \pm 0.22	9	5.39 \pm 0.23	9
	8 Week	5.15 \pm 0.24	9	5.15 \pm 0.16	9
Insulin (mU/l)	Pre-treatment	4.54 \pm 0.83	6	4.09 \pm 1.07	8
	4 Week	4.73 \pm 1.02	6	3.97 \pm 0.72	8
	8 Week	5.68 \pm 0.74	6	3.83 \pm 0.63	8
Leptin (ng/ml)	Pre-treatment	20.08 \pm 5.29	6	11.37 \pm 2.93	8
	4 Week	14.96 \pm 2.12	6	10.56 \pm 2.15	8
	8 Week	15.16 \pm 2.31	6	10.69 \pm 2.76	8
NEFA (μ mol/l)	Pre-treatment	967.27 \pm 117.92	6	820.88 \pm 67.95	8
	4 Week	941.87 \pm 145.34	6	847.32 \pm 109.84	8
	8 Week	833.02 \pm 105.50	6	850.38 \pm 88.52	8

There were no significant differences in any of the measured parameters; 4 participants' (3 in placebo group and 1 in L-Histidine group) plasma samples were lost due to breakage of eppendorf tubes during freezing.

Figure 3.9 - Mean total cholesterol (\pm SEM), high density lipoprotein (HDL; \pm SEM) concentrations pre-treatment and post ingestion of either placebo or L-Histidine for 4 and 8 weeks



n = 18 (9 in placebo group, 9 in L-Histidine group); There were no significant differences.

Table 3.18 - Mean low density lipoprotein (LDL; \pm SEM) concentrations pre-treatment and post ingestion of either placebo or L-Histidine for 4 and 8 weeks

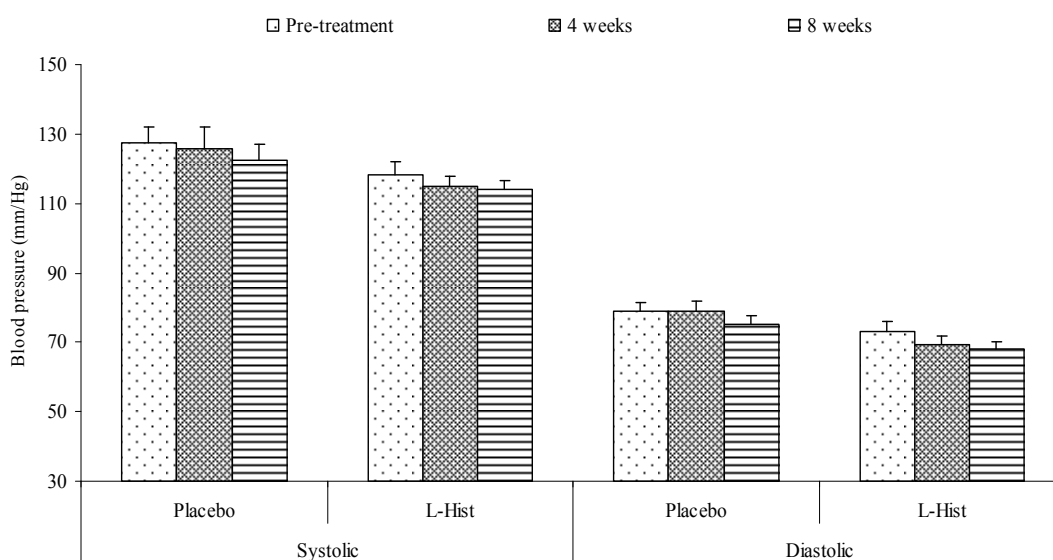
LDL (mmol/l)	Placebo	L-Histidine	Mean of both groups
Pre-treatment	3.9 \pm 0.5	2.6 \pm 0.3*	3.2 \pm 0.4
4 Week	3.4 \pm 0.3	3.6 \pm 0.4	3.5 \pm 0.4
8 Week	4.2 \pm 0.5	3.9 \pm 0.4	4.1 \pm 0.4 **

*n = 18 (9 in placebo group, 9 in L-Histidine group); * Significantly different from pre-treatment measurement of placebo group ($p = 0.023$); ** Significantly different from overall mean LDL concentration at pre-treatment ($p = 0.039$).*

There were no differences in total cholesterol or HDL concentrations between the placebo and L-Histidine supplemented groups (Figure 3.9). There was a significant difference in

LDL concentration between the pre-treatment measurements of the two groups. Participants in the L-Histidine group had a significantly lower concentration of LDL at the start of the study when compared to pre-treatment reading of participants in the placebo group (pre-treatment (placebo): 3.9 ± 0.4 mmol/l as compared to pre-treatment (L-Histidine): 2.6 ± 0.3 mmol/l; $p = 0.023$, Table 3.18). Mean overall LDL concentrations increased after 8 weeks when compared with pre-treatment values, irrespective of the kind of treatment (pre-treatment: 3.2 ± 0.4 mmol/l as compared to overall mean 8 weeks: 4.1 ± 0.4 mmol; $p = 0.039$; Table 3.18).

Figure 3.10 - Mean systolic and diastolic blood pressure (\pm SEM) following 4 and 8 weeks of either placebo or L-Histidine ingestion



n = 18 (9 in placebo group, 9 in L-Histidine group); There were no significant effects of L-Histidine on blood pressure; Overall ($n=18$) mean systolic BP measured at 8 weeks was significantly lower from overall ($n=18$) mean systolic BP measured pre-treatment ($p = 0.029$).

There was an overall decrease in systolic blood pressure in all participants after eight weeks, from overall mean pre-treatment measurement (pre-treatment: 122.72 ± 4.3 mm/Hg as compared to at 8 weeks for both placebo and L-Histidine combined: 118.13 ± 3.6 mm/Hg; $p = 0.029$). There was no effect of L-Histidine supplementation on either systolic or diastolic blood pressure (Figure 3.10).

3.4.6 Discussion

The results of the current study show that there were no significant differences in most of the measured parameters due to L-Histidine ingestion for a period of 8 weeks. However, there was an overall decrease in body weight, an increase in LDL concentrations and a decrease in systolic blood pressure among all participants at 8 weeks compared to pre-treatment.

Effect of 8 week L-Histidine ingestion on body weight and regulators of energy balance (RMR, MST, CT, RER, and dietary intake)

Mean overall body weight had decreased at 8 weeks when compared with pre-treatment measurements, irrespective of type of supplementation. Decrease in body weight is usually associated with changes in other parameters which influence weight loss such as RMR, MST, CT, RER and dietary intake. There were no changes detected in any of these parameters at 8 weeks compared to pre-treatment measurements. The question therefore arises as to whether this decrease in body weight is genuine or an artefact of study participation. Possibly, the methods employed in this study to measure the parameters associated with weight change were insufficiently sensitive to detect very subtle changes.

The effect of histidine on weight loss has been substantiated by studies conducted in animal models wherein it has been shown that intracerebroventricular infusion of histamine upregulates UCP1 mRNA in BAT and UCP3 mRNA in WAT and down-regulates *ob* gene expression: all contributing to weight loss (Masaki *et al.*, 2001; Masaki *et al.*, 2003). Previous animal studies have measured changes in EE via Northern blot analysis for UCP mRNA analysis (Masaki *et al.*, 2001; Yasuda *et al.*, 2004). No data for measurement of EE via indirect calorimetry post histidine or histamine supplementation among either animal or human studies is available in the literature. Future studies of this kind could perform mRNA analysis to detect up-regulation of UCPs to further elucidate the effect of oral ingestion of L-Histidine in humans and any possible increase in RMR.

Mean overall RER increased after 4 weeks in comparison with pre-treatment, indicating increased carbohydrate oxidation at 4 weeks. This could be due to the regulatory mechanism adapted by the body during the post-absorptive state. Perhaps performing

indirect calorimetry to calculate RMR and RER at different times of the day or after shorter periods of fasting might have led to differing results. No variation in MST, CT was observed over time or due to supplementations. There are also various other methods for measurement of CT which might detect changes more sensitively. One of these is the use of a radio sensitive pill which can be swallowed and then monitored by radio telemetry. A disadvantage of this is the unpleasant task is its retrieval once it has travelled the full course of the digestive tract. An alternate to this is the use of disposable pills (remains in an individual's system for 18 to 30 hours, before passing out of the body safely. This need not be retrieved as it wirelessly transmits core body temperature as it travels through the human digestive tract. A sensor within the pill sends a signal that passes harmlessly through the body to the data recorder outside of the body) and a data logger worn by the subject for recording the transmitted signals (Edwards *et al.*, 2002). Another option is to measure the temperature of mid-stream urine which gives reasonable indication of internal body temperature. This method can be unreliable if urine volume is small (Reilly and Waterhouse, 2005).

There was no change in nutrient intake between the placebo and L-Histidine groups. It has been shown that dietary L-Histidine of 50 g/kg body weight for 8 days significantly reduced dietary intake in rats (Kasaoka *et al.*, 2004) (compared to dose used in the current study - 0.024 g/kg body weight) and was attributed to activated histamine neurones in the brain. It is possible that an increase in L-Histidine over and above the dose in the current study might have shown reduction in dietary intake, but it is uncertain whether this dosage might show the same deleterious effects in humans as in rats (Aoyama *et al.*, 1990).

Effect of L-Histidine on fasting blood glucose, insulin, leptin, NEFA and lipids concentrations

There were no differences in fasting glucose, insulin, leptin and NEFA concentrations in circulation between the placebo and L-Histidine groups over time. Others have previously shown hypoglycaemic and hypolipidaemic effects of histidine and/or histamine. One way in which histamine exerts a hypoglycaemic action may be via stimulation of zinc absorption, in turn regulating insulin receptor initiated signal transduction mechanisms and insulin receptor synthesis (as discussed in Chapter 3.3) an effect well established in animal models (Arquilla *et al.*, 1978; Song *et al.*, 1998). Hypoglycaemic and lipolytic effects of

histidine or histamine have been demonstrated to be a direct action of histamine via H₁ and H₂ receptors in animal studies.

Histamine action via its receptors in the PVN of the hypothalamus stimulates secretion of corticotrophin-releasing hormone (CRH). CRH raises concentrations of cAMP causing a release in adrenocorticotrophic hormone (ACTH). ACTH then acts on the adrenal glands, stimulating increase of glucocorticoids to increase lipolysis (Bugajski and Janusz, 1981; Grund *et al.*, 1975; Grund *et al.*, 1976; Yoshimatsu *et al.*, 2002b). Histaminergic neurones, via activation of SNS suppress and regulate *ob* gene expression and hence leptin production (Yoshimatsu *et al.*, 2001; Zhang *et al.*, 1994). As circulating leptin concentrations normally reflect the quantity of adipocytes in the body (Frayn, 2003b) there may also be changes in overall body fat composition. The above described mechanisms have implications of increasing insulin sensitivity and reduction in blood glucose concentrations due to increase in lipolysis via decrease in body fat and circulating leptin concentrations. To investigate these phenomena further, measurements of cortisol (key glucocorticoid) or ACTH concentrations may be necessary in further studies of this nature. These measurements were not carried out during the course of this study due to technical and financial resource limitations.

There were no changes in total cholesterol and HDL concentrations following either placebo or L-Histidine ingestion during the 8 week period. There was however, a significant increase in mean overall LDL concentrations after 8 weeks when compared with mean overall pre-treatment values, irrespective of the kind of ingestion (pre-treatment: 3.2 ± 0.4 mmol/l as compared to overall mean 8 weeks: 4.1 ± 0.4 mmol; $p = 0.039$; Table 3.18). L-Histidine is involved in both copper and zinc absorption and hence levels in blood (Aiken *et al.*, 1992). *In-vitro* studies have also shown that histidine removes both copper and zinc from plasma albumin complexes with these metals (Henkin, 1974). Animal studies have previously shown the hyperlipidaemic effects of excessive dietary L-Histidine along with significant reductions in plasma copper and zinc concentrations (Harvey *et al.*, 1981), induction of hepatomegaly along with an increase in plasma cholesterol concentrations (Soloman and Geison., 1978), development of fatty liver due to increase in triacylglycerol (TAG) (Aoyama *et al.*, 1995); Kasaoka *et al.*, 2004). Measurements of changes in plasma copper or zinc concentrations could show distinct

mechanisms of hyperlipidaemic action of L-Histidine and needs to be considered as an outcome measure for future studies of this nature.

Effect of 8 week ingestion of L-Histidine on blood pressure

Systolic blood pressure significantly decreased from pre-treatment measurement after 8 weeks of both placebo and L-Histidine ingestion. Diastolic blood pressure remained unchanged over 8 weeks and there was no effect of either placebo or L-Histidine ingestion. As weight loss occurred during both supplementations, systolic blood pressure decreased in both groups and the effect of L-Histidine ingestion, if at all, was not apparent. It is debatable whether this decrease in blood pressure was due to the participants being comfortable with the test procedure over time (Gordon *et al.*, 1976). To establish whether this was indeed the case, the pre-treatment reading was omitted (presuming that participants were more anxious in the first test session) and only the second and third measurements during both treatments were analysed to observe any difference between the measures and there was no statistical changes between these readings.

3.4.7 Conclusion

Revisiting the study hypothesis, the effects of oral ingestion of L-Histidine for a period of 8 weeks at 25 mg/kg on metabolic indices of energy balance (RMR, MST, and CT) and blood glucose concentrations were inconclusive. The significant reduction in body weight and diastolic blood pressure by the end of the study period need to be further investigated as these results could be as a result of lifestyle changes brought about due to participation in a study and constant monitoring of body weight.

3.5 Summary and further direction

3.5.1 Summary of studies investigating oral intake of L-Histidine

The studies carried out and presented in this thesis were designed to investigate possible histaminergic effects on metabolic parameters which influence energy balance, glucose and lipid regulation. Favourable changes among these parameters perhaps can aid moderation of obesity and its associated disorders. The results showed promising trends towards decreasing weight and fasting blood glucose concentrations. The regulatory effects, which are well documented and defined in animal models such as significant increase in EE and a decrease in food intake, was not clearly shown in these studies.

The objective of the pilot study was to develop protocols of RMR measurement along with MST and CT measurements for all following studies. Temperature measurements, especially core temperature measurement was envisaged to be awkward and potentially invasive, but was found to be simple and uncomplicated. The simultaneous use of both the Deltatrac II and the Squirrel data logger for measurement of RMR and temperature measurements (CT and MST) were found to be easily achieved. A dose of 25 mg L-Histidine /kg body weight was well tolerated in all subjects. The effect of this dose on RMR, MST and CT was inconclusive over an acute period of 3.25 hours which lead to the design of the next study to measure the effect of the same dose over a longer period (10 days) of supplementation.

The next study conducted, investigated effects of 10-day chronic ingestion of L-Histidine on RMR, body weight, MST, CT, FBG, body fat composition and blood lipids. L-Histidine ingestion (25 mg/kg body weight/day) for an uninterrupted period of 10 days by healthy men brought about a significant decrease in body weight. Decrease in body weight might have occurred due to activation of the SNS by histamine receptors. However, this was also accompanied by a decrease in FBG concentrations which is not consistent with increased adrenergic activity. This study did not demonstrate any variation in MST or CT. This suggests that oral L-Histidine ingestion at the doses and durations used in the present studies do not induce measureable changes in body temperature. It would be of value therefore in future investigations to have markers of metabolism to further understand the

mechanism of action. For example, with respect to the essential amino acid tryptophan, relative rates of conversion to niacin (a pyridoxal-phosphate dependant pathway) can be elucidated by measuring the intermediary metabolite kynurenic acid thereby identifying with relative precision where the metabolic bottleneck for this pathway occurs (Ruddick *et al.*, 2006). The example of tryptophan metabolism is apro-po in this connection, as a separate transformation of this amino acid to serotonin occurs only after tryptophan has crossed the blood-brain barrier (Turner *et al.*, 2006). In order to quantify serotonergic activity within the CNS, it is necessary to obtain samples of cerebro-spinal fluid (the metabolite of serotonin being 5-hydroxy-indolacetate). Such sampling requires a spinal tap to be carried out and hence data is rarely forthcoming. With respect to the present study, similar considerations are in evidence as metabolites of histidine like tryptophan may reflect a number of different metabolic processes occurring at multiple sites in the body. Measurement of central histaminergic activity via biomarkers would require CNS specific markers. It remains therefore a research challenge to access a suitable subject base (undergoing spinal tap procedures for other reasons) and/or animal studies where real-time brain slice data could be obtained (Sakata *et al.*, 1997; Yoshimatsu *et al.*, 1999). Subsequently, the next study was designed to measure further effect of L-Histidine supplementation over a longer period (8 weeks) with additional analyses of key metabolic hormones – insulin and leptin along with NEFA to understand possible mechanisms of action.

The longer-term (8 weeks) study of L-Histidine ingestion was designed and conducted in overweight and obese men. By the end of this study, a significant decrease in weight was observed after 8 weeks irrespective of type of test supplement ingested. Systolic BP was significantly decreased after 8 weeks of both placebo and L-Histidine ingestion. On the whole, the effects of L-Histidine ingestion of 25 mg/kg body weight/day among overweight and obese men for 8 weeks were inconclusive among measured metabolic parameters. One consideration for the interesting decrease in body weight observed among all participants after 8 weeks could be the time of the final laboratory test. This laboratory session was carried out during the third and fourth week of January, following the festive period, possibly when people seem to make changes in their lifestyle pattern (healthier eating and increased physical activity). This could have led to decrease in body weight among both groups of participants. Future studies of this nature need to be aware of the time of year during which the study is conducted.

The sample size calculation for both the above studies was based on the study conducted in humans investigating the blood glucose lowering effect of the dipeptide His-Pro (L-Histidine and Proline; Song *et al.*, 1998), which at the time of commencement of these studies was the only human study conducted to test the effects of Histidine on one of the proposed study measures. Results from Chapter 3.3, have shown significant differences in body weight and FBG and these changes were taken to calculate sample sizes to determine the number of subjects needed to show significant differences. The calculations are as below:

Calculations based on the difference in body weight-

$$n > 2 \left\{ \frac{(z2\alpha + z2\beta) \sigma}{\delta_0} \right\}^2$$

Using the equation with $z2\alpha = 1.96$, $z2\beta = 0.842$, $\sigma = 3.6$, $\delta_0 = 1$, gives a result of 203.5. Future investigations on effects of oral ingestion of L-Histidine on changes in body weight may possibly need to recruit atleast 204 participants.

Similarly, calculations based on the difference in fasting blood glucose concentrations-

$$n > 2 \left\{ \frac{(z2\alpha + z2\beta) \sigma}{\delta_0} \right\}^2$$

Using the equation with $z2\alpha = 1.96$, $z2\beta = 0.842$, $\sigma = 0.5$, $\delta_0 = 0.6$, gives a result of 10.90. Therefore, for further trials investigating the effects of oral ingestion of L-Histidine on changes in FBG may need to recruit atleast 11 participants.

The sample size calculation done for L-Histidine studies used one parameter (blood glucose concentration) due to a lack of data from human trials investigating the metabolic effects of L-Histidine supplementation. Significant differences were observed in fasting blood glucose concentrations accordingly (chapter 3.3). If the calculation of sample size had been based on another parameter it is possible that a larger sample size may have been

required. It is possible therefore that due to this, different results may have been realised. Also, small sample size could be just one of the causes for inconclusive results from the current studies. Utmost care was taken to devise sample sizes in light of available relevant literature, but future studies need to recruit larger samples.

Only a handful of studies have been conducted using oral supplementation of L-Histidine intake in humans and as a result the dose of active ingredient was based on the recommended daily intake of this essential amino acid. Increasing the dose from 25 mg/kg/day might have shown more pronounced results but may perhaps result in side effects such as loss of taste and smell. The maximum supplementation period was 8 weeks and lengthening this phase (to > 3 – 6 months) may also show positive changes in metabolic factors.

3.5.2 Further directions

Inclusion criteria for future trials could include biochemical screening to identify clinically leptin resistant (> 31.3 µg/l) participants and to study the effects of L-Histidine ingestion in this group. As overweight and obesity increases, many people who are at a risk of developing chronic disorders (CVD, DM, hypertension etc) are thought to be leptin-resistant and numerous animal studies carried on leptin-resistant models have shown positive effects of histidine and histamine ingestion on energy balance (Morimoto *et al.*, 1999; Yoshimatsu *et al.*, 1999). Screening the participants before commencement among the present studies' participants was could not be performed due to financial limitation.

Previous animal and epidemiological human studies have shown that the anorexic effects of histidine are greater in females than in males (Nakajima *et al.*, 2001; Okubo and Sasaki, 2005) and that females are more sensitive to dietary histidine-induced anorexia than males as it is believed that oestrogen upregulates mRNA expression and/or activity of HDC (Martin and Bulfield, 1984). HDC is the primary enzyme regulating histamine biosynthesis from L-Histidine. Hence, increased activation of histamine neurons suppresses food intake more in females (Kasaoka *et al.*, 2005). Inclusion of female subjects for the above studies might have shown differences in the measured parameters. On the other hand, the L-Histidine studies conducted herein are the first to record MST and CT following L-Histidine ingestion in order to investigate its potential thermogenic property. The variation in CT among women due to changes during the menstrual cycle would have led to

erroneous recordings; hence women were not included in the L-Histidine studies. The anorexic effects of L-Histidine ingestion among women need to be investigated further and future studies not measuring MST and CT following L-Histidine ingestion could include female participants.

Any future studies should also consider the 24-hour rhythm of hypothalamic histamine concentrations. Currently, literature regarding this diurnal effect has been observed (Orr and Quay, 1975) in animals but needs to be further investigated in humans. This was not considered in the current studies as access to overnight testing facilities (to accommodate the participants for 24 hours) was unavailable to the researcher.

Regulation of dietary histidine or histamine-inducing foods along with monitoring of physical activity over the course of the study period will eliminate any confounding factors for any potential metabolic changes observed. Further studies could utilise the USDA nutrient database or INTERMAP database which allows quantification of amino acids in a limited number of foods to prescribe quantity of dietary histidine ingestion. Prescription of dietary intake was not performed as the objective of the studies was to test L-Histidine ingestion among free-living subjects with their normal dietary intakes, but future studies might want to consider controlling dietary histidine intake.

The use of Northern blot analysis to analyse mRNA for upregulation of UCPs was beyond the scope of this study, but if performed in future trials might be informative about promising data from WAT and facilitate correlation of MST and CT to its up-regulation.

In conclusion, oral ingestion of L-Histidine requires further study before it can be concluded if it has the potential to affect components influencing energy balance. Once this is determined it will be necessary to carry out further experiments in humans to understand the exact mode of action in terms of the underlying mechanisms and the implications of long – term L-Histidine supplementation.

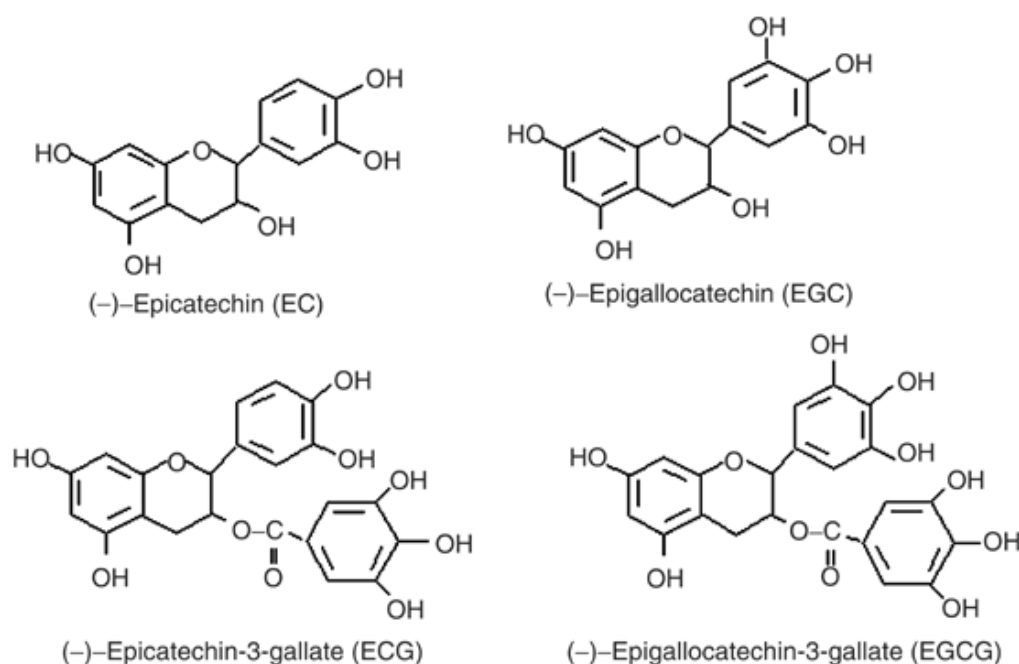
4 Metabolic implications of epigallocatechingallate ingestion

Keeping with the overall theme of investigation of novel dietary intervention which may possibly influence energy balance and blood glucose regulation, the following two studies investigating the supplementation of EGCG among healthy men were carried out. The pharmacokinetics of EGCG *in-vivo* are well established as shown in the literature however, intervention studies are few among humans. The proposed studies are the first to examine the effect of EGCG on thermogenesis following varying periods of supplementation. Below is a review of literature of EGCG dietary quantification, its bioavailability, mechanism of action (in particular effects on energy balance, blood glucose and blood lipid concentrations) and finally data on possible toxicity are explored.

4.1 Review of Literature

Green tea produced from *Camellia sinensis* is a major source of dietary polyphenols, particularly flavonoids. During the process of manufacturing, fresh tea leaves are dried and steamed which inactivates the enzyme polyphenol oxidase, preventing oxidation from occurring and this non-fermentation process preserves the polyphenols in green tea (Wan *et al.*, 2008). The main flavonoids present include catechins and the four major catechins are epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC) (Cabrera *et al.*, 2006). Figure 4.1 illustrates the structure of green tea catechins.

More than 50% of the total catechins present in green tea is composed of EGCG and a vast body of scientific research suggests that EGCG and other catechins are responsible for the potential health benefits (discussed in detail in the following paragraphs) attributed to green tea consumption (Nagle *et al.*, 2006). Green tea polyphenols have also been proven to be potent antioxidants (Henning *et al.*, 2004) which in turn benefit health and well-being by preventing widespread cellular destruction and maintaining a healthy immune system. There are also other reported benefits such as anti-mutagenic and anti-cancerous effects of green tea consumption (Cabrera *et al.*, 2006). The anti-oxidant, anti-mutagenic and anti-cancerous effects of green tea will not be discussed here as is beyond the scope of the study.

Figure 4.1 - Structure of green tea catechins

Reference: (Bigelow and Cardelli, 2006)

4.1.1 Quantification of catechins

Tea consists of over 2000 different substances and flavonoids are the most abundant. Flavonoids are a vast group of low molecular weight, polyphenolic phytochemicals that are found in the leaves, flowers and outer parts of plants. Over 4000 flavonoids have been described to date and are sub-classified into flavanols, flavones, catechins, flavanones, anthocyanidins and isoflavanoids according to the level of oxidation of the C-ring (Wheeler and Wheeler, 2004). Catechins are abundantly found in different foods and beverages. High-performance liquid chromatography (HPLC) with ultra violet (UV) and fluorescence detection have been used to quantify the concentrations of catechins. A few examples of foods with high catechin content are Cox Apple (109 mg/kg), raw broad beans (493.7 mg/kg), dark chocolate (Albert Heijn (459.8 mg/kg)) and Bordeaux (Rineau, 1997 (95.5 mg/l). The various sources of catechins from fruits, vegetables, foods and beverages are further shown in Appendix 17.

Table 4.1 shows the difference in catechin content between caffeinated and decaffeinated green tea leaves. Green tea differs from black and oolong tea in the processing and manufacturing stage, which results in different properties and preservation of polyphenols. Catechins are lost during decaffeination due to exposure of tea leaves to ethyl acetate

solution which is used as a solvent. Apart from binding to the caffeine molecules, ethyl acetate also binds with the catechin molecules and following the rinsing and cleansing process, the catechins are washed out (Chang *et al.*, 2000). A comparison of catechin content between black, oolong and green tea is shown in Table 4.2.

Table 4.1 -Variation in flavanoid content of dried green tea following decaffeination

Description	Flavonoid	Amount (mg/100g)
Tea leaves, green, dry	EC	791.46
	ECG	1701.59
	EGC	1695.02
	EGCG	8294.91
Tea leaves, green, dry, decaffeinated	EC	423.02
	ECG	522.01
	EGC	1153.49
	EGCG	1843.64

EC: (-)-epicatechin; ECG: (-)-epicatechin 3-gallate; EGC: (-)-epigallocatechin; EGCG: (-)epigallocatechin 3-gallate; Reference: (USDA, 2003).

Table 4.2 - Comparison of flavonoid content among brewed green, black and oolong teas

Description	Flavonoid	Amount (mg/100g)
Tea, green, brewed	EC	8.47
	ECG	20.95
	EGC	17.08
	EGCG	82.89
Tea, green, brewed, decaffeinated	EC	6.16
	ECG	7.57
	EGC	16.02
	EGCG	26.05
Tea, black, brewed, prepared with tap water	EC	2.33
	ECG	7.24
	EGC	10.43
	EGCG	11.48
Tea, black, brewed, prepared with tap water, decaffeinated	EC	0.49
	ECG	0.64
	EGC	0.55
	EGCG	1.01
Tea, oolong, brewed	EC	2.59
	ECG	6.73
	EGC	6.00
	EGCG	36.01

EC: (-)-epicatechin; ECG: (-)-epicatechin 3-gallate; EGC: (-)-epigallocatechin; EGCG: (-)epigallocatechin 3-gallate; Reference: (USDA, 2003).

4.1.2 Bioavailability and absorption

There are a limited number of studies published which measure the absorption, distribution and elimination of green tea and its catechins. Chen and colleagues (1997), found that injecting male Sprague - Dawley rats with decaffeinated green tea (DGT, containing 73, 68 and 27 mg/g of EGCG, EGC and EC respectively) intravenously, increased the distribution rate constants between the extracellular fluid (ECF) and the intracellular fluid (ICF) for EGCG and were found to be three fold larger than the distribution rate constant from the ICF to the ECF, indicating that EGCG tends to distribute in the ICF. EGCG was retained in the body for a longer period of time (212 minutes) when compared with EGC and EC (45 and 41 minutes) as it had a longer half-life and smaller clearance rate (Chen *et al.*, 1997). The group also observed that EGCG had a lower bioavailability (0.1%) when compared to EGC (13.7%) and EC (31.2%) when given as part of DGT or as pure EGCG indicated by higher C_{max} (maximum plasma concentration) values for EGC and EC than that for EGCG. EGC and EC, but not EGCG was recovered from animal urine samples. The area under the curve (AUC) was found to be higher for EGCG in the intestinal fluid samples after intravenous injection, suggesting that EGCG is excreted mainly through the bile. EGC and EC are likely to be excreted through both urine and bile as the AUC values of EGC and EC were similar in both kidneys and intestine in rats (Chen *et al.*, 1999).

A similar study investigating the bioavailability of green tea (GT, containing 213.6 mg/day EGCG, 76.5 mg/day EC, 269.6 mg/day EGC), black tea (BT, containing 230.8 mg/day EGCG, 39.8 mg/day EC, 103.4 mg/day EGC) and a green tea extract supplement (GTS, containing 193.3 mg/day EGCG, 38.3 mg/day EC, 24.9 mg/day EGC; provided via gelatine capsules) in thirty healthy humans showed that plasma bioavailability of EGCG (C_{max} = 80 nmol/l) was lower than that of EGC (C_{max} = 740 nmol/l) and EC (C_{max} = 330 nmol/l). The study investigators noticed that the absorption of polyphenols from the GTS was delayed, probably due to the gelatine capsule used to deliver the supplement, but was still higher than GT or BT administration. They hypothesised that GTS increases the bioavailability of tea flavanols in the absorption phase. It was concluded that polyphenols supplemented in the form of GTS showed increased bioavailability when compared with GT or BT consumption (Henning *et al.*, 2004). An investigation of dose-dependant incorporation of tea catechins (EGCG and EGC) in human plasma found that oral ingestion of green tea extract (capsule form) dose-dependently increased plasma concentrations of

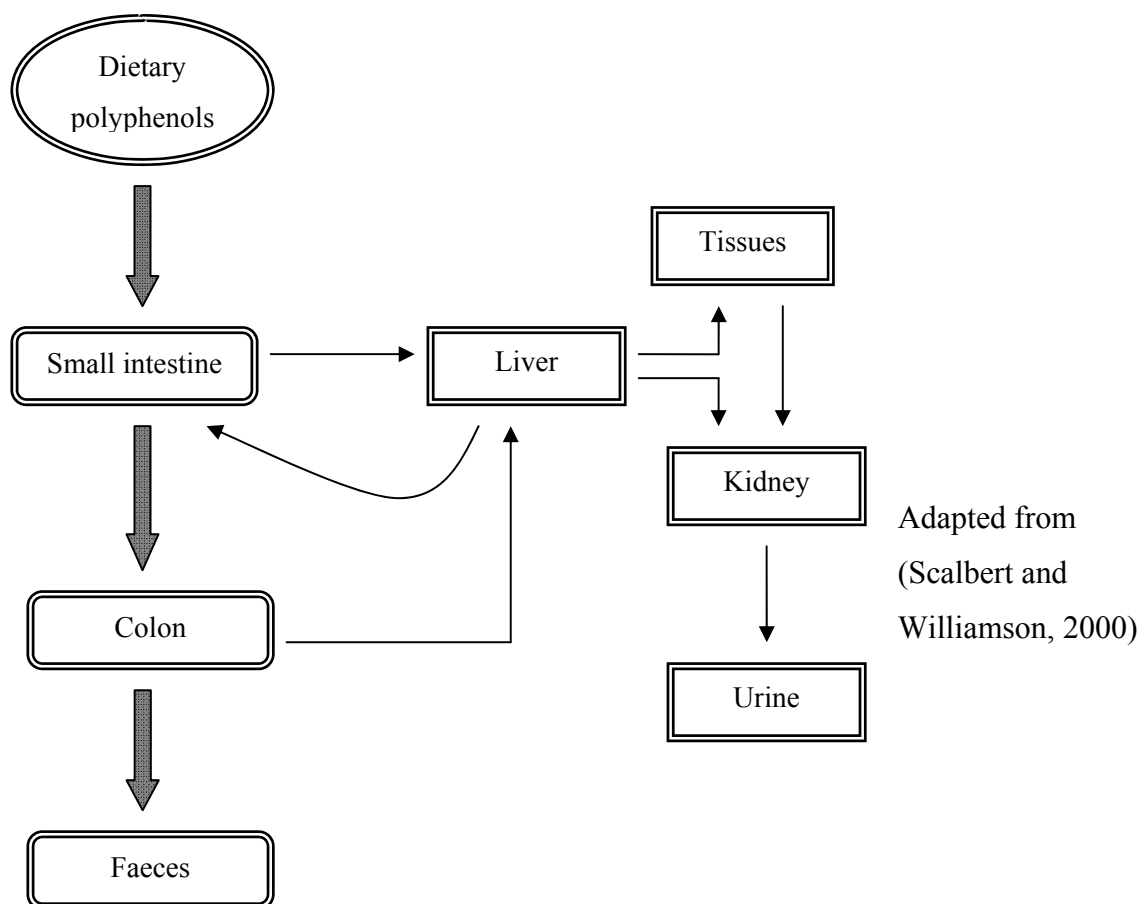
EGCG (Nakagawa *et al.*, 1997). The study employed 3 doses of 225, 375 and 525 mg EGCG and found that EGCG concentrations detected in plasma corresponded to 0.2 - 2% of the ingested amount.

Lee and colleagues (2002) studied the pharmacokinetics of tea catechins after ingestion of green tea (GT) and EGCG in humans. A single oral dose (20 mg/kg) of GT solids (containing EGCG, EGC and EC, 13.9, 11.0 and 3.2% of respectively) was given dissolved in 200 ml warm water. The plasma and urine concentrations of total EGCG, EGC and EC were quantified by HPLC. Peak plasma concentrations of these catechins were reached 1-2 hours post ingestion and reached undetectable levels in 24 hours. T_{max} (time when maximum plasma concentration is reached) for these catechins was in the range of 1.3 – 1.6 hours. The half-life of EGCG was found to be 3.4 ± 0.3 hours. Urinary excretion of catechins were high in the first 3-6 hours and it was found that >90% of the urinary catechins were excreted in the first 8 hours after ingestion. In all their experiments EGCG was present in trace or undetectable amounts in the urine. They conclude that most of the ingested EGCG does not get into the bloodstream and the absorbed EGCG is preferentially excreted through the bile to the colon. Interestingly, a similar study investigating the levels of tea catechins in human blood and urine samples after ingestion of different amounts of green tea observed C_{max} values at 1.4 - 2.4 hours post ingestion of 1.5 g/kg of decaffeinated green tea solids (DGT). When the dosage was increased to 3 g/kg of DGT, C_{max} values increased 2.7 - 3.4 folds and increasing the dose to 4.5 g/kg DGT did not increase the C_{max} value significantly. This suggests a saturation phenomenon. The half life of EGCG in this study was found to be 5.0 - 5.5 hours and this was higher than the half-life of EGC and EC (2.5-3.4 hours) (Yang *et al.*, 1998). The doses used in the above studies vary considerably (approximately 0.014g/kg of green tea solids for an average weight of subject (70 kg) used in the study by Lee and colleagues (2000) as compared with a minimum dose of 105g/kg for the same average weight in the study conducted by Yang and colleagues (1998). It can be concluded that the half-life of EGCG to vary with ingestion dose and in the range of 3.4 – 5.5 hours.

The potential *in vivo* bioactivity of tea flavanoids is dependant on the absorption, metabolism, distribution and excretion after ingestion. The GI tract plays an important role in the metabolism and conjugation of polyphenols before they reach the liver. In the small intestine, in particular in the jejunum and ileum, extensive glucuronidation by the action of

UGT (UDP-glucuronyltransferase) enzymes and O-methylation by the action of catechol O-methyltransferase (COMT) occurs. Unabsorbed, and taken up flavanols which are metabolised in the small intestine and liver are then transported to the large intestine where they are further metabolised into smaller phenolic acids by the gut microflora. These phenolic acids can sometimes be further conjugated and metabolised in the liver (Figure 4.2). However, the extent of their absorption in the colon and the extent of absorption of the glucuronides and O-methylated compounds by cells and tissues are unknown. The remaining compounds pass out into the faeces (Spencer, 2003).

Figure 4.2 - Possible routes of absorption and elimination of dietary polyphenols in humans



Green tea and EGCG supplementation has been purported to augment key metabolic pathways involved in weight management as well as improving associated co-morbidities such as lipid imbalances and cardiovascular risk factors. Below is a detailed discussion of effects of green tea and EGCG supplementation in animal and human studies.

4.1.3 Discovery of an EGCG receptor

The anti-carcinogenesis effect of green tea consumption, and in particular of EGCG, is one of the earliest known metabolic implications and is well documented. A study conducted to identify the means through which EGCG inhibits cell growth in cancer cells led to the discovery of a receptor for green tea polyphenol-EGCG. This was a single target which allowed EGCG to bind to the cell surface and was found via subtraction cloning strategy involving DNA analysis (Tachibana *et al.*, 2004). The cell surface candidate was found to be the 67-kDa laminin receptor (67LR). This putative receptor, 67LR, is expressed on a variety of tumour cells and its expression was strongly correlated with the risk of tumour invasion and metastasis. Cells transfected with 67LR were treated with two concentrations of EGCG (0.1 and 1.0 μM ; concentrations similar to the amount of EGCG found in human plasma after drinking more than two or three cups of tea at one time) to investigate whether the 67LR can bring about sensitivity to EGCG at physiologically relevant concentrations (Tachibana *et al.*, 2004). It was found that the growth of these transfected cells was inhibited at both of these concentrations. This growth-suppressive effect was completely eliminated upon treatment with anti-67LR before the addition of EGCG. Also, the ability of 67LR to mediate a response to other tea constituents (caffeine and other tea polyphenols) was tested and it was found that none of these other compounds affected the growth of 67LR-expressing cells, nor could they bind to the cell surface. Together, these observations demonstrate that the cell surface 67LR is the target for EGCG. The investigation on the precise structure-activity relationship of green tea catechins with their cell-surface binding site has suggested that the galloyl moiety and the B-ring hydroxylation pattern contribute to the exertion of 67LR-mediated biological activities of tea catechins (Fujimura *et al.*, 2008).

The 67 Kda protein is not just found on tumour cells: muscle cells, macrophages, neutrophils, epithelial cells, interstitial cells, endothelial cells and hepatocytes all have a protein in this size range which binds laminin (the extracellular glycoprotein known to mediate cell attachment, movement, differentiation and growth) (Mecham, 1991). This widespread localisation of the receptor in the cells with various isoforms may explain numerous biological effects of EGCG (Figure 4.3).

4.1.4 Animal studies investigating effect of tea catechins on parameters of energy balance, glucose and lipid regulation

Effects on parameters of energy balance: Rats fed on a high fat diet for a period of 14 days showed that supplementation with green tea extract (20 g/kg diet) not only increased energy expenditure but also prevented any increase in body weight as a consequence of the high fat diet (Choo, 2003). The administration of propranolol (a β -adrenoceptor antagonist) inhibited this weight suppressive effect of green tea, indicating that the increased thermogenic effect of green tea may exert its effects through β -adrenoceptor activation or consequences of such activation.

In animal models tea catechins have been shown to exert a hypolipidaemic effect by increasing faecal excretion of total lipids and cholesterol (Muramatsu *et al.*, 1986; Raederstorff *et al.*, 2003). Supplementation of 1 and 2% tea catechins to lard-cholesterol fed rats, decreased plasma total cholesterol and cholesterol ester concentrations. The liver weight, liver total lipids and cholesterol concentrations in rats fed the lard-cholesterol diet increased more than in the control rats, but the addition of tea catechins to the lard-cholesterol diet decreased those parameters, indicating that tea catechins exert a lipid lowering effect (Muramatsu *et al.*, 1986).

Evidence of a mixture of EGCG and ECG as a potent inhibitor of fat utilisation has been described in animal models when compared with other catechins. These catechins were more effective in reducing cholesterol absorption and also tended to decrease lymphatic absorption of triacylglycerols. When purified EC, EGC, ECG and EGCG (0.8%, 4.9%, 20.6% and 44.1% respectively in a dose of 10g/kg of diet) were used, EGCG was more effective in precipitating cholesterol solubilised in mixed bile salt micelles as observed during *in vitro* study on micellar solubility of cholesterol (Ikeda *et al.*, 2005). These results show that tea catechins, in particular EGCG, effectively reduce cholesterol absorption from the intestine by reducing solubility of cholesterol in mixed micelles. The observation accounts for the hypocholesterolemic effect of tea catechins (Ikeda *et al.*, 1992). Yet another confirmation for the lipid lowering effect of green tea has been shown by a study supplementing tea catechins (at 1% of diet level) for 23 days (Ikeda *et al.*, 2005). It was found that visceral fat deposition and the concentration of hepatic triacylglycerol were significantly lower in the tea catechin group than in the control group. The activities of

fatty acid synthase and the malic enzyme in the liver were significantly lower in the catechin group than in the control group. In contrast, the activities of carnitine palmitoyltransferase (CPT) and acyl-CoA oxidase in the liver homogenate were not significantly different among groups. The authors conclude that the reduction in activities of enzymes related to hepatic fatty acid synthesis by the feeding of tea catechins causes reductions of hepatic triacylglycerol and possibly of visceral fat deposition.

Hypoglycaemic effect: *In vitro* study investigating the effect of EGCG on high glucose-induced apoptosis showed significant suppression of apoptotic features such as DNA fragmentation and damage to mitochondrial function (Oh *et al.*, 2008). It is presumed that this is due to the scavenging of reactive oxygen species (anti-oxidant effect of EGCG). It is supposed that EGCG therefore might play a role in reduction of free radicals and oxidative stress occurring due to hyperglycaemia. A study examining the effects of green tea supplementation in rats on glucose tolerance and insulin sensitivity demonstrated that 12 weeks supplementation of 0.5 g/day green tea lowered both fasting plasma glucose and insulin concentrations, when compared with the control group (Wu *et al.*, 2003). This was a result of increased insulin sensitivity by increasing the glucose uptake and insulin binding of adipocytes. A similar study exploring the hypoglycaemic effects of Teavigo® (purified EGCG) in male diabetic (*db/db*) mice supplemented EGCG for 5 weeks at concentrations of 2.5, 5.0, or 10.0 g/kg of diet (Wolfram *et al.*, 2006). Oral glucose tolerance test was performed post supplementation and it was found that EGCG supplementation improved glucose tolerance and elevated insulin secretion in a dose-dependant manner. The authors suggested that these effects maybe due to a reduction of endogenous glucose production as they observed an upregulation in the glucokinase mRNA expression in the liver and an increase in glucose-induced insulin secretion.

Effects on cardiovascular risk factors: A study conducted to determine whether black and/or green tea polyphenols can lower blood pressure in stroke-prone hypertensive rats (Negishi *et al.*, 2004) found that both green and black tea polyphenols attenuated blood pressure increases through their antioxidant properties. They found that green tea polyphenols significantly increased catalase expression (the enzyme catalase helps maintain defence against reactive oxygen species). The study also found that both black and green tea polyphenols significantly decreased phosphorylated myosin-light-chain (MLC-p) expression. The major regulatory mechanism of smooth muscle contraction is

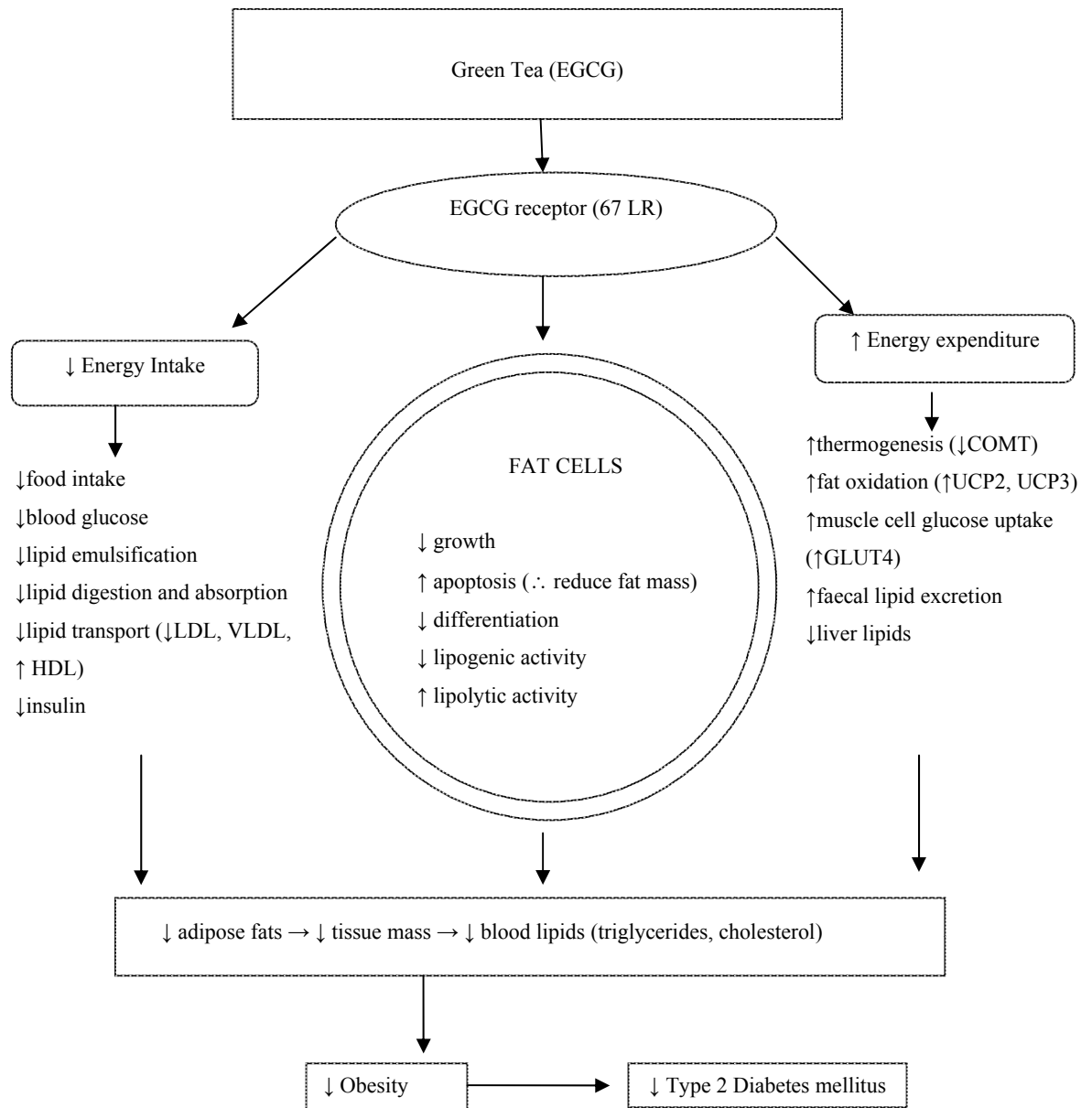
phosphorylation/dephosphorylation of the MLC. A marked reduction in plasma nitric oxide (NO) concentration and urinary NO excretion was observed among rats ingesting either green or black tea polyphenols. This data suggest that alleviation of oxidative stress by tea polyphenols reduces reactive oxygen species (ROS) -mediated NO inactivation and raises the bioavailability of NO in the black and green tea polyphenol groups. An increase in the NO bioavailability enhances NO-mediated vasodilatory tone, which could account for the observed decrease in blood pressure. It is evident from the results obtained in this study that the hypotensive properties of green tea polyphenols could be attributed to their anti-oxidant properties. As the amounts of green tea polyphenols used in this study (3.5 mg/l) correspond to those in approximately in 1 litre of green tea, the authors suppose that regular consumption of green tea may provide some protection against hypertension in humans.

4.1.5 Human studies investigating the effect of tea catechins on parameters of energy balance, glucose and lipid regulation and blood pressure

Effects on parameters of energy balance: Metabolic effects of tea catechins have been ascribed to activation of the SNS. Green tea extract (containing 90 mg EGCG and 50 mg caffeine, 3 times/day) was more effective than an equimolar concentration of caffeine in increasing the 24 hour energy expenditure in humans and increased lipolysis, thus influencing body weight and composition (Dulloo *et al.*, 1999). The authors reasoned that the polyphenols inhibit COMT enzyme which degrades noradrenaline. Thus its inhibition results in an increase in noradrenaline release and/or prolongation of its effect on thermogenesis and fat metabolism thereby resulting in increased energy expenditure. Long term supplementation (3 months) of EGCG (270 mg/day) along with caffeine (150 mg/day) among overweight and moderately obese men and women brought about greater weight maintenance after weight loss, when compared to placebo (Westerterp-Plantenga *et al.*, 2005). This supplementation of EGCG along with caffeine also increased thermogenesis, fat oxidation and reduced fasting blood parameters of glucose, insulin, triacyl glycerol and leptin, which are all important risk factors for metabolic disorders. The ingestion of green tea (375 mg catechins + 75 mg caffeine) for 87 days among overweight females brought about a significant increase in desire to eat when compared with placebo (Diepvens *et al.*, 2005). Leptin concentrations were not measured in this study but the authors conclude that this effect could be due to down-regulation of leptin release through

stimulation of SNS by green tea catechins. Figure 4.3 shows the possible mechanisms of action of EGCG.

Figure 4.3 - Possible anti-obesity mechanism following EGCG ingestion



Adapted from Kao et al (2006).

Hypoglycaemic effects: The hypoglycaemic effects of green tea catechins have been attributed to the promotion of insulin's action, improved glucose tolerance and possibly increased insulin secretion. The effect of green tea ingestion on OGTT was studied among

healthy human volunteers (Tsuneki *et al.*, 2004). It was found that glucose tolerance substantially improved after drinking a suspension of 1.5 g green tea powder (containing 84 mg of EGCG) when compared with hot water administration. The authors attributed this anti-hyperglycaemic effect to the promotion of insulin's action in the peripheral tissues such as skeletal muscles and adipocytes. Ingestion of 300 mg EGCG (Teavigo) in subjects exercising at moderate intensity at 45 minutes thrice a week, for 12 weeks reduced plasma glucose in overweight women with impaired glucose tolerance (Hill *et al.*, 2007). It is thought that this effect is due to the effect of EGCG in increasing the uptake and translocation of the GLUT4 protein in skeletal muscle (Ashida *et al.*, 2004). However, a more recent study has shown that ingestion of 800 mg EGCG for 8 weeks did not influence insulin sensitivity or secretion in men with overweight and obese men (Brown *et al.*, 2009).

Effects on cardiovascular risk factors: The cardiovascular protective action of green tea ingestion has been attributed to its anti-oxidant effects. Other human studies investigating effects of green tea polyphenols on cardiovascular risk factors are mainly epidemiological in nature. Regular green tea consumption of 120 ml/day or more significantly reduced the risk of developing hypertension among a Chinese population (Yang *et al.*, 2004). A similar study observing the effect of tea ingestion in older women (>70 years) found that habitual intake had a favourable effect on blood pressure (Hodgson *et al.*, 2003). Both these studies showed an inverse dose dependent effect of green tea polyphenols on blood pressure and found that the higher the green tea intake (>150 ml/day), the lower was systolic and diastolic blood pressure. Consumption of 583 mg of green tea catechins per day for 12 weeks had a greater reduction in systolic BP when compared to participants ingesting a lower dose (96 mg/day) (Nagao *et al.*, 2007). Ingestion of pure EGCG (800 mg/day) in men has recently shown to decrease diastolic BP after 8 weeks (Brown *et al.*, 2009). Long-term, regular tea (black and green (daily intake exceeding 500 ml) ingestion has shown to attenuate increases in blood pressure in both animal and human studies (Hodgson *et al.*, 2003; Negishi *et al.*, 2004). The cardio-protective effect of habitual green tea consumption is ascribed to the anti-oxidant property of tea catechins by authors of observational studies as above. It is necessary to consider potential confounding factors of lifestyle and dietary factors along with socioeconomic factors associated with habitual tea drinking before accepting these findings.

4.1.6 Toxicity

The safety and tolerability of single doses of EGCG ranging from 50 mg to 1600 mg was studied in healthy humans and it was found that doses of up to 1600 mg purified EGCG were well tolerated (Ullmann *et al.*, 2003). A pharmacokinetic study was performed to determine the systemic availability of green tea catechins after a single oral dose administration of EGCG and Polyphenon E (decaffeinated green tea catechin mixture) on twenty healthy subjects (Chow *et al.*, 2001). The subjects were randomly assigned to one of the dose levels (200, 400, 600, and 800 mg based on EGCG content). Throughout the study, all side effects experienced by study subjects were recorded. Both tea polyphenol formulations administered as a single oral dose over the dose range studied were well tolerated by the study participants. Some subjects experienced mild headache and fatigue, possibly related to the study products. The authors suggest that these adverse events could also have been consequences of the procedures and restrictions that the subjects encountered on the study days (such as refraining from beverages containing caffeine). They concluded that both tea polyphenol formulations administered as a single oral dose over the dose range studied were well tolerated by the study participants.

A study was conducted to investigate the inhibitory effects and toxicity of green tea polyphenols on N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)-induced duodenal carcinogenesis in mice, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced carcinogenesis of the glandular stomach in rats and azoxymethane-induced colon carcinogenesis in rats (all three chemicals are potent carcinogens used to induce colon cancer in rats and mice) (Yamane *et al.*, 1996). It was found that EGCG the doses of (EGCG supplemented in mice correspond to 500-600 mg actual intake) and green tea extract (1g of green tea extract/day) inhibited the chemical carcinogenesis of the gastrointestinal tract in rodents. The study concluded that a dose of 1g of green tea extract per day is an effective dose for clinical use in humans and non-toxic.

The genotoxic potential of concentrated EGCG (Teavigo[®]) was tested in mouse lymphoma cell assays (Isbrucker *et al.*, 2006a). Oral administration of 500, 1000 or 2000 mg EGCG/kg (actual intake of 25, 50 and 100 mg) to mice did not induce micronuclei formation in the bone marrow cells, an indication of reduced genotoxicity. Even

intravenous injection of 10, 25 and 50 mg EGCG/kg/day to rats showed no genotoxic effects.

A single oral dose of 200 mg EGCG/kg in rats induced no toxicity whereas a single oral dose of 2000 mg EGCG/kg (corresponds to 400 mg of actual intake) was lethal. Chronic dietary administration of 500 mg/kg/day to rats for 13 weeks (corresponds to 100 mg/day of actual intake) was however non toxic. Similarly, no adverse effects were noted when 500 mg EGCG preparation/kg/day was administered to pre-fed (dose provided 1 hour after feeding) dogs in divided doses (Isbrucker *et al.*, 2006b). Another safety study investigated the potential effects of EGCG preparation of >91% purity on pregnant rats during organogenesis (Isbrucker *et al.*, 2006c). Subcutaneous infusion (40 mg/kg/day EGCG) (actual doses correspond to 8 mg/day of subcutaneous infusion) did not induce direct embryo-fetal toxicity. Feeding pregnant rats diets supplemented at 1400, 4200 or 14000 ppm (delivering average doses of 100, 300 and 1000 mg EGCG/kg/day respectively, actual doses correspond to 20, 60 and 200 mg/day) during organogenesis was non-toxic and showed no adverse effects on reproduction and fertility.

4.1.7 Rationale for the studies

There is a major interest in the study and development of novel, dietary non-pharmacological compounds which have the potential to produce physiologically significant effects on thermogenesis, EE, satiety, fat oxidation and reduction of hyperglycaemia. Green tea and its principal catechin, EGCG, are believed to play an important role in energy regulation. The data in the literature regarding the effect of EGCG supplementation in humans and its mechanism of increasing EE and lipolysis is inconclusive. Further studies are therefore required to explore and understand the full metabolic potential of EGCG supplementation in humans. The second part of this thesis seeks to investigate some of the proposed effects of EGCG supplementation (increased RMR, thermogenesis, suppression of food intake and appetite, glucose and blood lipid regulation along with changes in key metabolic hormones insulin and leptin).

The following two studies were designed and carried out to achieve the above mentioned purpose.

- A study designed to measure the acute effects of pure EGCG intake after a glucose challenge. This study aimed to test the insulin potentiating and hyperglycaemic actions of EGCG along with its efficacy on metabolic rate over a 2.5 hour period (Chapter 4.2).
- A study to investigate the effect of varying dose of pure EGCG ingestion over a 7 day period. This study aimed to measure chief metabolic parameters involved in energy balance (RMR, body weight, insulin, leptin, NEFA) and cardiovascular well-being (blood pressure, heart rate and plasma lipids) (Chapter 4.3).

4.2 Acute effect of epigallocatechingallate supplementation on metabolic indices of energy balance

A comprehensive review of literature shows that green tea and its principal polyphenol, EGCG, has several metabolic actions (Chapter 4.1). Research concerning the absorption, distribution and elimination of polyphenols has been well documented in animal models (Chen *et al.*, 1997). The pharmacokinetics of tea catechins in humans has only been studied, in detail, in recent times to comprehend the action and interaction of tea catechins, especially of EGCG and its metabolic effects (Lee *et al.*, 2002; Yang *et al.*, 1998). A previous human study has found positive correlations with green tea extract ingestion and both increased EE and enhanced fat oxidation over a 24 hour period (Dulloo *et al.*, 1999). EGCG ingestion has also been shown to enhance insulin activity (Anderson and Polansky, 2002). No previous human studies have attempted to investigate the above mentioned effects of the principle green tea catechin (EGCG) alone without the interaction with other catechins and caffeine on components of EE, especially thermogenesis. The present study was designed to test whether intake of 150 mg pure EGCG during an acute period of ingestion, would reproduce any of the previously documented metabolic effects. This study and the study described next (Chapter 4.3) are novel as they also attempted to study the effects of pure EGCG ingestion on thermogenesis.

4.2.1 Hypothesis

Ingestion of 150 mg/day pure EGCG will increase RMR, MST and CT over an acute period (2.5 hours) and also lead to euglycaemia following an oral glucose tolerance test more rapidly when compared to placebo.

4.2.2 Aim

This study aimed to investigate the acute effects of oral supplementation of EGCG following an oral glucose challenge on RMR, MST, CT, plasma concentrations of insulin, leptin, NEFA and indicators of appetite regulation.

4.2.3 Methods

4.2.3.1 Participant recruitment and profile

The sample size calculation (key parameter considered-weight change; section 2.22.1) showed that a minimum of 8 subjects were needed to show statistical significance at 95% confidence interval in the study parameters. Subjects were recruited by advertising in the London Evening Standard and London Metro newspapers along with posters and flyers displayed and distributed in the University of Westminster, Cavendish campus, London, UK. Participants with chronic disorders such as diabetes, hypertension and heart disease were excluded. Smokers and professional athletes or people with daily training schedules were also excluded from taking part in the study (refer to section 2.2 for detailed inclusion and exclusion criteria). Eleven, healthy men were recruited for the study to offset any attrition during the study (3 failed to complete the study). A study-specific inclusion criterion was the habitual non-consumption of green tea. This was to avoid possible confounding effects of chronic intake of high levels of EGCG. Data was however gathered from eligible participants regarding habitual black tea consumption. It should be noted however that EGCG levels in black tea are over 7 times lower in black tea than in green tea (see Table 4.2) and would be unlikely to impact on any changes elicited via the EGCG doses utilised in the present study. EGCG is only known to occur in measurable quantities in *Camellia Sinensis* plant and therefore no other food-stuffs ingested could influence outcomes of the present study.

Table 4.3 - Mean anthropometric data (\pm SEM) of only participants who completed the study at pre-treatment

Parameter	Measurement	Range
Age (years)	31.5 \pm 3.1	18 - 47
Height (metres)	1.8 \pm 0.02	1.70 - 1.94
Weight (kilograms)	73.7 \pm 2.0	66.2 – 86.5
BMI (kg/m ²)	23.6 \pm 1.0	18 - 25
<i>n</i>	8	

4.2.3.2 Study design

The study protocol was approved by the University of Westminster Ethics Committee (application number 06/07/27, Appendix 8). This study was a randomised, cross over,

single blinded study. None of the participants recruited for the study consumed green tea habitually. The current study was designed to assess the acute effects of EGCG ingestion in men over a short period of time (2.5 hours). The dose of 150mg was chosen (as detailed in section 2.4) to be tested during the acute ingestion phase. Pure EGCG was obtained from DSM nutritional products, Switzerland, marketed under the brand name Teavigo®. This is 94% purified EGCG, caffeine free and is available as a white powder.

Participants arrived at the laboratory fasted (overnight, minimum of 12 hours) for two test sessions, separated with a wash out period of 1 week in between the test sessions. On arrival at the laboratory for the first test session, informed consent (Appendix 9) was sought from the participants and the study protocol explained in detail with the aid of research study information sheet (Appendix 10).

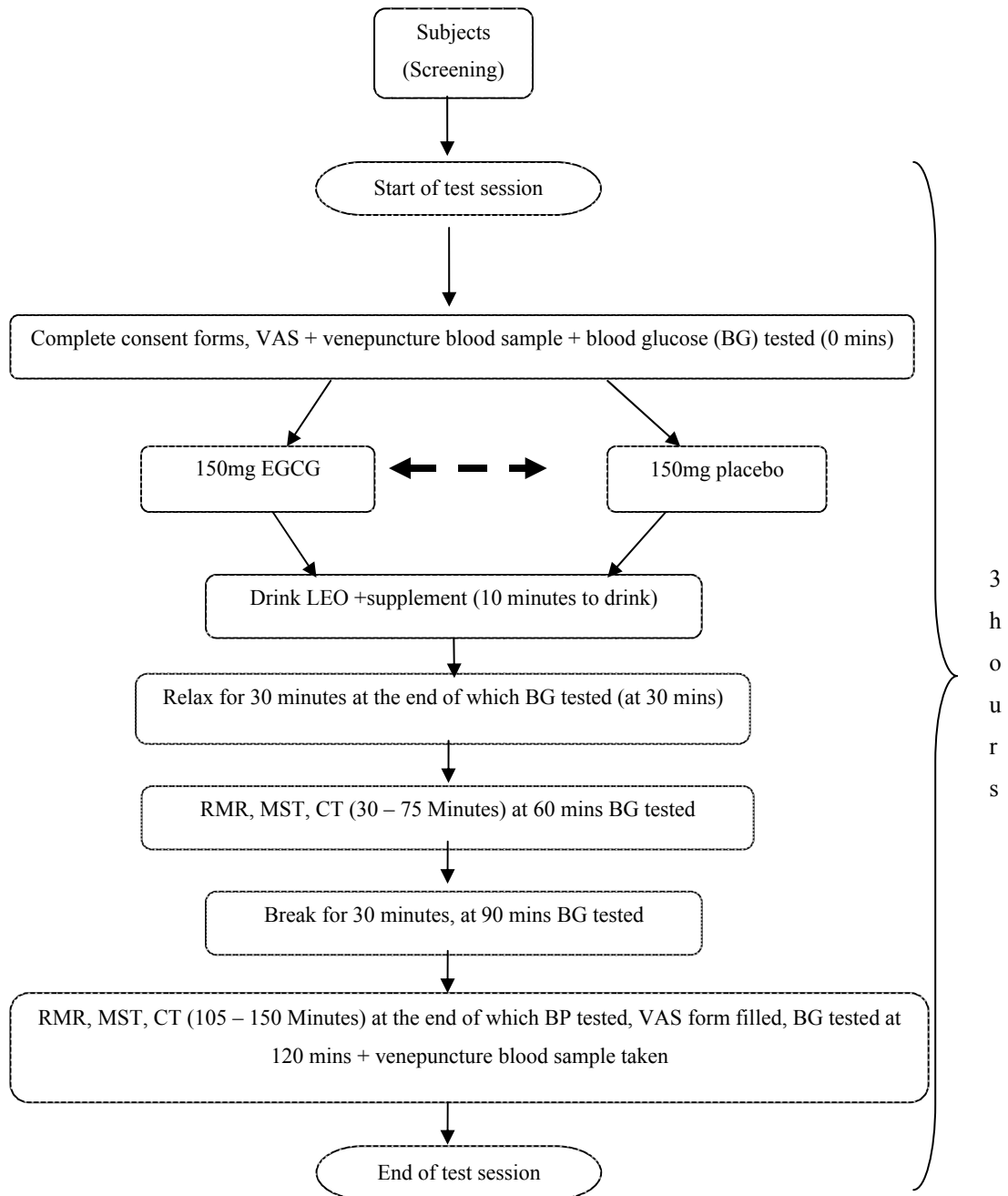
Participants were randomly assigned to either the placebo or the EGCG supplementation during the first test session. During these test sessions either 150mg of placebo (Casilan 90, Complian foods ltd, UK) or 150mg of Teavigo® (DSM nutritional products, Switzerland) powder was mixed with 410ml Lucozade Energy Original (LEO, GlaxoSmithKline Company, UK; providing 75 g of glucose). EGCG was chosen to be given with a fluid as it has been shown that EGCG was better absorbed when given through drinking fluid (Chen *et al.*, 1997). Also, although LEO contains a small amount of caffeine, the same amount was delivered whether EGCG was ingested or not (hence, any differences noted in parameters measured would be superimposed on an equal baseline). One of the study aims was to test the participants' blood glucose response to a glucose load therefore an oral glucose tolerance test (OGTT) was performed. OGTT protocol was taken from those set with the American Diabetes Association (ADA), whose guidelines specify testing the blood glucose response every 30 minutes to an oral load of 75 g glucose (LEO drink) over a span of 2 hours (ADA, 2002).

Participants filled in a visual analogue scale (VAS) in order to assess hunger and satiety before the supplementation and at the end of each test session. Blood glucose concentrations were measured via HemoCue® at the start of the test session and every 30 minutes upto 2 hours after ingestion (section 2.11). Indirect calorimetry (Deltatrac II) was used to measure RMR for two 45 minutes intervals (30-75 minutes and 105-150 minutes post ingestion) with measurement beginning 30 minutes post ingestion. A break of 30

minutes was given in between the two sampling periods (section 2.7). Heart rate was recorded using a Polar heart rate monitor throughout the test session (section 2.18). The participants wore comfortable, light indoor clothes during the test sessions and had soothing music played via headphones to help them relax as well as keep out any unwanted noise stimulus. Blood pressure was monitored towards the end of each test session to detect any changes due to supplementation (section 2.19). Whole blood samples were collected by venepuncture in heparin containing tubes (BD Vacutainer® Plasma Tube, Becton Dickinson UK Limited) once at the beginning and again at the end of the test session to detect any changes in insulin, NEFA and leptin concentrations (Figure 4.4). The filled vacutainers were immediately centrifuged at 3500 rpm for 12 minutes and the plasma supernatant was transferred into 1.5 ml eppendorf tubes and stored at -80°C until further analysis (sections 2.16, 2.15, 2.17 and appendices 19, 20, 21 for further details of assays)

4.2.4 Data analyses

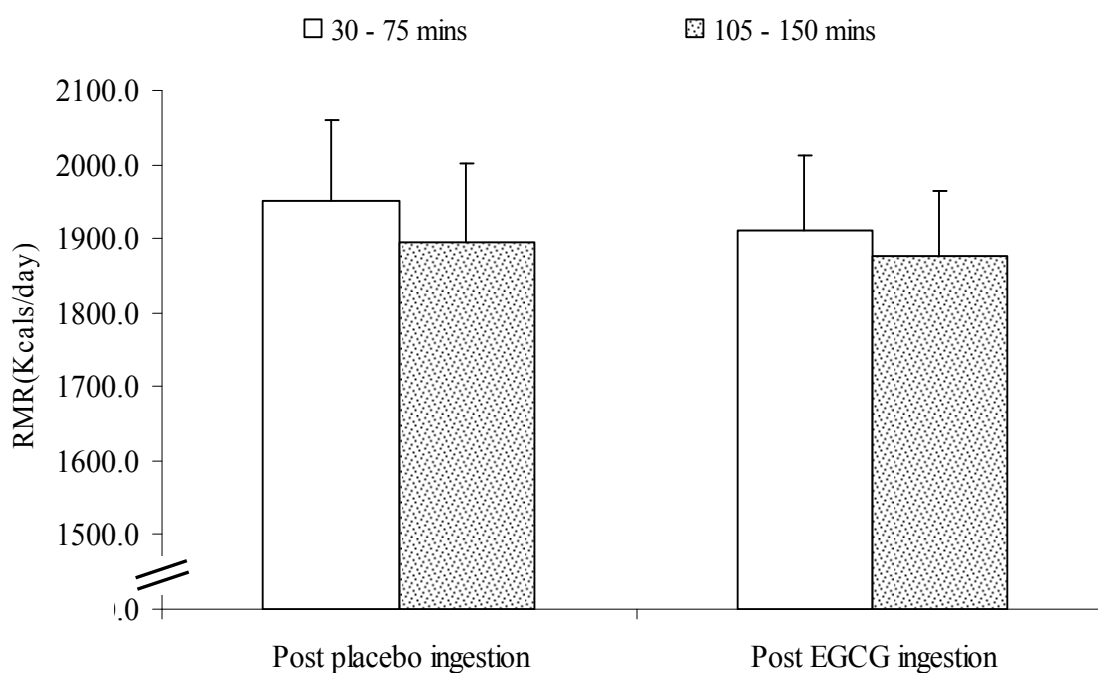
Data are presented as mean \pm standard error of mean (SEM). SPSS 14 was used to analyse data. Paired samples t-tests were performed between groups to detect any statistical significance. The results were concluded as statistically significant when $p \leq 0.05$. Pearson's correlation co-efficient analysis was performed to determine any significant correlation between inter-dependant factors (e.g. correlation between plasma leptin and body fat composition).

Figure 4.4 - Schematic representation of test session

4.2.5 Results

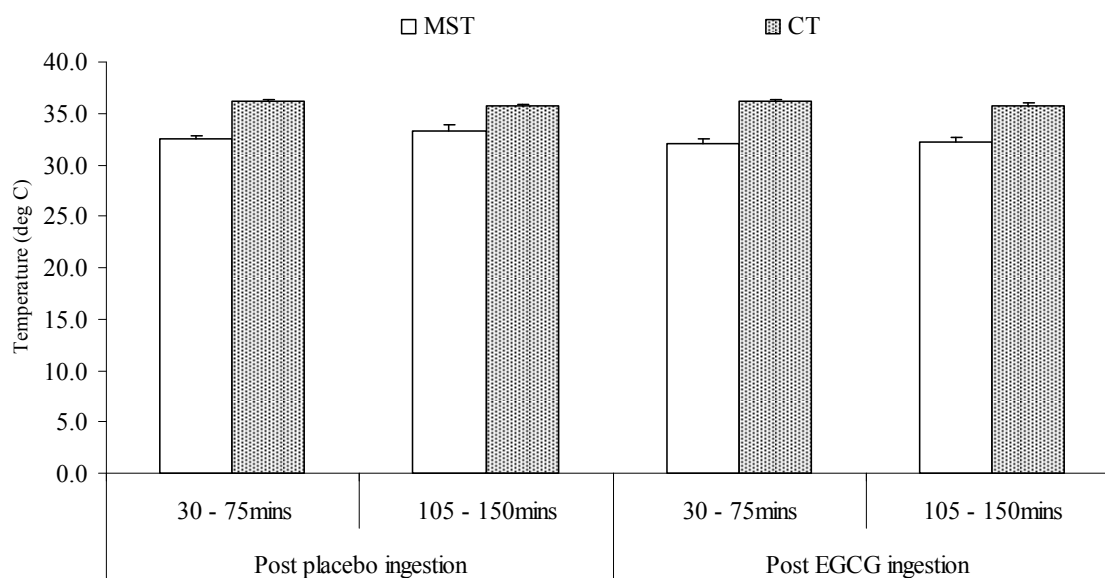
There were no significant differences between RMR, MST and CT measured at 30 – 75 and 105 – 150 minutes post placebo and post EGCG ingestion (Figure 4.5 and Figure 4.6 respectively). The ambient temperature was maintained from $22.5 \pm 0.4^{\circ}\text{C}$ to avoid inducing shivering thermogenesis (Figure 4.6). There were no differences in RER measured between 30 – 75 and 105 - 150 minutes following placebo or EGCG ingestions over a period of 2.5 hours (Table 4.4). There were no changes between macronutrient, total calorie and tea consumption prior to the two test sessions (Table 4.5, Table 4.6).

Figure 4.5 - Mean resting metabolic rate (RMR; \pm SEM) at 30 - 75 minutes and 105 - 150 minutes post placebo and post EGCG ingestion



n = 8; There were no significant differences.

Figure 4.6 - Mean skin (MST; \pm SEM), core (CT; \pm SEM) temperatures at 30 – 75 and 105 - 150 minutes following ingestion of either placebo or EGCG



n = 8; There were no significant differences; Ambient temperature (AT; \pm SEM) for 30 – 75, 105 – 150 mins post placebo intake was 22.7 ± 0.5 , 22.6 ± 0.4 °C respectively; post EGCG ingestion was 22.4 ± 0.3 , 22.4 ± 0.5 °C for 30 – 75, 105 – 15 mins respectively.

Table 4.4 - Mean respiratory exchange ratio (RER; \pm SEM) at two intervals (30 - 75 and 105 - 150 minutes) post placebo and post EGCG ingestion

	RER	
	30 – 75 mins	105 - 150mins
Post placebo ingestion	0.89 ± 0.02	0.93 ± 0.03
Post EGCG ingestion	0.91 ± 0.02	0.91 ± 0.02

n = 8; There were no significant differences.

Table 4.5 - Mean daily dietary intake (\pm SEM) and nutrient intake expressed as a percentage of total energy intake (%TEI)[†] 3 days prior to the placebo ingestion test session compared to mean dietary intake 3 days prior to the EGCG ingestion test session

Ingestion	Energy (kcal/day)	Protein %TEI	Fat %TEI	Carbohydrate %TEI
Placebo	2256.50 \pm 224.02	20.7 \pm 1.5	37.4 \pm 2.2	40.3 \pm 3.4
EGCG	2606.50 \pm 508.71	18.2 \pm 2.1	40.3 \pm 4.5	38.9 \pm 5.5

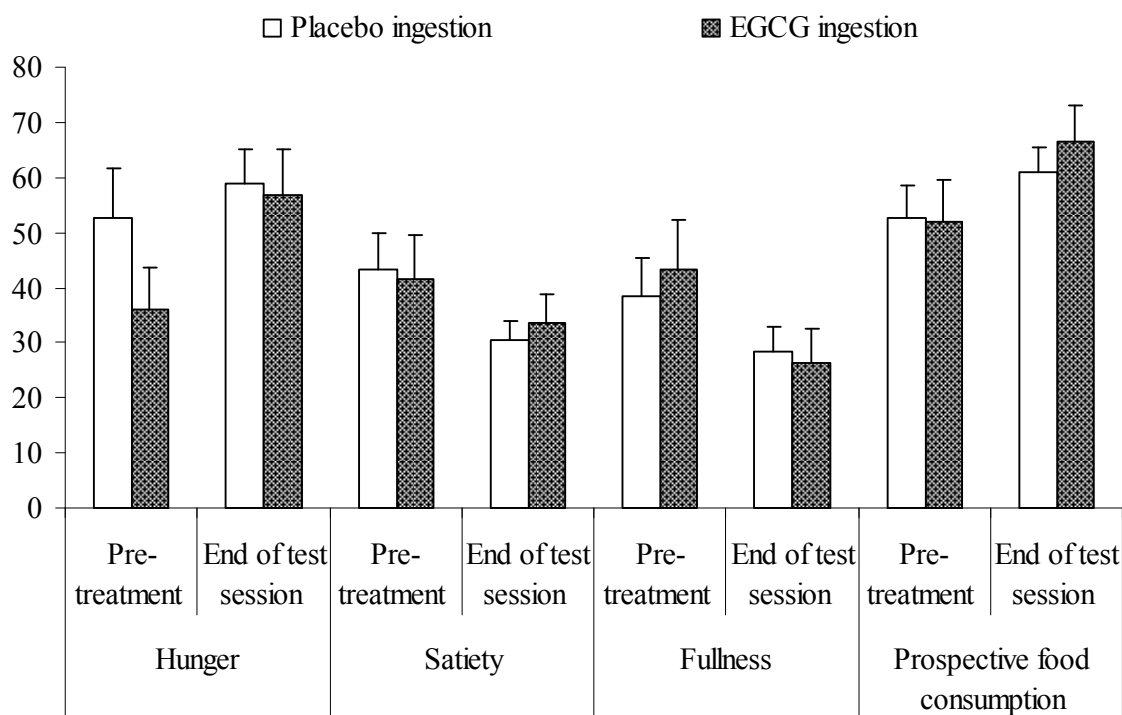
n = 8; There were no significant differences; [†] Likely validity of reported kcal intake discussed in appendix 13.

Table 4.6 - Mean black tea intake (ml \pm SEM) 3 days prior to the placebo ingestion test session compared to mean black tea intake 3 days prior to the EGCG ingestion test session

Tea consumption prior to placebo test session (ml)	Tea consumption prior to EGCG test session(ml)
551.3 \pm 250.3	481.3 \pm 236.6

N.B: *n* = 5 as 3 participants reported nil tea consumption over these days, There were no significant differences.

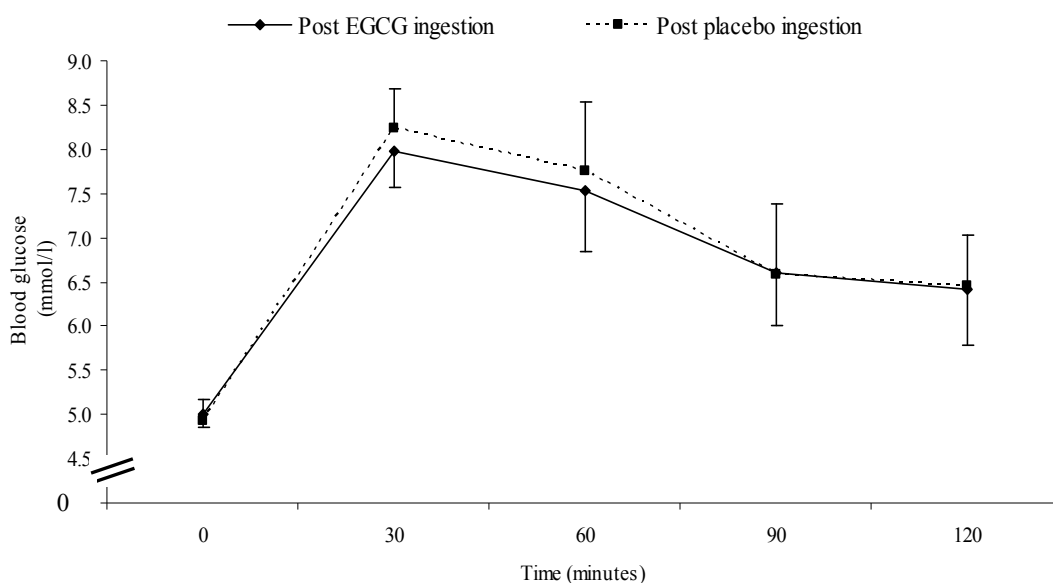
Figure 4.7 - Mean percentage change in visual analogue scale (\pm SEM) rating measure over a period of 2.5 hours for hunger, satiety, feeling of fullness and prospective food consumption from pre-treatment post placebo and post EGCG ingestion



n = 8; There were no significant differences.

At the end of the EGCG test session participants tended to appear to be more hungry ($p = 0.053$). Ratings of feelings of satiety, fullness and prospective food consumption were not different post placebo and post EGCG ingestion and also over time (2.5 hours) (Figure 4.7).

Figure 4.8 - Mean blood glucose response (\pm SEM) to oral glucose tolerance test over 2 hours following placebo and EGCG ingestion



$n = 8$; There were no significant differences.

Table 4.7 - Mean plasma insulin (\pm SEM), plasma leptin (\pm SEM) and plasma non-esterified fatty acid (NEFA; \pm SEM) concentrations measured pre-treatment and at the end of both placebo and EGCG test sessions

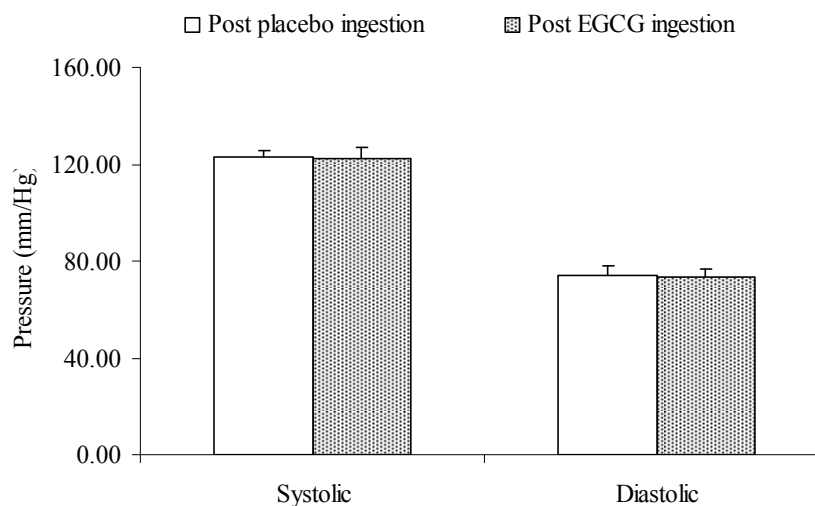
	Insulin (mU/l)		Leptin (ng/ml)		NEFA (μ mol/l)	
	Pre-treatment	End of test session	Pre-treatment	End of test session	Pre-treatment	End of test session
Placebo	5.50 \pm 1.27	13.40 \pm 5.07	12.88 \pm 10.09	14.07 \pm 11.22	755.19 \pm 105.63	474.65 \pm 69.15*
EGCG	7.64 \pm 2.89	14.55 \pm 5.20	7.03 \pm 3.56	6.36 \pm 3.55	721.83 \pm 105.61	433.21 \pm 75.83**

$n = 8$; *Significantly different from pre-treatment ($p = 0.0002$); **Significantly different from pre-treatment ($p = 0.0005$).

There was no difference in blood glucose concentrations measured every 30 minutes following an oral glucose challenge along with either placebo or EGCG (Figure 4.8). There were no differences in plasma insulin and leptin concentrations pre and post ingestion of either placebo or EGCG (Table 4.7). Correlation analysis revealed a significant correlation ($p = 0.016$; $r = 0.806$) between mean fasting blood glucose with fasting insulin concentration at the beginning of the EGCG test session. There were however decreases in

NEFA post placebo ($p = 0.0002$) and post EGCG ($p = 0.0005$) ingestion when compared with pre-treatment concentrations (Table 4.7).

Figure 4.9 - Mean systolic and diastolic blood pressure (\pm SEM) following either placebo or EGCG ingestion



$n = 8$; There were no differences between the two groups.

Table 4.8 - Mean heart rate (HR; \pm SEM) for a 2 hour period after ingestion of either placebo or EGCG

Ingestion	Mean HR (beats/minute)	Maximum HR range (beats/minute)
Placebo	60.63 ± 3.01	68 - 117
EGCG	63.75 ± 2.25	62 - 123

$n = 8$; There were no significant differences.

There were no differences in either systolic or diastolic blood pressure post placebo or post EGCG ingestion and heart rate recorded over 2.5 hours of each test session (Figure 4.9 and Table 4.8 respectively).

4.2.6 Discussion

The current study was designed to investigate the effect of an acute ingestion of 150 mg EGCG in healthy humans on RMR, MST, CT, post prandial glucose regulation, and index of lipolysis (NEFA) as markers of energy regulation and on the energy balance regulatory hormones- insulin and leptin.

Effect of EGCG on RMR, MST, CT and RER

There were no differences in RMR measured in two 45 minute sampling periods for up to 2.5 hours post ingestion of either placebo or EGCG. This period of RMR measurement coincided with the maximum plasma concentration (C_{max}) of EGCG, which has been observed to be 1.4 to 2.4 hours after ingestion in humans, with a half – life of 5 – 5.5 hours (Yang *et al.*, 1998). Green tea ingestion increases 24-hour EE (Dulloo *et al.*, 1999), but no data exists for any changes observed with green tea or EGCG ingestion following an acute period of ingestion in humans. It is possible that changes in RMR over a short period of time (2.5 hours) is not evident or is very subtle. It may be that addition of other catechins or caffeine may show measurable changes in RMR. Green tea unless decaffeinated, contains caffeine which may lead to increased EE. Caffeine content in the LEO drink used in this study was 46mg and as the same amount was ingested during both test sessions, any changes in RMR should be attributed to EGCG. Examining the data regarding metabolic effects of caffeine shows indefinite actions. Caffeine intake of 50mg did not affect EE or RER (Dulloo *et al.*, 1999) whereas 4 mg/kg body weight (approximately 280mg for a 70 kg man) increased metabolic rate significantly (Acheson *et al.*, 1980). Caffeine content used in the current study was an average dose of 0.5 mg/kg body weight and reviewing the data from the above mentioned study, it is believed that this quantity would not lead to any increase in RMR.

There were no changes observed in MST, CT and RER following placebo or EGCG ingestion over the period of 2.5 hours. Possibly, the effects were subtle and the measurement techniques employed were unable to detect minute changes. Analysis of mRNA for expression of any increases in UCPs might indicate changes in thermogenesis and should be considered by other researchers for future studies of this nature. Also, in light of the above previously discussed half-life and C_{max} of EGCG it can be hypothesised

that with measurement of RMR for a further 60-90 minutes, a probable variation could have been observed.

Participants maintained 3-day diet diaries prior to each test session so as to ascertain that the diet consumption remains similar prior to both test sessions and any significant changes in any macronutrients do not play a part to any changes in any metabolic indices. Results of dietary analysis revealed no significant differences in dietary intake prior to both test sessions. None of the participants consumed green tea and this criterion was purposeful during recruitment for the study as the confounding effect of habitual green tea intake was eliminated. Dietary intake diaries showed that consumption of black tea intake remained similar prior to both test sessions in participants who habitually drank tea. Therefore, catechin intake from this source was consistent throughout the study period.

Background and hence baseline intakes of caffeine (a dietary component with possible effects on some of the measured parameters) did not to change during the study as subjects were instructed to continue their normal eating patterns. Therefore, in the same manner that the mode of delivery (LEO) of EGCG was uniform in caffeine delivery so was daily caffeine ingestion. Deviations in measures of RMR and glucose tolerance that may have been observed would then be attributable to the EGCG intervention. It would not have been appropriate to attempt to change habitual caffeine intake, as long-term adaptation to this compound would mean that abrupt cessation to participate in the study would have produced a withdrawal syndrome (Ozsungur *et al.*, 2009). Such a syndrome would induce changes likely to confound study findings. In addition and in common with other pharmacologically active agents, sudden changes in intake could mask or distort the frequently reported synergy between caffeine and EGCG with respect to its physiologic and metabolic activity (Dulloo *et al.*, 1999; Dulloo *et al.*, 2000).

Effect of EGCG on blood glucose, insulin, leptin and NEFA concentrations in circulation

There was no difference observed in post prandial glucose following either placebo or EGCG ingestion. Several studies have investigated the effect of green tea supplementation on insulin sensitivity and glucose tolerance in both animals and humans. Green tea ingestion (containing 84 mg of EGCG in an acute dose) improved glucose tolerance compared to placebo in humans and this effect has been observed to be due to promotion

of insulin action in the peripheral tissues such as skeletal muscles and adipocytes (Tsuneki *et al.*, 2004). In diabetic mice, glucose tolerance and insulin secretion was improved due to reduction of endogenous glucose production and increase in glucose-induced insulin secretion after ingestion of Teavigo® (Wolfram *et al.*, 2006). Improved insulin action was not evident in this study and it is plausible and debatable that this mechanism could be better observed if EGCG was supplemented for a few weeks before the OGTT challenge. Plasma leptin concentrations were not different between the placebo and EGCG groups. There is a lack of data regarding leptin changes following acute intake of EGCG in the literature. It is also arguable whether any leptin changes occurs or could be observed over a 2.5 hour period at all. A statistically significant reduction from baseline was observed in plasma NEFA during both test sessions. This reduction was expected as a consequence of OGTT, as glucose provision promotes glucose oxidation and glucose and lipid storage, and inhibits fatty acid oxidation (Randle, 1998). This is attributed to a decrease in glucagon concentrations with increasing glucose concentrations in the blood, in turn impeding ketogenesis.

Effect of EGCG intake on appetite regulation

The present study assessed in participants, a range of hunger-satiety related subjective impressions over a period of 2.5 hours. The tests were carried out following an overnight fast and with all subjects missing their normal breakfast and reporting hunger (via VAS) at the start of the test period (all the participants reported regularly consuming three meals daily, including breakfast). Subjective impressions (VAS) given prior to testing (ingestion of test fluid) did not differ statistically significantly for any of the parameters measured. Therefore, no influence of prior fluid ingestion (only water permitted) was evident.

All subjects consumed isocaloric/isovolumetric test drinks (287 kcals/410 ml) therefore post-prandial differentials in subjective sensations could be related to EGCG ingestion. By the end of the test session (2.5 hours later), gastric emptying would be complete (Stenson, 2006) and any effects would likely stem from post absorptive events according to test drink composition (ie EGCG content). Analysis revealed a strong trend for participants to feel more hungry post EGCG ingestion ($p = 0.053$) when compared with post placebo ingestion although this did not reach statistical significance. Similar observation of increased appetite was observed in a study investigating long-term supplementation of green tea in humans related to down-regulation of leptin release through stimulation of SNS by green

tea catechins (Diepvens *et al.*, 2005). No other significant differences according to test group were observed.

Results obtained from animal studies have shown EGCG and/ or green tea supplementation may modulate leptin-independent appetite pathways and reduce food intake (Kao *et al.*, 2000). This mechanism is uncertain in humans and needs to be further investigated. Yet again, no data exists from previously conducted animal or human studies regarding appetite regulation following an acute ingestion of green tea or EGCG and further studies will clarify acute effect of EGCG in humans.

Effect of acute EGCG intake on blood pressure and heart rate

Blood pressure measured at the end of two test sessions showed no difference after either supplementation. Heart rate monitored through out the test session also showed no major difference between supplementations. Habitual green tea intake is believed to have hypotensive effects in Chinese medicine and its ingestion has been extensively researched for this purpose (Cabrera *et al.*, 2006). This blood pressure attenuation effect of green tea is attributed to its anti-oxidant properties (Negishi *et al.*, 2004). Reduction in risks of cardiovascular disease is important for overall well being and it may well be obvious that these cardiovascular effects are more apparent following long-term ingestion rather than an acute intake.

4.2.7 Conclusion

Results obtained from the present study indicate that there are no effects on glucose tolerance, RMR, MST, CT, RER, insulin secretion and lipolysis of ingestion of a single dose of 150 mg EGCG over a 2.5 hour period. This study is novel as there have been no studies previously conducted to measure the acute effects of 150 mg pure EGCG over 2.5 hours among healthy humans. Due to this, data obtained from the current study cannot be compared with previous findings. Several studies do indicate metabolic effects (increased BMR, lipolysis, reduced blood glucose concentrations) of habitual green tea and/or EGCG ingestion. Therefore, a longer term study was designed to further evaluate effect of EGCG at both differing doses (< and > 150mg) and over a longer period of supplementation (7 days). It was hypothesised that an increase in the dose of EGCG and / or the extension of period of supplementation might show notable changes in metabolic parameters such as RMR and lipolysis, which were not apparent in the present study. Consequently, a one

week supplementation study with differing doses was designed and is described in the following chapter (4.3).

4.3 Dose response effects of epigallocatechingallate supplementation over a 7 day period on metabolic indices of energy balance

Studies have previously demonstrated that ingestion of EGCG increases plasma EGCG concentrations dose-dependently (Nakagawa *et al.*, 1997). Following on from the previous study (Chapter 4.2) of acute oral ingestion of pure EGCG, this study was designed to investigate the effects of pure EGCG ingestion over a longer period at varying doses. By extending the intervention period the induction of metabolic processes of significance may become manifest through increases in flux through relevant pathways not possible in a two and a half hour period.

4.3.1 Hypothesis

Ingestion of EGCG (75, 150 and 300 mg/day, each for a period of 7 days) daily will dose dependently decrease body weight, increase RMR, increase lipolysis, reduce fasting blood glucose concentrations and decrease lipid concentrations in circulation.

4.3.2 Aim

The aims of this study were to measure the dose-dependant responses of EGCG ingestion on body weight, RMR, MST, CT, % body fat, fasting blood glucose and fasting lipid concentrations, blood pressure and heart rate, NEFA along with key metabolic hormones - insulin and leptin.

4.3.3 Methods

4.3.3.1 Participant recruitment and profile

The sample size calculation was based on a difference in body weight (section 2.22.1) and showed that at least 8 participants were needed to observe a change in body weight. Eleven, healthy men between the ages of 20-55 years were recruited for the study by advertising in local newspapers, the Metro and Evening Standard (section 2.2 for inclusion and exclusion criteria). Out of the eleven participants, 8 completed the study and 3 participants dropped out during the course of the study. The study protocol was approved by the Ethics Committee of University of Westminster, London, UK (Application number 06/07- 27, Appendix 8).

Table 4.9 - Mean anthropometric data (\pm SEM) of only participants who completed the study

Parameter	Measurement	Range
Age (years)	39.0 \pm 2.9	31 - 54
Height (centimetres)	175.7 \pm 2.2	168.1 – 182.2
Weight (kilograms)	75.2 \pm 2.6	65.9 – 87.1
BMI (kg/m ²)	24.4 \pm 0.5	22.2 – 24.9
n	8	

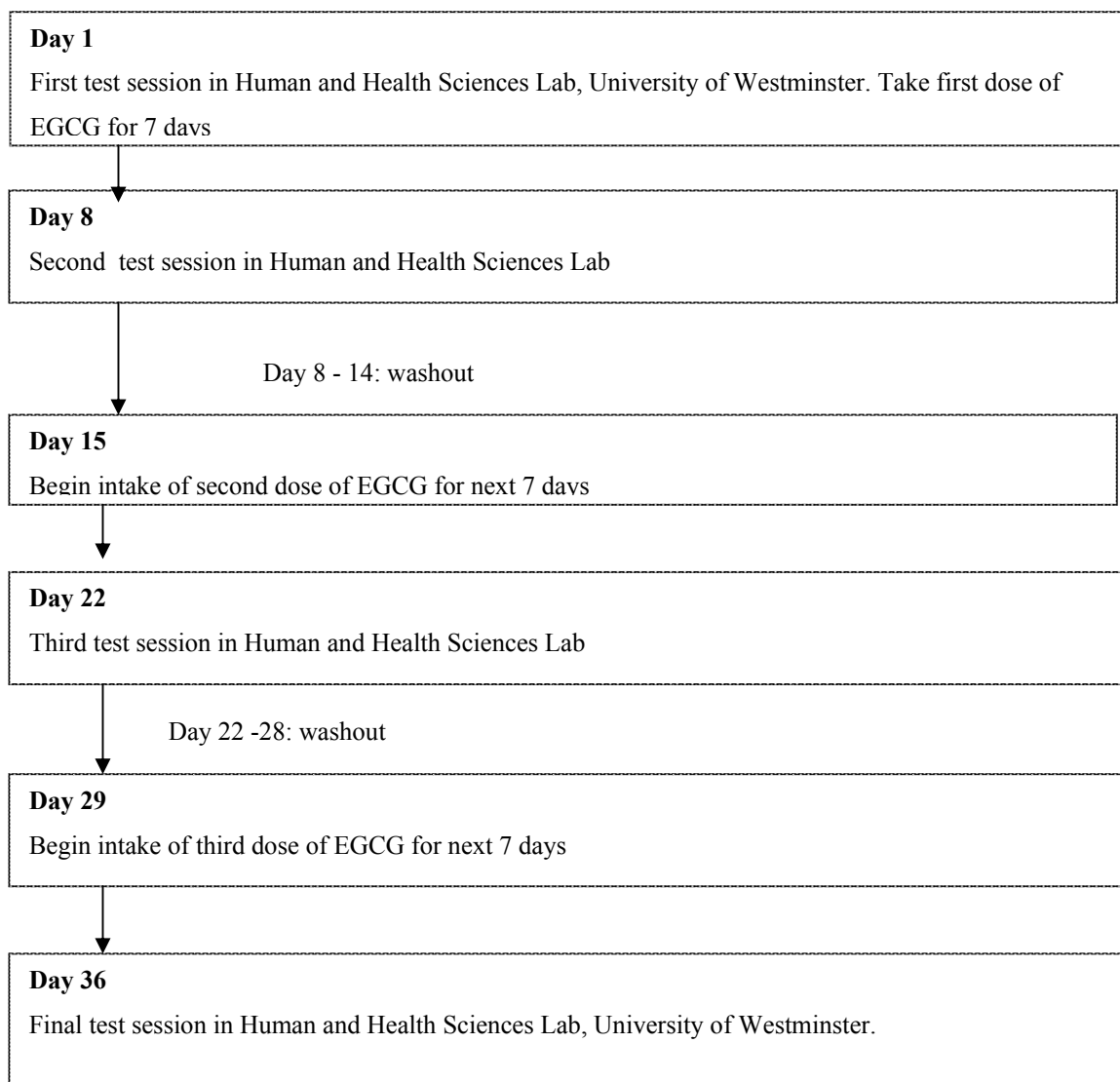
4.3.3.2 Study design

This study was single blinded and crossed-over in design. Each participant acted as their own control and the order in which they took each dose over the three test sessions was randomised. Each supplementation dose was for a period of 7 days with a wash out period of 7 days between each dose. All participants were randomly assigned to 75, 150 or 300 mg/day of pure EGCG (Teavigo®, DSM nutritional Ltd, Switzerland; section 2.4 for dose calculation) made up as gelatine capsules (size 0: G. Baldwin & Co, London). It was carefully explained to participants that it was important to take the capsules daily at the time advised (with breakfast). All reasonable measures to ensure compliance were carried out, including checking via a telephone call to each participant mid-way through each study week, and verification of compliance again at the end of the study. Subjects were trusted to be honest with the investigator as biomarker assays of compliance were beyond budgetary constraints of the study.

The participants visited the laboratory for 4 test sessions over a period of 35 days (Figure 4.10). At the start of each test session, body weight was recorded and body composition measured via skinfold measurement (section 2.6). Participants' RMR was then measured with the use of Deltatrac II (section 2.7). During this time, participants wore a Polar heart rate monitor and were connected to the Squirrel data logger, recording their heart rate and MST and CT respectively (section 2.18, 2.9). At the end of each test session, blood pressure was measured (section 2.19), fasting blood glucose was analysed by a finger prick blood sample with the use of HemoCue® analyser (section 2.11) and venepuncture blood samples were obtained to measure fasting blood lipids, plasma insulin, leptin and NEFA concentrations (section 2.13). The filled vacutainers were immediately centrifuged at 3500

rpm for 12 minutes. The plasma was transferred into eppendorf tubes and stored at -80°C until further analysis (section 2.16, 2.17 and 2.15 for further details of each of the assays). Following on, the previously completed 3-day diet diaries (section 2.20) procured from them. The second, third and final test sessions followed the same protocol of measurements (Figure 4.10). At the end of the first, second and third test sessions, participants were given the next of the randomised doses and further instructions regarding the wash out period and the start of EGCG ingestion were discussed.

Figure 4.10 - Schematic overview of dose dependant EGCG study design



4.3.4 Data analyses

All data in tables and figures are presented as mean \pm standard error of mean (SEM). Data analyses were performed using SPSS 14.0 for Windows (SPSS Inc. Illinois). Data were

analysed using a repeated measures ANOVA and a p value of ≤ 0.05 was considered significant. Correlation analyses between inter-dependant variables (e.g. insulin and fasting blood glucose concentrations) were performed using Pearson's correlation coefficient. A p value of ≤ 0.05 was considered as significantly correlated (section 2.22.2).

4.3.5 Results

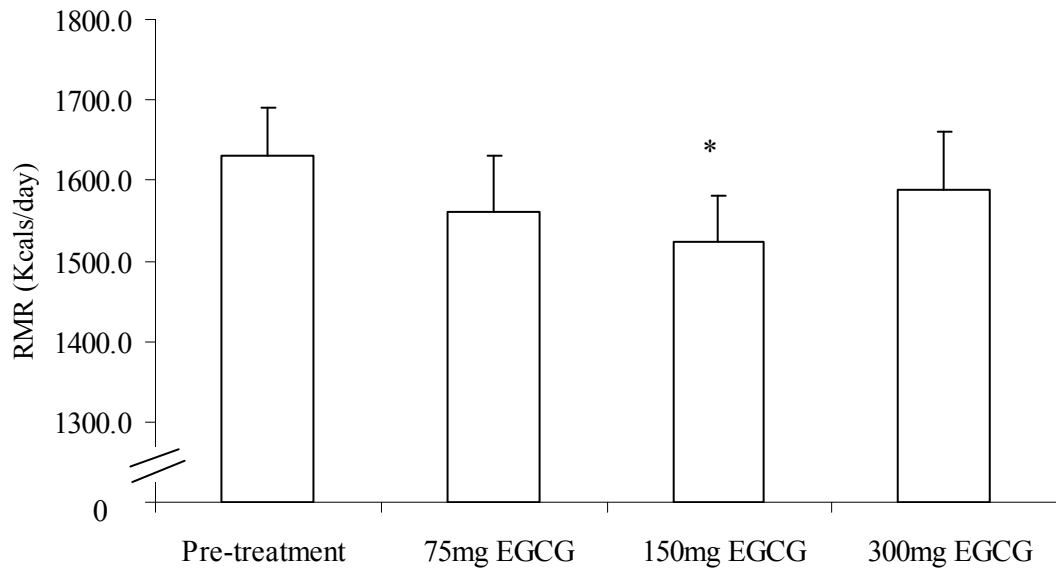
There were no differences in body weight following ingestion for 7 days of 75, 150 or 300 mg/day EGCG from pre-treatment (Table 4.10). Mean RMR measured following ingestion of 150mg EGCG was significantly lower than pre-treatment measurement ($p = 0.008$; Figure 4.11). There were no differences between RMR measurements following ingestion of 75 and 300mg EGCG in comparison with pre-treatment (Figure 4.11). MST and CT remained unchanged from pre-treatment readings, after 75, 150 and 300mg EGCG ingestion (Figure 4.12). Ambient temperature was maintained at $22.3 \pm 0.4^{\circ}\text{C}$ during all test sessions (Figure 4.12).

Table 4.10 - Mean body weight (\pm SEM) pre-treatment and following 7 day ingestion of 75, 150 and 300mg EGCG

Test session	Weight (kg)
Pre-treatment	75.15 ± 2.58
75mg EGCG	75.11 ± 2.46
150mg EGCG	74.85 ± 2.68
300mg EGCG	74.76 ± 2.63

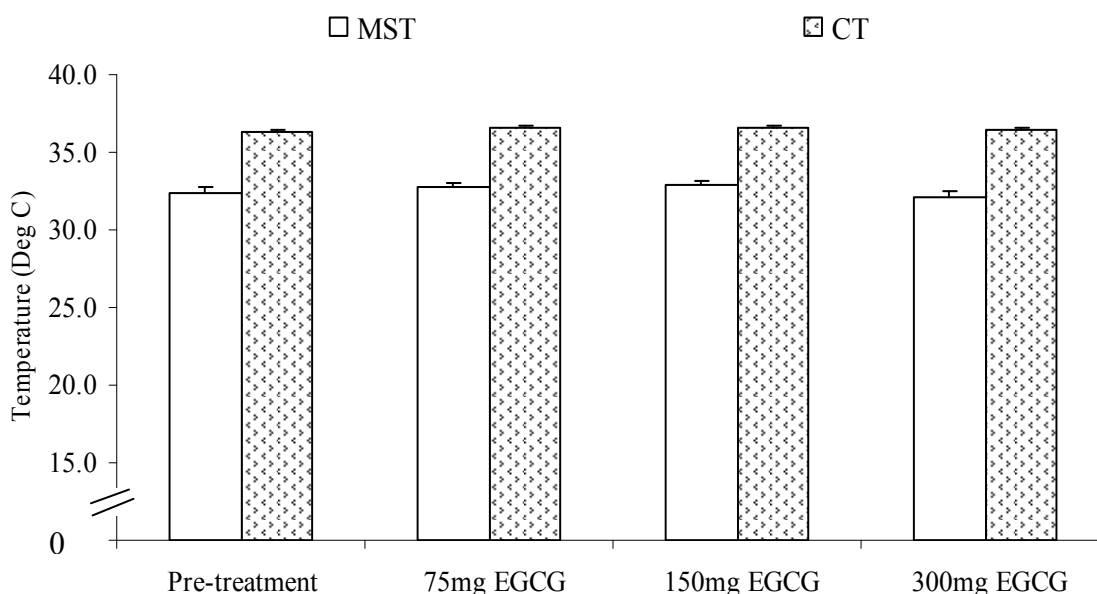
n = 8; There were no significant differences.

Figure 4.11 - Mean resting metabolic rate (RMR; \pm SEM) pre-treatment, post ingestion of varying doses of EGCG (75, 150 and 300mg; for 7 days each)



*n = 8; *Significantly different from pre-treatment measurement ($p = 0.008$).*

Figure 4.12 - Mean skin (MST; \pm SEM) and core temperatures (CT; \pm SEM) measured pre-treatment and following 75, 150 and 300mg EGCG ingestion



n = 8; There were no significant differences; Ambient temperature (AT; \pm SEM) measured pre-treatment and following 75, 150 and 300mg EGCG ingestion was 22.5 ± 0.6 , 22.2 ± 0.3 , 21.9 ± 0.4 , $22.7 \pm 0.3^\circ\text{C}$ respectively.

There were no differences in RER post 75, 150 and 300mg EGCG ingestion from pre-treatment (Table 4.11). Percentage body fat remained stable from pre-treatment measurements following ingestion of 75, 150 and 300mg EGCG (Table 4.11).

Table 4.11 - Mean respiratory exchange ratio (RER; \pm SEM) and % body fat pre-treatment, post 75, 150 and 300mg EGCG ingestion

Test session	RER	Body Fat (%)
Pre-treatment	0.84 ± 0.03	19.7 ± 1.5
75mg EGCG	0.80 ± 0.03	20.2 ± 1.6
150mg EGCG	0.84 ± 0.03	19.7 ± 1.5
300mg EGCG	0.82 ± 0.02	19.8 ± 1.5

n = 8; There were no significant differences.

Table 4.12 - Mean daily dietary intake (\pm SEM) and nutrient intake expressed as a percentage of total energy intake (%TEI)[†] 3-days prior to each test session compared to 3-days prior to pre-treatment

Test session	Energy (Kcal/d)	Protein %TEI	Fat %TEI	Carbohydrate %TEI
Pre-treatment	2236.7 \pm 350.6	16.2 \pm 1.1	31.7 \pm 3.9	44.1 \pm 3.2
75mg EGCG	2470.3 \pm 308.6	17.2 \pm 2.2	30.9 \pm 3.0	51.3 \pm 2.7
150mg EGCG	2314.5 \pm 399.4	18.0 \pm 3.1	32.2 \pm 3.6	48.8 \pm 2.8
300mg EGCG	2827.67 \pm 348.1	14.9 \pm 2.8	30.6 \pm 4.9	49.0 \pm 3.5

n = 8; There were no significant differences; [†] Likely validity of reported kcal intake discussed in appendix 13.

Table 4.13 - Total black tea intake (ml) 3-days prior to each test session among study participants during the course of the study

Test session Subjects	Pre-treatment	75mg EGCG	150mg EGCG	300mg EGCG
1	250	250	0	250
2	400	200	0	0
3	600	1000	1000	600
4	0	750	1250	800
5	500	500	800	600
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0

N.B: Due to disparity in reported tea intake over the sampling period, total intake has been reported without the calculation of SEM; complete data set for all 4 test sessions *n*=2; all other data sets were excluded during statistical analysis due to missing values; therefore, due to very small sample size no statistical inference made.

Consumption of total calorie intake and macronutrients remained unchanged during all three periods of EGCG supplementation from pre-treatment (Table 4.12). None of the participants recruited for the study consumed green tea, but a few participants reported drinking black tea habitually. Only 2 participants reported intakes of tea during the entire course of the study (subject numbers 3 and 5 in Table 4.13). Three participants did not

report tea intake at any period of the study (subject numbers 6, 7 and 8 in Table 4.13) and three remaining participants reported tea intake prior to only 3 test sessions (subject numbers 1, 2 and 4 in Table 4.13).

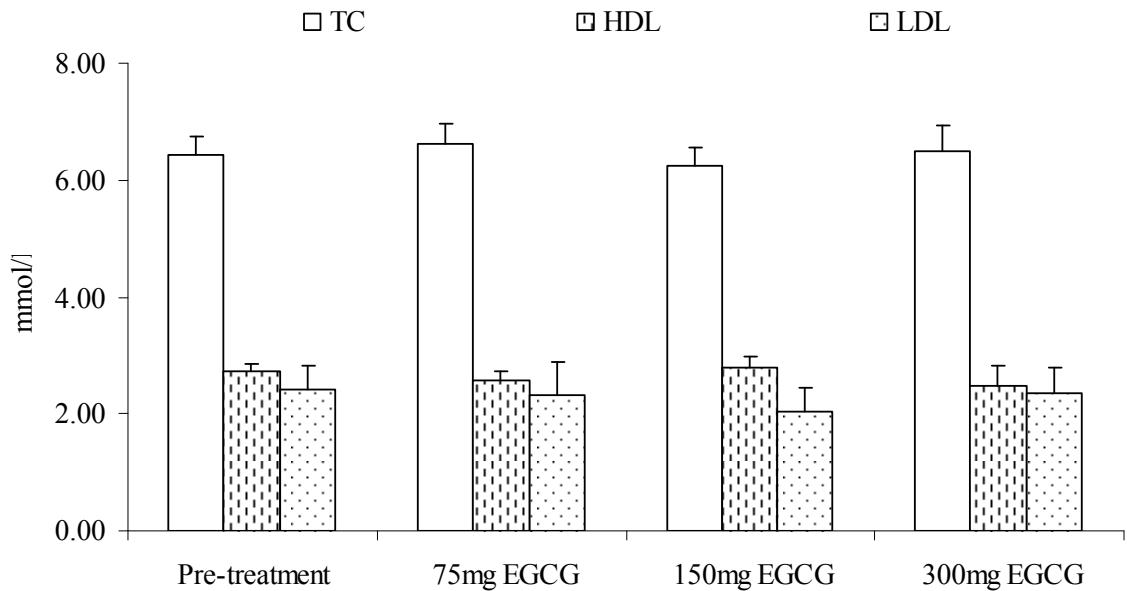
Table 4.14 - Mean fasting blood glucose (FBG; \pm SEM), plasma insulin (\pm SEM), plasma leptin (\pm SEM) and plasma non-esterified fatty acid (NEFA; \pm SEM) concentrations measured pre-treatment and after 75, 150 and 300mg EGCG ingestion

Test session	FBG (mmol/l)	Insulin (mU/l) [#]	Leptin (μ g/l)	NEFA (μ mol/l)
Pre-treatment	4.72 \pm 0.17	3.40 \pm 0.91	4.68 \pm 1.44	568.23 \pm 59.50
75mg EGCG	4.81 \pm 0.19	6.5 \pm 2.15 ^	4.39 \pm 1.15	484.33 \pm 79.67*
150mg EGCG	4.72 \pm 0.25	3.22 \pm 0.68	4.47 \pm 1.25	626.86 \pm 69.17
300mg EGCG	4.93 \pm 0.19	4.88 \pm 1.51	4.90 \pm 1.33	655.73 \pm 95.57

*n = 8 for FBG, leptin and NEFA; # n = 7 for insulin (one subjects' plasma sample was lost due to breakage of eppendorf tube during storage at -80 °C); ^ Range – 1.6-18.4; * Statistically significant from 150mg (p = 0.014) and from 300mg (p = 0.026).*

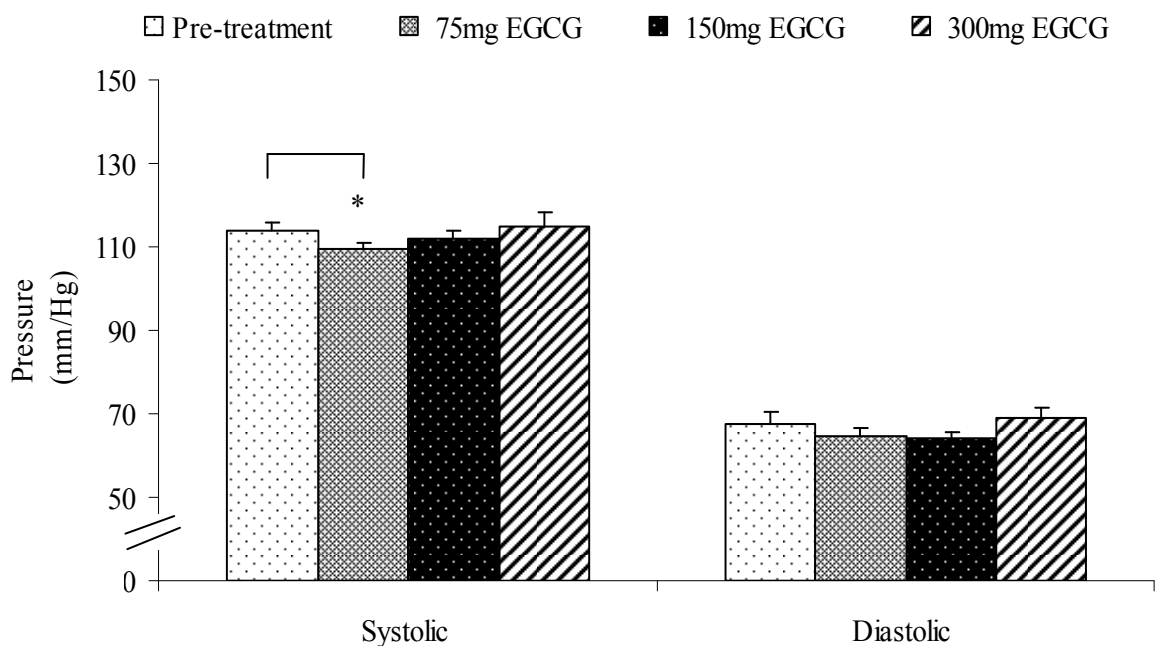
There were no differences in fasting glucose, insulin and leptin concentrations in circulation between pre-treatment and varying doses of EGCG (75, 150 and 300mg) (Table 4.14). Plasma NEFA concentrations post 75mg EGCG ingestion were significantly different (lower) from plasma NEFA concentrations post 150mg EGCG ingestion (p = 0.014; Table 4.14) and post 300mg EGCG ingestion (p = 0.026). Plasma NEFA concentrations post 75mg, 150 and 300mg EGCG did not differ from pre-treatment measurements (Table 4.14). There were no differences in total cholesterol, HDL and LDL concentrations between pre-treatment and varying doses (Figure 4.13).

Figure 4.13 - Mean total cholesterol (TC; \pm SEM), high density lipoprotein (HDL; \pm SEM) and low density lipoprotein (LDL; \pm SEM) concentrations pre-treatment and post ingestion of 75, 150 and 300mg EGCG



n = 8; There were no significant differences.

Figure 4.14 - Mean systolic and diastolic blood pressure (\pm SEM) pre-treatment and following 75, 150 and 300mg EGCG ingestion



*n = 8; *Significantly different from pre-treatment value ($p = 0.042$).*

Table 4.15 - Mean heart rate (HR; \pm SEM) pre-treatment and following 75, 150 and 300mg EGCG ingestion

Test session	Mean HR (beats/minute)	Maximum HR range (beats/minute)
Pre-treatment	63 \pm 2	64 - 88
75mg EGCG	61 \pm 2	67 - 92
150mg EGCG	61 \pm 4	66 - 91
300mg EGCG	62 \pm 4	59 - 88

n = 8; There were no significant differences.

Systolic blood pressure measured post 75mg EGCG ingestion was lower than pre-treatment ($p = 0.042$; Figure 4.14). There were no changes in systolic blood pressure following 150 and 300mg EGCG ingestion compared to pre-treatment measurements. There were no effects of varying EGCG dose on diastolic blood pressure and mean heart rate when compared with pre-treatment measurements (Figure 4.14 and Table 4.15, respectively).

4.3.6 Discussion

This study investigated the dose dependant effect of pure EGCG in healthy men. It was hypothesised that oral intake of EGCG at 75, 150 and 300 mg/day, each for a period of 7 days, would dose dependently affect energy balance by increasing RMR resulting in a decrease in both body weight and body fat composition. The variation in EGCG dose would also lower blood glucose concentrations, lipid profile and blood pressure (BP) following increase in EGCG doses along with variations in plasma insulin, NEFA and leptin concentrations. The results obtained from the study showed no difference in body weight, MST, CT, RER, body composition, dietary intake, lipid profile and heart rate following ingestion of varying doses of EGCG. There were significant decreases compared to pre-treatment measurement in RMR following 150 mg/day EGCG, decrease in systolic BP following ingestion of 75 mg/day EGCG and decrease in NEFA concentrations following 75 mg/day EGCG ingestion.

Effect of EGCG on energy balance and thermogenesis

Mean body weight did not change with varying EGCG dose from pre-treatment measurements. A decrease in body weight would be observed with an increase in RMR and/or decrease in total body fat percentage. Ingestion of 150 mg/day EGCG for 7 days significantly decreased RMR when compared with pre-treatment measurement ($p = 0.008$; Figure 4.11), but RMR measurements following other doses were not different from pre-treatment. One would expect to see a decrease in RMR following a loss of lean body mass but this was not evident from the results of this study. There was no change in body fat composition throughout the period of study, for that reason no change in lean tissue was expected. There was no change observed in thermogenesis to enhance RMR. The reason for the decrease in RMR following 7 days of ingesting 150 mg EGCG/ day is inexplicable. This study is the first to study dose response effect of pure EGCG ingestion among healthy humans. The chemistry of pure EGCG *in vivo* over a 7 day period might vary from the *in vivo* reactions following ingestion of all green tea catechins. The effects and actions of isolated pure EGCG in humans are also not fully understood as most of the previous studies have used green tea supplementation which contains the four major catechins. It is possible that isolated EGCG ingestion might have varying effects on RMR. RMR

measurements following ingestion of 150 mg/day EGCG needs to be investigated further to conclude its effect fully.

Data in the literature also provides inconclusive evidence towards increased metabolic rate following green tea extract (containing all catechins) and EGCG ingestion. Previous studies among obese subjects have demonstrated the effect of green tea extract (containing 100 mg EGCG) on weight loss following supplementation for 8 to 12 weeks due to increased EE and lipolysis (Auvichayapat *et al.*, 2008). Dulloo and others (1999) demonstrated the BMR enhancing effect of 90 mg/day EGCG over a 24 hour period while a 2 day supplementation of 300 mg/day of EGCG did not elevate BMR (Boschmann and Thielecke, 2007). In addition, a longer period of green tea extract supplementation (570 mg/day and for 2 months) did not increase BMR over the period of study (Ota *et al.*, 2005). Two main mechanisms have been proposed to explain the effect of green tea catechins and EGCG on increased metabolic rate. First, the effect of green tea catechins on the inhibition of COMT, the enzyme that degrades noradrenaline (Borchardt and Huber, 1975). This inhibition results in prolonged effect of noradrenaline on thermogenesis and fat oxidation (Dulloo *et al.*, 2000). Second, it is suggested that the increase in metabolic rate might be due to a synergistic effect between EGCG and caffeine. Caffeine inhibits the phosphodiesterase-induced degradation of intracellular cAMP, thus elevating cellular concentrations of cAMP: a critical intracellular mediator for catecholamine action on thermogenesis (Acheson *et al.*, 1980). The mechanism and rationale for both these observations is still unclear and needs to be further studied. Possibly, longer period of supplementation (> 3 months) may confirm elevation in BMR/RMR in humans following EGCG ingestion.

Inspection of the background intake of tea showed that those participants who drank tea, maintained an intake of tea similar to their usual intake throughout the study period. Hence, it is presumed that dietary intake of tea would not influence RMR during the study. As the completed data set without any missing values was only 2, this was insufficient to perform statistical tests and draw inferences. Although this was disappointing, it is worth noting that three of the eight study participants were non-tea drinkers and one other subject failed to record only their baseline tea intake value. The remaining two subjects did however fail to record tea intake during EGCG ingestion phases of the study. It was not deemed appropriate to attempt to extrapolate intake levels from available data, rather

report the complete data where it was available. With respect to the impact that regular caffeine consumption per se in the form of tea might have on responses to intervention with EGCG, the data gathered suggests that this was not physiologically a significant factor (no differential in response magnitudes was observed between tea drinkers and non-tea drinkers in terms of RMR).

The purpose of MST and CT measurements were to detect any thermogenic effects of EGCG ingestion, possibly expending energy via heat release. There have been no studies conducted in humans to test effects of EGCG on body temperature and this study is the first to do so. Data obtained from the current study showed no changes in skin and core temperatures. Few studies have been conducted on animal models to measure changes in UCPs and thermogenesis following green tea or EGCG ingestion. Choo and others (2003) found that rats fed an extract of green tea at 20 g/kg diet for 14 days (approximately 600 mg/day) had increased BAT thermogenesis through β -adrenoceptor activation. Klaus and others (2005) found that 0.5 and 1% (approximately 150 mg and 300 mg/day) supplementation of pure EGCG in the diet for a 4 week period resulted in an increase in UCP2 in the liver of rats, but observed that oral administration of EGCG (500 mg/kg; approximately 100 mg/day) over 3 days had no effect on body temperature. The studies conducted on animal models supplemented EGCG or green tea extract in higher quantities and comparatively, the maximum dose used in the current study is low. Moreover employing detection techniques of mRNA in WAT might detect dose-dependant changes of the effect of EGCG consumption on UCPs and should be included in future studies carried out by other researchers of this nature.

Effect of EGCG on fasting blood glucose, plasma insulin, leptin concentrations

There were no changes in fasting blood glucose (FBG) and plasma insulin and leptin concentrations from pre-treatment following varying doses of EGCG. A study conducted on rats fed a high fructose diet demonstrated that green tea extract (1-2 g/kg diet) regulates gene expression of the glucose uptake and insulin signalling pathway (Cao *et al.*, 2007). This study is one among many animal studies which have shown anti-diabetic effects of polyphenols. Rats fed 1 and 2 g of green tea solid extract/kg diet for 6 weeks had higher levels of GLUT4 mRNA in liver and muscle. An increase in GLUT4 mRNA in muscles is physiologically significant as the GLUT4 protein is the insulin-responsive GLUT in the

muscle (Shepherd and Kahn, 1999). This increase in GLUT4 mRNA and ensuing transport of glucose into cells, thereby maintaining euglycaemia was achieved after ingestion of approximately 60 mg of green tea extracts/kg body weight per day (equivalent to humans drinking approximately 5 cups of green tea per day). Insulin enhancing properties of tea and its components were assayed in rat epididymal adipocytes. It was found that EGCG and ECG had a direct effect on insulin activity and had insulin-potentiating effect (Anderson and Polansky, 2002). However, there are no studies published to date at the molecular level in humans to analyse the effects of green tea extract or EGCG ingestion on glucose transport or insulin and further work is necessary to elucidate these actions. All participants in the current study were euglycaemic at the start of the study. It is uncertain whether any changes would be expected if pre-treatment blood glucose measurements were within normal levels. It would be interesting to study the variation in blood glucose concentrations if hyperglycaemic subjects are recruited.

Leptin concentrations were unchanged from pre-treatment measurements following ingestion of varying doses of EGCG in the current study. Data obtained from mice trials investigating the effects of pure EGCG (Teavigo®) intake have found significant decreases in plasma leptin concentrations over a period of 3-4 weeks (Klaus *et al.*, 2005; Wolfram *et al.*, 2005). This effect has been similarly observed in long-term human trials (Auvichayapat *et al.*, 2008; Diepvens *et al.*, 2006). In these studies participants had significant weight losses which were reflected in reductions in plasma leptin concentrations. A reduction in plasma leptin concentration is observed following loss of weight or body fat. In the current study, no change in body weight was observed following ingestion of varying doses of EGCG therefore no change was measured in plasma leptin concentrations. Moreover, as no variations in leptin concentrations were measured, there was no variation observed in dietary intake throughout the test period.

Effects of EGCG on biomarkers of lipid metabolism

Fresh green tea extract infused intraduodenally (doses equivalent to 1-2 cups of tea) in ovariectomised rats significantly lowered the lymphatic absorption of cholesterol in a dose dependant manner (Löest *et al.*, 2002). Ovariectomised rats were used here to mimic the physiologic conditions of ovarian hormone deficiency or the postmenopausal state in which serum levels of cholesterol are elevated with increased risk of CVD (Rosenberg *et al.*, 1981). Raederstorff and others (2003) found that a single oral dose of EGCG (0.1 g/kg body weight; approximately 20 mg/day) lowered the absorption of cholesterol in a dose

dependant manner in rats by increasing faecal excretion of total lipids and cholesterol. In the current study, ingestion of 75, 150 and 300 mg EGCG daily for 7 days did not bring about any change in total cholesterol, LDL and HDL concentrations in comparison with pre-treatment measurements. NEFA concentrations significantly decreased following ingestion of 75 mg EGCG indicating a reduction in lipolysis when compared to measurements following 150 and 300 mg EGCG ingestion ($p = 0.014$; Table 4.14). Numerous animal and human studies have shown an increased effect of EGCG and green tea consumption on fat oxidation after prolonged periods of supplementation (Klaus *et al.*, 2005; Venables *et al.*, 2008; Wolfram *et al.*, 2005). An optimum dose of EGCG or green tea extract (containing other catechins) needs to be deduced in order to observe potential lipid lowering and lipolytic effects of EGCG in humans. Perhaps, increasing the dose or supplementing for a longer period than in the current study would show significant differences.

Effects of EGCG on blood pressure and heart rate

The reduction of cardiovascular risk factors is vital for the prevention of CVD. Hypertension is one of the risk factors and is characterised by increased peripheral vascular resistance and oxidative stress in and around vascular endothelial cells (Nakazono *et al.*, 1991). Yang and others (2004) found that habitual tea consumption exerted a protective effect on the development of hypertension. Habitual consumption of green tea (120-599 ml/day) decreased the risk of developing hypertension by 46% and was further reduced by 65% for those who drank 600 ml/day for 1 year. This corresponds to an intake of approximately 170 – 200 mg of EGCG/day for a year. A possible hypothesis explaining the decrease in the incidence of hypertension due to green tea consumption could be that the polyphenols in green tea exert antioxidant and vasodilatory effects (Fitzpatrick *et al.*, 1995; Rice-Evans *et al.*, 1995). In the present study, systolic BP was significantly decreased following supplementation with 75 mg EGCG/day for 7 days whilst diastolic BP remained unchanged when compared with pre-treatment measurements. The significant decrease in systolic BP observed in the current study following ingestion of the lowest dose of EGCG could be a false positive and a type 1 error. Analysis of data regarding randomisation of doses showed that 5 participants out of 8 received 75mg EGCG dose as their final dose and the measurements to record this doses' effects were carried out in the final laboratory test session. It is possible that participants felt more relaxed and were more accustomed to the test sessions, which could have lead to lower BP. There were no

differences in mean heart rates measured following varying doses of EGCG from pre-treatment values. Low HR has been shown to decrease progression of CVD (Giannoglou *et al.*, 2008; Palatini, 2007; Palatini and Julius, 2004). Randomised controlled trials conducted by two research groups investigating the effect of green tea extract (containing 100 and 200 mg EGCG, respectively) supplemented for 12 weeks among women (46 and 60 subjects, respectively) found no significant changes in HR over time (Auvichayapat *et al.*, 2008; Diepvens *et al.*, 2005). The EGCG quantities employed in these studies ranged from 67 – 100 mg/day which might be very low or insufficient to induce any noticeable changes. Perhaps, ingestion of doses used in the current study over a longer period might demonstrate significant differences as well as further protective physiologic effects on hypertension and heart rate.

4.3.7 Conclusion

This study aimed to test the *in vivo* dose response to ingestion of pure EGCG for a period of 7 days. Results of the current study did not show any major significant differences in key metabolic parameters such as RMR, body fat percentage and dietary intake, all of which can affect/are components of, energy balance and therefore may influence risk factors associated with obesity. Further trials need to be carried out to observe the action of pure EGCG in humans to see if longer periods of supplementation and/or use of green tea extract (interaction with other catechins and caffeine) with pure EGCG would increase any measured metabolic parameters.

4.4 Summary and future directions

4.4.1 Summary of studies investigating oral intake of EGCG

The anti-oxidant effect of green tea intake has been widely studied, but few human studies have been performed to investigate the effect of its principal catechin, EGCG, on metabolism and its potential to influence components of energy balance. The second part of this thesis report pertains to studies conducted to investigate the effects of EGCG ingestion (75 – 300 mg/day) on weight, RMR, FBG, appetite, MST, CT, heart rate, blood pressure, associated regulatory hormones (insulin, leptin) and NEFA in healthy men.

The first study investigated the effects of ingestion of an acute dose of 150 mg EGCG, over 2.5 hours, in healthy humans. An improvement in glucose tolerance following 150 mg EGCG ingestion was not detected when compared with placebo. Plasma insulin, leptin and NEFA concentrations were not different following either placebo or EGCG ingestion. It may be a possibility that an acute dose of 150 mg was not sufficient to induce changes in measured study parameters. Following on from the results of this study, the next study was designed to measure the effects of a longer period of EGCG ingestion at varying doses on RMR, MST, CT and other metabolic parameters.

The effects of chronic ingestion (7 days) of EGCG was examined, with the aim to study effects of differing doses (75, 150 and 300 mg/day) on body weight, thermogenesis, RMR, blood glucose and lipid regulation. There were no effects of varying EGCG dose on body weight, FBG, body fat composition, MST, CT, dietary intake and leptin. A dose of 75 mg EGCG brought about a statistically significant decrease in NEFA concentrations and systolic BP. A dose of 150 mg EGCG ingested for 7 days decreased RMR lower than pre-treatment measurement. These results are difficult to interpret as no changes were measured in any of the associated parameters (such as an increase in food intake or weight due to decrease in RMR). Perhaps the changes associated with these parameters might have been subtle and have had physiological significance but were not statistically significant. In conclusion, the effects of EGCG ingestion need to be studied further in detail to arrive at conclusions regarding their efficacy in influencing components of energy balance and blood glucose regulation.

There is some discrepancy between the results from human studies and cultured cell and animal models with respect to the effects of EGCG or green tea intake. Two possible causes towards the differing activities of EGCG and other catechins according to study type, may relate to issues of metabolism and bioavailability *in vivo*. Studies have demonstrated that green tea catechins undergo methylation, glucuronidation and sulfation in *in-vitro* systems and in both animals and humans (Spencer, 2003). Several processes including intestinal, microbial and hepatic metabolisms and chemical degradation have been found to be involved in the fate of green tea, and to be responsible for its low availability in animals, presumably occurring also in humans (Feng, 2006).

Other studies have shown that the low systemic availability of orally ingested tea catechins could be a result of slow absorption, high first pass effects, and wide tissue distribution (Zhu *et al.*, 2000). Studies investigating dosing conditions and oral bioavailability in healthy men found that there was >3.5-fold increase in the average maximum plasma concentration of free EGCG when a green tea extract (up to a dose that contains 800 mg EGCG) was taken in the fasting condition than when taken with food (Chow *et al.*, 2005). Greater oral bioavailability of free catechins can be achieved by taking the EGCG capsules on an empty stomach after an overnight fast. In the dose dependant study (Chapter 4.3), participants were instructed to take the capsule at least 30 minutes before breakfast. Half of study participants reported taking the EGCG capsule along with or before lunch and reported that taking it mid-day helped them remember the intake.

Data regarding role of dietary proteins in the absorption and bioavailability of tea flavonoids are inconclusive. One study reports that proteins can bind with flavonoids effectively and could inhibit absorption of tea flavonoids (Shi and Kakuda, 2006). Other studies report data that proteins do not inhibit the absorption and bioavailability of tea flavanols in humans, as results obtained from investigations in healthy men revealed that catechins from green and black tea were rapidly absorbed and addition of milk did not impair the bioavailability of tea catechins (Hof *et al.*, 1998; Hollman *et al.*, 2001). As the intake of dietary protein did not vary significantly during the course of both studies, the usual intake appears not to have hindered the absorption of catechins in these studies.

4.4.2 Further directions

The challenges of investigating single or composite dietary components in ‘intervention’ mode are multiple. The present thesis has attempted to move a step forward in the investigation of qualitative changes in diet with the aim of improving known risk parameters in human metabolism.

While there is clearly a significant further body of work required in this field, the decision to start with food chemistry having a reasonable theoretical expectation of impact was taken. Within the time-frame and technical and financial limitations, real progress was made. Firstly, methodologically and in terms of protocol development and secondly in challenging the assumption (in the case of EGCG), that an isolated dietary component will necessarily have the quasi-pharmacologic effect that prior investigation might suggest. Such work is vital to avoid false claims regarding extracted ‘natural’ products, but equally allows for constructive thought towards new avenues for research. For example, examining the synergies and antagonisms that exist within the components of the biochemical milieu of complex foodstuffs, and also by establishing baseline dose and duration data, known to be or not to be of useful effect.

Dose: Due to scarcity of data from human studies regarding optimum dose of pure EGCG, it is difficult to conclude the most potent dose. One human study tested the effects of a single dose of 1600 mg EGCG, which was well tolerated (Ullmann *et al.*, 2003). Other animal studies have supplemented single doses of upto 400 mg EGCG, which was well tolerated in mice, but lethal in rats (Isbrucker *et al.*, 2006a; Isbrucker *et al.*, 2006b). These studies have tested single dose administrations and no data for longer periods of supplementation at these doses exist. The maximum dose used in the study might be too cautious and possibly doubling this dose may result in significant differences.

Longer periods of supplementation: Epidemiological and longer term studies have shown that green tea consumption (>500 mg catechins) for >3 months appears to reduce body weight and fat in humans (Chantre and Lairon, 2002; Nagao *et al.*, 2005). When subjects take supplements for >3 months it is possible that they may also incorporate other health promoting lifestyle changes into their routine such as inclusion of high fibre, low fat diets and increased daily physical activity to complement health benefits of tea catechin

supplementation. There are no studies performed to investigate the effects of long-term ingestion of pure EGCG and as previously conducted green tea supplementation studies have influenced components of energy balance, further investigation on green tea's principle catechin over a prolonged period of ingestion is necessary.

Use of green tea extract: Chen and others (1997) observed that EGCG displayed different pharmacokinetic behaviours when it was given to rats as one of the tea catechins in decaffeinated green tea (DGT) in comparison to when it was given as pure EGCG alone. Based on the AUC and Cmax produced per unit of EGCG, the authors concluded that DGT seemed to deliver EGCG into the bloodstream more effectively than when EGCG was given as a pure compound. They reasoned that this effect was largely due to the property of catechins to bind with proteins (Hagerman, 1989). Other tea catechins in DGT compete with EGCG for binding to plasma and tissue proteins, thus changing the EGCG pharmacokinetic behaviour and therefore prolonging EGCG action in the system. Possibly, interaction with other catechins is essential to cause metabolic changes and the effects of this interaction need to be investigated future studies.

Use of caffeine with pure EGCG: The thermogenic effects of caffeine via the sympathetic system are well documented (Acheson *et al.*, 1980; Astrup *et al.*, 1990). Caffeine inhibits adenosine and phosphodiesterases and therefore intracellular concentrations of cAMP remain elevated for longer. cAMP is a critical mediator for the thermogenic effects of noradrenaline. EGCG inhibits COMT thus prolonging effect of noradrenaline and thus increasing cAMP and thermogenesis. When both these agents are combined during ingestion, there might be a perceivable increase in EE even after shorter periods of supplementation (Dulloo *et al.*, 2000; Westerterp-Plantenga *et al.*, 2006). As the focus of the present studies was to investigate the effects of pure EGCG in isolation, caffeine and other tea catechins were not supplemented to the study participants.

In both the EGCG supplementation studies, metabolic rate was measured by indirect calorimetry using the Deltatrac, which most subjects find tiresome since it involves lying still under a hood, after a period of 50-60 minutes. Possibly, the use of a respiratory chamber over 24 hours or doubly labelled water might have lead to different results, particularly in the acute ingestion study. Both these techniques were unavailable for the above conducted studies.

Dietary intake before the start or during the study was not stipulated as the study was designed to test free-living conditions. A possible prescription and/or analysis of percentage intake of macronutrients intake along with maintenance of polyphenol content in the diet throughout the study might have shown interesting and differing results from the current data. However, the analyses of dietary data from the studies do not show any statistically significant difference in dietary intake throughout the study period from baseline.

Future studies could involve the collection and analysis of urine and faeces collected over 24-hour to measure catecholamines and to verify concentrations of EGCG by using high performance liquid chromatography to gather details regarding bioavailability and absorption of EGCG in humans over different phases of time and also to detect diurnal changes if any. Use of Northern blot analysis to study mRNA in order to explore the expression of UCPs in WAT would provide more explanations towards the mechanisms of EGCG actions in the human body.

5 Final conclusion

The impact of foodstuffs and their constituent ingredients on human health is of interest to a wide range of scientists and clinicians, and increasingly to those budgeting health delivery systems. With an apparent burgeoning of health problems related to positive energy balance, much research (including the present investigations) is being carried out, and is needed to elucidate the effects of dietary composition on human well-being. With respect to the specifics of energy intake and expenditure, prior basic research into the composition of food (in bioenergetic terms) and the components of EE, have allowed progress in subtler investigations into the relative impact of individual dietary components on energy balance. It is in this context that the present research was carried out. A review of the extant literature in the area revealed much interesting work suggesting that there indeed may be potential to influence energy intake/expenditure in humans via dietary manipulation. The studies outlined in this thesis have been concerned with the effect of putative active agents, aimed at influencing aspects of metabolism in normal and overweight individuals; specifically to assess effects on EE and body weight. In addition, measures of blood glucose regulation were of interest as hyperglycaemia is frequently a dysregulation associated with chronic positive energy balance.

While as stated, there is data to suggest that certain dietary agents may be of utility in the present context, there is also a clear paucity of well controlled human studies. This is in part due to the relatively new arena of nutritional intervention as a quasi-clinical science, and also due to the relatively recent emergence of overweight as a clinical as well as nutritional challenge (DOH, 2009; NICE, 2006). Having pared down the possible compounds meriting further investigation, it was decided as has been described, to evaluate the metabolic effects of two dietary interventions, L-Histidine and EGCG. Their effects on EE, lipolysis, modulation of blood glucose and cholesterol concentrations were the chief focus and measurements representing them were carried out in the studies. The stability of energy balance in the human body is regulated in large-part by neurochemical activity and both the dietary agents in question have the potential to influence neurochemical activity and affect various facets of energy balance and were therefore chosen to be studied.

It was hypothesised that the supplementation with L-Histidine and EGCG among healthy subjects would increase EE, decrease fasting blood glucose and lipid concentrations and

affect other parameters of energy balance (such as reduction in dietary intake and increased lipolysis). The effects of both L-Histidine and EGCG on these parameters however were inconclusive, as shown by the results of the studies. This is perhaps surprising in view of the animal data available (Kasoaka *et al.*, 2004; Masaki *et al.*, 2001; Morimoto *et al.*, 1999; Sakata *et al.*, 1997; Yasuda *et al.*, 2004a; Yoshimatsu *et al.*, 2002b; Yoshimatsu *et al.*, 1999) which clearly suggests potential for modulation of these parameters.

The pilot study into L-Histidine did however demonstrate that RMR, MST and CT measurements can be performed simultaneously. Following ingestion of L-Histidine for 10 days, there was a significant decrease in body weight and fasting blood glucose levels but this was not apparent when the same dose was ingested for a longer period (8 weeks). In addition it has been shown that (at the dose used) there is no 'sensitisation' to Histidine with a longer duration (10 days/8weeks) failing to produce a more marked response in the parameters of interest. Upregulation of systems to enhance sensitivity (e.g. during nicotine addiction, sensitivity to nicotine is augmented by upregulation of nicotinic acetylcholine receptors which modulate the release of dopamine (Govind *et al.*, 2009)) is not uncommon, but was not apparent in the present study of L-Histidine in connection with the parameters measured.

Human studies have not previously been carried out to investigate effects of oral ingestion of L-Histidine on the above mentioned parameters and there are doubtless other avenues to explore with respect to this essential amino acid. Such ideas have been outlined in the discussion of the findings of the L-Histidine studies (section 3.5.1 and 3.5.2). It is likely that Histidine works synergistically with other compounds (e.g. with zinc (Song *et al.*, 2001) or agonistically (rather than its metabolite histamine acting directly as a neurotransmitter) at specific neural sites to enhance the effect of other energy regulating substances (e.g. involvement with leptin-induced decrease in food intake (Fulop *et al.*, 2003; Itateyama *et al.*, 2003; Morimoto *et al.*, 1999; Toftegaard *et al.*, 2003; Yoshimatsu *et al.*, 1999). Such interactions will probably be the focus of future work as neurobiological investigative techniques progress, allowing clearer insights into CNS system specific processes and adaptations.

EGCG supplementation in humans has been studied somewhat more in humans than has L-Histidine supplementation. EGCG in animals and humans has demonstrated effects in

terms of increased EE and improved blood glucose and lipid profiles (where dysregulation was present), probably acting at its receptors (e.g. in adipocytes, liver and skeletal muscle; Fujimura *et al.*, 2008). Some of the effects previously demonstrated in humans are: increased EE and lipolysis, thus aiding decrease in body weight by inhibiting COMT and prolonging the effect of noradrenaline (Dulloo *et al.*, 1999; Westerterp-Plantenga *et al.*, 2005); promoting insulin action in skeletal muscles and adipocytes, by this means, exerting a relative hypoglycaemic effect (Hill *et al.*, 2007; Tsuneki *et al.*, 2004). Most of these studies however supplement other tea catechins along with EGCG, some also with caffeine. For these reasons, the exact dose and supplementation period required to affect parameters of energy balance remains unknown.

The EGCG studies presented in the current text aimed to address these two aspects. Importantly, results from the present studies do not provide confirmation of previously demonstrated effects. Ingestion of 150mg EGCG over a period of 2.5 hours did not affect RMR or blood glucose response following OGTT. The ingestion of varying doses of EGCG (75, 150 and 300mg/day), each for a period of 7 days, did not bring about variations in body weight, RMR, blood glucose or lipid concentrations. The effects of pure EGCG on the parameters studied herein have not previously been studied, therefore is quite likely that previous studies (Dulloo *et al.*, 1999; Tsuneki *et al.*, 2004; Westerterp-Plantenga *et al.*, 2005) that have observed significant increases in EE and/or reductions in blood glucose, have done so through the action of other components of green tea. Frequently these include caffeine which is known to increase EE and lipolysis (Dulloo *et al.*, 1999; Westerterp-Plantenga *et al.*, 2005).

EGCG intake is safe and non-toxic and as is available as a white, odourless powder which could be used as an additive to many foodstuffs. If its effects on regulation of energy balance are established, it could potentially be a candidate for the status of a 'functional food' additive. However the present study has raised the caveat that when EGCG is dissected out from the biochemical milieu of its plant origin, much of the potency of expected effects seems to be lost. It remains the case however that EGCG appears capable of increasing insulin sensitivity as demonstrated by Tsuneki *et al* (2004) and demonstrates significant anti-oxidant capacity (Shi and Kakuda, 2006). It is probable that other important functional capacities will emerge through careful study of its action alone but also in combination with other compounds. Examples of nutritional compounds that 'fail to

deliver' when extracted and isolated are not uncommon, (e.g beta carotene and its anti-oxidant effects (Palozza et al., 2003)). Thus the challenge for nutritional scientists and biochemists is great, inasmuch as dose and duration and also precise compositional considerations need painstaking consideration in terms of study design and implementation.

Concluding Remarks

The studies described in this thesis are novel. The effect of oral ingestion of L-Histidine on parameters of energy balance has not been studied previously in humans. Equally the methodological development and application of a novel multi-compartment model of energy expenditure, measured in real time post nutritional intervention, has been realised. Such measurements have potential application in any investigation where energy expenditure and its differential and site specific expression are of research interest.

With respect to EGCG, the present data strongly suggest that as an isolate from *Camellia Sinensis*, this catechin may have physiological and clinical importance, but in which precise capacity remains unclear. The translation from epidemiological observation of dietary intake and related health status, to a reductionist biopharmacological model capable of clinical and/or public health application is clearly a lengthy and (frequently) iterative process.

Nutritional biochemistry and its potential clinical utility are in a relative state of infancy compared to traditional physiology and clinical chemistry. It is hoped that research such as is presented in the present document represents a step towards the inclusion and recognition of human nutritional science as a key theme in the human physical and health sciences.

6 References

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7 Appendix

Appendix 1 - Dietary supplements to aid in weight reduction, available without prescription

Described below are the representations of a few of the dietary supplements available to be purchased on the high street. The purported mechanism of action is described and quoted in the exact language of the manufacturers. Each supplement is also usually a combination of products and there is little evidence to support their efficacy in weight reduction

Brand name	Active compound	Recommended dosage	Purported action/mechanism*
Lipobind fat binder	Patented fibre complex from organic plant source	2-3 tablets immediately after each meal	Lipobind actively binds to fat as soon as they come into contact in the stomach, creating a fat-fibre. As the fat-fibre is formed, a gel surrounds it, creating a barrier from stomach acids and the break-down process. It is purported to decrease food cravings, suppresses the appetite and lowers blood cholesterol.
Formoline L112 Weight Management Tablets	β-1,4 polymer of D-glucosamine and N-acetyl-D-glucosamine from shells of crustaceans	2 tablets, twice daily	A naturally sourced fibre with an extremely high fat-binding capacity, which binds to (or adsorb) a substantial amount of dietary fats from food in the digestive tract. The active ingredient is not digestible and is excreted naturally, together with the bound fats, significantly reducing the calories your body can process from the food you have just eaten.
Slimthru® Natural Weight Loss Liquid	Fat emulsion (water 26.7%, fractionated palm oil 2%, fractionated oat oil)	7.5ml serving at breakfast and once at lunchtime	Provides greater satiety and less hunger feeling between meals.
Zotrim	Yerba Mate Guarana (contains caffeine) Damiana	1 tab tds	These ingredients are designed to slow the rate at which the stomach is emptied. It makes one feel full more quickly and therefore eat less. This may help develop a regime for better management of the amount of food consumed.
Adios	Butternut 20mg, Dandelion root 30mg,	1 tablet 3 or 4 times a day at	Adios help lose weight naturally by speeding up your body's metabolic rate and stimulating fat metabolism.

Brand name	Active compound	Recommended dosage	Purported action/mechanism*
	Dry extract boldo 4:1 34mg, Dry extract of fucus 5:1 45mg	mealtimes	
Adios Max	Contains 120mg dry fucus extract (the equivalent of 600mg fucus - a species of sea weed)	1 tablet tds	Adios Max contains a natural herbal active ingredient called fucus dry extract which derives from a type of algae. The fucus dry extract helps loose weight by speeding up the body's metabolism as long as it is taken along side a calorie controlled diet.
Lamberts Green Tea Extract 2750mg	250mg of catechins from whole green tea leaves and L-Theanine.	1-2 tablet with every meal	Increases metabolic rate. It is also a powerful antioxidant and free radical scavenger.
Maximuscle Weight Management System CLA - 1000	800mg of CLA, Extracted from high quality safflower oil, Contains natural levels of Capric acid	3- 6 caps daily, according to your requirements	Supports lean muscle growth and weight management goals, contains naturally occurring fatty acids.
Bioforce Helix slim tablets	Extract of Helianthus tuberosus (Jerusalem Artichoke), microcrystalline cellulose and hydrated cotton seed oil.	1 tablet, tds	Helianthus slows down the rate at which sugars are broken down in the body prolonging the digestion of food, slowing down hunger pangs. Better metabolism of sugars also balances out swings in blood sugar levels, reducing sugar cravings that come when blood sugar levels drop. Helianthus contains plant sugars that feed friendly bacteria in the gut, and these bacteria break down undigested food particles. As Helianthus also contains water soluble plant fibres, it helps to bulk up waste matters travelling through the colon and thus improves bowel regularity.
Dandelion 50ml	Tinctures of organically grown fresh leaves and roots of Taraxacum officinalis (Dandelion), extracted in alcohol (50% v/v).	5 drops in water, 3 times daily	Dandelion is an excellent cleansing agent, being one of the most effective detoxifying herbs. It possesses a wide range of active constituents and is also rich in minerals and nutrients. The root of the plant stimulates liver function. This has a primary use in improving digestion as a result of an increase in digestive juices. In addition, Dandelion leaves have diuretic and mild laxative properties.

Brand name	Active compound	Recommended dosage	Purported action/mechanism*
Lamberts chromium complex	100mg Vitamin C 5mg Thiamine 5mg Niacin 5mg Vitamin B6 300mg Magnesium 15mg Zinc 500µg Copper 200µg GTF Chromium	1 tablet daily with a meal	Chromium helps the body to use insulin, so helps the body maintain normal blood sugar levels. In people with insulin resistance (syndrome X) it may help prevent the onset of diabetes, by allowing the body to use insulin more effectively. Because chromium has an effect on blood sugar levels it can also relieve headaches, irritability and mood swings caused by low blood sugar levels. It is also important for the break down of fat and protein in the body. Because chromium helps to break down fats, it may also lower total blood cholesterol levels, reduce LDL cholesterol and increase HDL cholesterol.
Lamberts 5-HTP	L-5-Hydroxytryptophan 100mg (natural extract from the seeds of Griffonia simplicifolia)	3 tablets daily, half an hour before meals or half an hour before bedtime	5-HTP is converted, in the brain, into serotonin, which influences everything from appetite, to mood, to sleep.
Fat stripper	Choline 150mg Inositol 150mg Betaine 150mg Methionine 150mg L-carnitine 10mg Chromium Piccolinate 50µg	1-2 tablets 3 tds (just before meals)	Fat Stripper's unique fat burner formula contains L-Carnitine which is a very strong fat oxidiser and fat burner, great for weight loss and fat loss; this product removes unwanted fat from the body.
Nobese	Octopamine 100mg Pomegranate Extract 80mg L-tyrosine 50mg Guarana 50mg L-carnitine 50mg Green Tea Extract 50mg Ginger 10mg	1-2 tablets, tds, 15 minutes before meals	Nobese is a fat metaboliser. It breaks fat down. Nobese is a diuretic. Once fat has broken down, it accelerates its exit through the water channels. Nobese is also a thyrothermogenic formula in that it assists your body in coming out of its lazy state and kicks it into high gear. Nobese leaves absolutely nothing to chance. It will work for anyone from very fat individuals to people who want to get rid of love handles to people who just want to tone up.

Brand name	Active compound	Recommended dosage	Purported action/mechanism*
	Sea Vegetable Extract 30mg		
Shaper	100% Pure Pharmaceutical Grade Calcium Pyruvate 250mg	2-4 tablets, 2-4 times a day with a meal or a full glass of water	Two turns of the Krebs cycle metabolises each glycogen molecule (for energy). The faster the Krebs cycle can turn, the more energy you will have. Shaper will accelerate the turning of the Krebs cycle, meaning you will have more energy, safely and naturally. Shaper gives you more energy, less fat and better performance.
Anti fat	Betaine 75mg Cayenne 5mg Choline 75mg Chromium Piccolinate 50µg Cinnamon Powder 75mg Inositol 75mg Methionine 75mg Mustard Seed Powder 75mgs	1-2 tablets, tds just 10-15 minutes before meals	Anti-Fat is a super-strong fat metaboliser and a thermogenic agent all in one! This product is similar to LA Muscle's FatStripper in that it is a fat "metaboliser". Anti-Fat contains many of the ingredients in FatStripper at a smaller dosage. Anti-Fat also contains 3 unique and extremely effective fat-burning herbs.
Sida Cordifolia by Reflex	White Willow Bark (Salicin (Aspirin)) 100mg Sida Cordifolia 125mg Guarana (Caffeine) 450mg	2 caps, tds	A thermogenic fat burner. This product may suppress appetite, help burn body fat, and increase energy levels.
Xenadrine EFX	Vitamin C 100 mg Vitamin B6 10 mg Pantothenic Acid 12mg Magnesium 10 mg Proprietary Thermodyne Complex 1415 mg Tyroplex (proprietary blend of l-tyrosine and	2 caps, tds	It is the first product ever to be powered by an ultra-potent blend of natural, clinically proven metabolic compounds that work synergistically to produce unprecedented fat-burning results - without containing ephedrine. It has an ability to increase metabolism and promote the fastest possible fat-loss, muscle-sparing results. Xenadrine-EFX is much more effective at promoting rapid fat loss because it directly targets a multitude of key metabolic pathways, simultaneously. And on the way, this one-of-a-kind supplement also greatly enhances mental focus and acuity, increases energy, suppresses

Brand name	Active compound	Recommended dosage	Purported action/mechanism*
	acetyl-l-tyrosine), Green Tea Extract (standardized for EGCG and caffeine), Seropro (proprietary cocoa extract standardized for PEA (phenylethylamine), tyramine and theobromine), Yerba Mate (standardized for caffeine), di-Methionine, Ginger Root (standardized for gingerols), Isotherm (proprietary blend of quercetin and fisetin), Bitter Orange (standardized for synephrine), DMAE (2-dimethylaminoethanol), Grape Seed Extract.		appetite and provides valuable antioxidant activity, all at the same time.
Potter's Femmeherb slim aid	Dry Extract Boldo (5:1 concentrate) 27mg (Equivalent to 135mg Boldo, containing 0.5% alkaloids) Butternut Bark 10mg Dandelion Root 30mg Dry Extract Fucus (5:1 concentrate) 45mg	1-2 tabs, tds	Slim Aid is a traditional herbal remedy used as an aid to slimming. Fucus (seaweed) is a mild thyroid stimulant so helping to increase your metabolic rate. Boldo and Dandelion support liver function which is a vital organ in the digestive process. Butternut acts as a mild laxative helping you to gently remove waste products from the body.

Brand name	Active compound	Recommended dosage	Purported action/mechanism*
	(Equivalent to 225mg Fucus)		
Lean system 7	Green Tea leaf extract 300mg Yerba mate 500mg Guarana 700mg Chromium 200µg Dandelion 250mg Bioperine 5mg	3 capsules, twice a day, 30 minutes prior to meals and/or exercise.	Lean System 7 is a dietary supplement to promote safe, effective fat loss (not to be confused with weight loss, which is normally mostly water and muscle loss). Lean System 7 provides a precise synergistic blend of seven powerful "fat-fighting" ingredients that have been shown in recent studies to help you get and stay lean - safely and effectively, without the use of harmful stimulants (like ephedrine).
Eat and cheat	100% Pharmaceutical Grade Chitosan 250mg	4 tablets, 30 minutes before fatty foods	Eat & Cheat is a great supplement for anyone who wants to eat the occasional fatty food without getting fat. Eat & Cheat contains an exclusive active ingredient which binds to fat and removes it from your body before it gets stuck.
Nordic BioFirm	Chicory root (radix chichorii), 2000 mg, Citrus pulp, 780 mg, Nettle (urtica dioica), 740 mg, Dandelion (taraxacum officinalis), 600 mg, Citrus fibres (biopectin®), 600 mg, Psyllium (semen isphagula), 600 mg, Asparagus (asparagus officinalis), 510 mg, Globe artichoke (cynara scolymus), 444 mg, Celery seed (apium graveolens), 300 mg, Thyme (thymus vulgaris),	2 tablets, tds	Nordic BioFirm has developed a special form of pectin. This important ingredient ensures that there is a good supply of nutrients to the skin and helps to reduce the waste and fluid stored between the cells in the tissues. Therefore, BioFirm also helps to reduce cellulite and at the same time strengthens the connective tissue. BioFirm formula cleanses the body, firms the skin and tones the system.

Brand name	Active compound	Recommended dosage	Purported action/mechanism*
	125 mg		

*tds- three times a day; *Explanation of purported mechanism taken from description given with the supplement (their own words); Data obtained from internet searches from www.expresschemist.co.uk/category_1762_weightloss.html & http://www.boots.com/onlineexperience/flexible_template_2006.jsp?classificationid=1045793*

Appendix 2 -Ethics application for L-Histidine study**University of Westminster
Ethics Committee**

Application for Approval of a Proposed Investigation, Demonstration, Research or Experiment

Section 1**To be completed by all applicants****1.1 PROJECT TITLE:**

Effects on blood sugar dysregulation of resistance exercise and macro and micro nutrient manipulation.

1.2 APPLICANT DETAILS

Name: Varsha Rajashekar	Email: V.Rajashekar01@wmin.ac.uk
Address: B09-4, Alexander Fleming Halls of Residence, University of Westminster, 3 Hoxton Market, London N1 6HG	Telephone Number: 07774516248
Please tick relevant box: <input type="checkbox"/> Undergraduate <input type="checkbox"/> Postgraduate <input checked="" type="checkbox"/> PhD Student <input type="checkbox"/> Staff	

Section 2**To be completed when applicable. Please note all applicants with a supervisor(s)
must ensure the supervisor signs the declaration.****2.1 SUPERVISOR DETAILS**

Name: Dr. Adam Cunliffe	Email: A.Cunliffe@westminster.ac.uk
School/Department: School of Biosciences	Telephone Number: 020-7911-5000 ext 3580
Declaration: In accordance with the University's Code of Practice Governing the Ethical Conduct of Investigations, Demonstrations, Research and Experiments I agree that the applicant named in 1.2 above should submit their proposal to the Ethics Committee for consideration.	
Signed:	Date:

Section 3

3.1 Does your work relate to any of the following areas? Please tick box:

Human Participants in Health and Community settings

Work with prescription drugs

Work involving foetal tissue

Drug Studies on human participants

Pre marketing drug trials

3.2 Are you proposing work using participants from any of the following categories:

Prisoners sectioned under the Mental Health Act

Prisoners or arrestees

Persons with severe mental illness

Persons with learning difficulties or brain damage

Persons with a reduced level of consciousness

3.3 Any work where a qualified clinical person is required to:

Be responsible for all work carried out

Be in attendance when certain procedures are carried out

Ensure that facilities for emergency medical care are at hand

If you have ticked one or more boxes in Section 3, please contact your supervisor and discuss sending your proposal for external approval. More information can be found at: <http://intranet.wmin.ac.uk/academicregistrars/staffinfo/ethics/Default.htm>

In addition please complete this form up to section 3, sign the final declaration in section 12 and send it to: Matt Such, Assistant Registrar (Student Information), Academic Registrar's Department 9-18 Euston Centre, London, NW1 3ET.

If you have not ticked any boxes in section 3 please continue to section 4

Section 4**4.1 Is your work related to any of the following areas? Please tick relevant box:**

- Any work involving patients
- Non clinical work involving bodily fluids
- Administering of a non food substance
- Work with children
- Deception of Participants
- Data not already in the public domain that bears on the issues of criminality
- Work which involves participants to reveal medical history

If you have ticked one or more boxes in Section 4, your proposal will need approval from the University Ethics Committee please continue to fill out the rest of this form giving as much detail as possible Proceed to Section 5. Send the completed form to: Matt Such, Assistant Registrar (Student Information), Academic Registrar's Department 9-18 Euston Centre, London, NW1 3ET.

If you have not ticked any boxes in Section 3 or 4 does not need approval. Please consult the University of Westminster Code of Practice governing the Ethical Conduct of Investigations, Demonstrations, Research and Experiments and consult with your supervisor.

Section 5

5.1 Please provide a brief description of your proposed work below:

In the year 2000, the estimated figures of people suffering from diabetes worldwide were 171,000,000 and the estimated figures for the year 2030 is 366,000,000 (http://www.who.int/diabetes/facts/world_figures/en/index.html).

Several pathogenetic processes are involved in the development of diabetes. These include processes that destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin.

Treatment of DM2

The primary concern in the control of DM2 is the reduction of fasting hyperglycaemia, which accounts for most of the secondary complications. Behaviour modification coupled with pharmaceutical intervention can both delay the onset of diabetes, as well as ameliorate the disease state. Obesity is primary risk factor for DM2, and diet restrictions coupled with moderate exercise has been proven significantly to enhance insulin sensitivity in both insulin-resistant and diabetic patients. However, these treatments only slow the progression to frank diabetes, and do not fully restore patients to normal plasma glucose and insulin levels (Saltiel 1996).

Several drugs are also currently used to improve glucose metabolism in DM2 sufferers. Insulin secretagogues, such as sulfonylureas, are used to increase insulin release and reduce plasma glucose levels. Metformin and thiazolidinediones are also being used in the treatment. However, none of these treatments individually cures DM2 (and are associated with side effects, anorexia, nausea, vomiting) diarrhoea although a combination of behaviour and drug therapies has given new hope that the disease can be controlled (Brady, J *et al.* August 1999).

The uptake and oxidation of fatty acids is diminished in skeletal muscle of type 2 diabetic subjects during the post absorptive state and during β -adrenergic stimulation. A lowered capacity of skeletal muscle to take up or oxidise plasma fatty acids may divert fatty acids toward increased storage in skeletal muscle, which is strongly linked to insulin resistance or may divert fatty acids toward other tissues like adipose tissue (promoting large adipose tissue stores) or liver (increasing VLDL and glucose output) (Blaak, van Aggel-Leijssen *et al.* 2000).

Generally, plasma FFA oxidation provides the majority of the energy needs during low-intensity exercise [$<30\%$ of maximal oxygen uptake capacity (O_2 max)]. Total fat oxidation has been shown to increase with the duration of exercise. The latter has been attributed to a progressive increase in peripheral lipolytic rate with concomitant increases in plasma FFA, R_a , R_d , and/or R_{ox} . There is evidence to show that peripheral lipolysis was high during low intensity exercise (25% VO_2 max) and did not increase further with more intense exercise (Romijn, Coyle *et al.*, 1993).

Resistance training (which can be classified as low to moderate intensity exercise) may induce beneficial changes in insulin sensitivity via muscle mass development,

effectively increasing glucose storage, facilitating glucose clearance from the circulation, and reducing the amount of insulin required to maintain a normal glucose tolerance (Cuff, Meneilly *et al.*, 2003).

Dietary intervention and fat oxidation:

Fat and carbohydrate are the principal substrates that fuel aerobic ATP synthesis in skeletal muscle (van Loon 2004). FA levels are strong predictors of muscle insulin resistance. Muscle fat content is increased in obesity and more so in type 2 diabetes. FFA levels are increased in most obese individuals and acute elevations in FFAs cause insulin resistance in muscle and the liver (Bloomgarden 2003). It has been argued that a shift to an increased oxidation of fat and decreased CHO use is desirable during exercise.

There is adequate data to suggest that increase in fat availability and fat oxidation decreases CHO oxidation during exercise (Helge, Watt *et al.* 2001). Increasing fat availability may increase NADH and buffer the fall in the cellular energy charge, resulting in reduced glycogenolysis and PDH (pyruvate dehydrogenase) activation (Cuff, Meneilly *et al.* 2003; Spriet and Watt 2003; Jacobs KA, Paul DR *et al.* 2004). Therefore a high fat diet, hypocaloric diet can bring about increased fat oxidation which should have favourable consequences on insulin sensitivity as well as body weight (Kim, Park *et al.* 1999; Spriet and Watt 2003) and blood lipids (Cha, Sohn *et al.* 1999; Helge, Watt *et al.* 2001).

Novel interventions:

Drug treatments for hyperglycaemia and hyperlipidaemic states are costly and not without side effects. There is therefore ongoing interest in developing non-pharmacologic nutritional interventions, which additionally may have low associated costs to healthcare providers. Following extensive literature research in this area, two potentially efficacious dietary agents have been identified and these are introduced below.

Histidine and histidine containing compounds supplementation:

L- Histidine is one such essential amino acid which is a precursor of neuronal histamine. Recent study findings indicate that L- histidine suppresses food intake through its conversion into histamine in the hypothalamus, which in turn interacts with leptin and key neuropeptides involved in the hunger – satiety cycle (Yoshimatsu, Chiba *et al.* 2002). A study conducted at the Penn state University on birds to test the appetite- suppressant effect of excessive dietary amino acids in reducing feed intake and, in turn, restricting the early rapid growth to minimize metabolic disorders, showed that high levels of methionine and histidine caused the greatest depression in appetite and that it is possible to use excessive individual amino acids in diets to suppress the appetite and early rapid growth to alleviate or minimize metabolic disorders (Acar, Patterson *et al.* 2001).

There is also evidence to indicate that histidine accelerates lipolysis in white adipose tissue through activation of the sympathetic nervous system. The regulation of lipolysis appears to involve histamine neurons in the brain, probably through the

conversion of L-Histidine to histamine in the hypothalamus (Yoshimatsu, Tsuda *et al.* 2002).

A further study examined the acute and long-term effects of cyclo (his-pro) (CHP) plus zinc and L-Histidine treatment on glucose metabolism in genetically obese (ob/ob), type 2 diabetic mice. L - Histidine was effective in decreasing blood glucose concentrations in genetically obese (ob/ob), type 2 diabetic mice. These data support the hypothesis that histidine may be an important anti-hyperglycaemic agent (Hwang, Go *et al.* 2003).

Histidine is considered an essential amino acid for children and conditionally essential amino acids for adults. This means that the body cannot produce (or produce sufficient) histidine, and it must be obtained from a dietary source. In children and under certain stress conditions the body's machinery is unable to generate adequate levels and supplemental dietary sources may be required in adults. If obesity is viewed as a (metabolic) stress state, a clearer understanding of histidine's effects as a dietary supplement is highly desirable. Histidine as a food supplement is not a drug, has not been associated with any toxicity, and is available for purchase in the high street without prescription in the UK and USA.

Cordyceps sinensis supplementation:

Cordyceps sinensis is yet another nutrient which is now under a lot of research worldwide. It is a fungus highly valued in China as a tonic food and herbal medicine (Zhu, Halpern *et al.* 1998). Cs-4 is a standardized mycelial fermentation product of Cordyceps sinensis, a fungus that has been used for various pharmacologic, metabolic, and ergogenic purposes. One study show that CordyMax Cs-4 is effective in lowering basal blood glucose and plasma insulin, improving glucose metabolism by enhancing insulin sensitivity, and improving oral glucose tolerance (Zhao, Yin *et al.* 2002). Another study using a polysaccharide (CS-F30) obtained from the cultural mycelium of Cordyceps sinensis showed potent hypoglycaemic activity in genetic diabetic mice after intraperitoneal administration, and the plasma glucose level was quickly reduced in normal and streptozotocin-induced diabetic mice after intravenous administration. Administration of CS-F-30 to normal mice significantly increased the activities of hepatic glucokinase, hexokinase and glucose-6-phosphate dehydrogenase, although the glycogen content in the liver was reduced. Furthermore, CS-F30 lowered the plasma triglyceride level and cholesterol level in mice (Kiho, Yamane *et al.* 1996; Zhu JS 1998).

Cordyceps is a safe, traditional Chinese herb. Investigators in numerous studies consider Cordyceps and its mycelial fermentation products very safe during clinical use (Zhu JS 1998). The present author has found no report of cordyceps toxicity in the literature to date.

In view of the above theories of histidine being an appetite suppressor and an anti-hyperglycaemic agent and the effect of cordyceps sinensis in enhancing insulin sensitivity, the study to check their supplementation in the diets of type II diabetic subjects is warranted. This kind of study has not been conducted previously and the proposed study will be one among the pioneering works done in this area.

Based on the above theories and results obtained, this study will provide non-pharmacological and relatively inexpensive preventive measures to help maintain a euglycaemic condition.

5.2 What are the specific aims of the work you plan to carry out?

1. To study the differences in rate of fat oxidation during and after different duration of resistance exercise.
2. To study the change in fat oxidation by altering the macronutrient (fat) intake.
3. To study the effect of L-Histidine and histidine containing compounds and Cordyceps Sinensis supplementation on energy expenditure, and blood sugar levels.

5.3 Please outline the design and methodology of your work

Aim:

To study the effects of resistance exercise and macro and micro nutrient manipulation on blood sugar regulation and energy expenditure.

Subject recruitment:

Initial investigations will be carried out in normal healthy volunteers, recruited from colleagues and students within the University of Westminster. Depending on the findings of these preliminary studies, further investigations will be carried out in populations with blood sugar dysregulation. Recruitment of such subjects will be of males and females with elevated fasting blood glucose and achieved via posters in all campuses of the university of Westminster, by advertising in daily papers (Evening Standard and Metro - this is a free service for researchers) and by referral from collaborating bodies (Royal London Hospital, Marylebone Health Centre, Weight Concern). The criteria for recruitment will also include BMI values of 26 and above, 25 years and above of age.

In addition, using questionnaires (e.g. PAR-Q, fitness for exercise screening tool) and different measurement techniques (e.g. blood pressure, cholesterol), subjects will be assessed for any health related issues, which might make them unsuitable as test subjects (chest pains, respiratory difficulties, heart disease, thyroid disease, orthopedic problems). Participants will be excluded if they are taking medications, which are expected to alter the results of the study (including medication that would affect metabolism or weight loss (psychiatric medication, appetite suppressants, and thyroid

medication). Furthermore, subjects who are obese due to a confounding diagnosed disease e.g. Cushing's syndrome will not be recruited. Any gross deviation from expected metabolic rate will quickly be identified by the study and appropriate referral made.

The number of subjects required will be determined by sample size calculations once pilot studies have been carried out to determine the size of significant differences in energy expenditure and metabolic responses that may be observed after ingestion of specific macronutrients and after exercise of different intensity and duration. For the study to have a 5% significance level (α , two sided), and 80% power (1- β) the sample size will be calculated. The standardised difference (sD) will be calculated as the physiologically significant difference (PSD) divided by the standard deviations of the measures in question, which is energy expenditure and substrate oxidation.

Plan of investigation:

Phase I: Assign the subjects randomly into two groups, control group (CG) and experiment group (EG). Develop a protocol of resistance exercise of differing duration as well as devise a high fat diet for the EG and check the effects of the exercise and diet as against the control group. The daily energy intake will be assessed and individual energy intake will be determined by calculating the energy needs. The diet developed for the EG will be high in fat content but also will be hypocaloric in nature reflecting the desired reduction in weight. The fat percentage would be 50% of the total energy intake. Of this intake, unsaturated fatty acids will equal to 70-80%, equally distributed between poly and monounsaturated fatty acids. (McArdle, Katch *et al.* 2001)

Phase II: Supplement L-Histidine in the EG and check its effect as an appetite suppressant, in blood sugar regulation and energy expenditure. The dosage supplemented will be 25mg/kg body weight.

Phase III: Supplement Cordyceps Sinensis in the EG group and check its effects on blood sugar levels and energy expenditure. The dosage for supplementation will be 2-3 g/day.

Methodology:

Determination of body weight (digital balance), height (stadiometer) and body composition (plethysmography, BOD-POD[®]) (Elia and Ward 1999; Miyatake, Nonaka *et al.*, 1999; Fields and Goran 2000), changes in abdominal, subcutaneous fat by anthropometry.

Assessment of oxygen consumption, post prandial thermogenesis (PPT) and fat/ carbohydrate oxidation (respiratory quotient, RQ) using metabolic monitors (Deltatrac II, Oxycon Delta) (Dabbech, Boulier *et al.*, 1996; Marques-Lopes *et al.*, 2001), body fat percentage calculated using the equations of Siri., 1956).

Blood samples for measurement of glucose (Accutrend), insulin, leptin, plasma lipids (multi-assay photometry), histidine, (Van Wymelbeke, Louis-Sylvestre *et al.*, 2001; Kousta *et al.*, 2002; Marques-Lopes *et al.*, 2003; Raben *et al.*, 2003)

Dietary recall for assessment of habitual diet.

Total fat oxidation = $1.67 \times \text{VO}_2 - 1.67 \times \text{VCO}_2$ with VCO_2 and VO_2 in litres per minute. The value 1.67 is derived from the volumes of oxygen consumed and carbon dioxide produced in oxidation of 1 g of fat. Because no estimation of protein oxidation is included in this calculation of fat oxidation, fat oxidation will be over

estimated. However, because fat oxidation is compared before and after exercise training, this over estimation will not influence the outcome (Van Aggel-Leijssen, Saris *et al.*, 2002).

Data analysis:

Analysis of data obtained will be carried out. It is envisaged that the general linear model of the analysis of variance will be used to assess the effects of exercise and its duration, macronutrient content of meal on variation in fat oxidation, effect of micronutrient supplementation i.e. L-Histidine and Cordyceps Sinensis on energy expenditure, substrate oxidation and blood sugar levels. These data will reveal which of the tested combinations of exercise and macro and micronutrient intake are most effective with respect to the parameters tested.

5.4

Start date: January 1 2005

Estimated duration of work: 24 months

5.5 If your work is a multi-centered study, please provide details of any other organisations involved

Contact Name	Contact Name:
Address	Address:
Telephone	Telephone:

Please attach a copy of any agreement between organisations

Section 6

6.1 Describe any potential physical/ emotional discomforts to participants in the investigation:

A momentary discomfort whilst taking blood samples (a qualified phlebotomist will be taking the blood samples).

Possible risk of slight bruising at site of venepuncture for up to 24 hours.

Possible discomfort because of slight muscle soreness after exercise training in

untrained subjects. This passes within a day or two and does not persist if training continues.

6.2 Aside from 6.1 above, describe potential hazards that may be suffered by the participants? Please give details of any measures taken eg. COSH, Risk Assessment etc

COSHH form submitted

6.3 Outline the degree to which these risks are balanced against potential benefits

There should be no risks. Subjects will be monitored at all times during testing. A qualified first aider will be present during the initial 3 sessions. Resistance exercise selected will be light, easy and without risk.

6.4 What criteria will be employed for deciding the end point at which:

- a) The investigation will stop because of unjustifiable further risks?**
- b) One method is declared the preferred option and the investigation terminates?**

Testing will be stopped immediately on request from subject. No risk should arise from the planned test procedures.

6.5 What monitoring mechanisms will be in place to decide when participants should be withdrawn from the research?

Testing will be stopped immediately on request from subject.

6.6 What procedures and subsequent observations are to be made on participants for the purpose of detecting any complication arising from the investigation?

No complications envisaged.

6.7 Do participants have any previous or existing professional relationship with the investigator?

Yes.

If yes, please explain the circumstances:

Colleagues, peers, students.

Section 7

Consent of Applicants

7.1 What type of consent will you seek?

Written (including email)

Verbal Only

Not applicable (Please give justification below as to why consent is not applicable)

7.2 How and where will you make contact with the participant in order to obtain consent?

Contact with the subjects will be made by advertising the study via posters in all campuses of the university of Westminster, the London area in general, by advertising in daily papers (Evening Standard and Metro (this is a free service for researchers)) and by advertising in the premises of the collaborating bodies.

7.3 Is there a subject information sheet?

Yes (Please enclose a copy with this application)

No

7.4 Is parental consent required?

Yes

No

Section 8**Confidentiality of Information****8.1 Will the sharing of information be communicated to others working on the project?**

Yes (Please attach a copy of the Participant Confidentiality Code of Practice which will be used)

No (apart from project supervisors who are full time lectures in the school of Biosciences)

8.2 Will the work include:

named participants

participants whose names have been separately coded

unnamed participants

8.3 Where will locked files of investigation materials be stored?

In the human performance laboratory at the University of Westminster, 115 New Cavendish Street, London W1W 6UW.

8.4 If the investigation involves storage of computerised data which might enable the participant to be identified, please name the investigator in charge of Computer Systems Security for the investigation.

Varsha Rajashekar

8.5 Does the investigation and any planned publication include the use of photographs or videos either of individuals or of tissues?

Tissues Yes No

Individuals: Yes No

If yes to either of these, please provide a copy of the consent form which participants will be asked to sign for this purpose.

Section 9

Finance

9.1 Will expenses be paid to participants?

Yes (Travel expenses will be paid)

No

9.2 Will a reward over and above expenses be made to participants?

Yes (If yes, please give more details)

No

9.3 Is this study initiated/sponsored by a pharmaceutical or other industrial company?

Yes (If yes, what is the name of the company?)

No

9.4 Detail any financial or other direct interest to you or to your department arising from this study.

None

9.5 Will this project increase work/cost to any other Department or School?

Yes (If yes, obtain and include the name and signature of the relevant Heads of School(s) concerned:

Name	Signature

No

Section 10
Insurance

6 (i) Are manufacturers providing insurance cover?

Yes No

If yes, please enclose a letter confirming insurance cover, including the names of all covered.

6 (ii) Are **all** of the investigators employees or student s of the University of Westminster?

Yes No

If no, please provide evidence of insurance cover, including:

- a) List of all people involved in the investigation;
- b) Details of the form this cover will take.

6 (iii) Does the investigation involve the use of equipment or medicines?

Yes No

If yes, please details of *manufacturer's indemnity*.

6 (IV) Does the investigations involve the use of equipment or medicines which are manufactured on site but are not covered by insurance?

Yes No

If yes, appropriate insurance cover must be arranged and written confirmation of such cover must be attached

Section 11

Declaration

To be completed by all applicants

Please Read and Sign: The information I have given on this form is true and to the best of my knowledge correct:

Signed:

Date:

Send the completed form to: Matt Such, Assistant Registrar (Student Information), Academic Registrar's Department 9-18 Euston Centre, London, NW1 3ET

Ms. Varsha Rajashekar
B09-4 Alexander Fleming Halls of Residence
3 Hoxton Market
London
N1 6HG

Direct Line 020 7 911 5000 ex 3933
Direct Fax 020 7 911 5118
Email kellyh@wmin.ac.uk
Our Ref 04/05-62
Date 7 June 2005

Dear Ms. Rajashekar

University Ethics Committee – Application No. 04/05-62

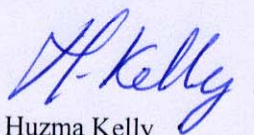
'Effects on blood sugar dysregulation of resistance exercise and macro and micro nutrient manipulation'.

I am pleased to inform you that following receipt of the information requested from you, your Application has been approved.

I am advised by the Committee to remind you of the following points:

1. Your responsibility to notify the University Ethics Committee immediately of any information received by you, or of which you become aware, which would cast doubt upon, or alter, any information contained in the original application, or a later amendment, submitted to the University Ethics Committee and/or which would raise questions about the safety and/or continued conduct of the research.
2. The need to comply with the Data Protection Act 1998
3. The need to comply, throughout the conduct of the study, with good research practice standards
4. The need to refer proposed amendments to the protocol to the University Ethics Committee for further review and to obtain University Ethics Committee approval thereto prior to implementation (except only in cases of emergency when the welfare of the subject is paramount).
5. You are authorised to present this University of Westminster Ethics Committee letter of approval to outside bodies, e.g. LRECs, in support of any application for further research clearance.
6. The requirement to furnish the University Ethics Committee with details of the conclusion and outcome of the project, and to inform the University Ethics Committee should the research be discontinued. The Committee would prefer a concise summary of the conclusion and outcome of the project, which would fit no more than one side of A4 paper, please.
7. The desirability of including full details of the consent form in an appendix to your research, and of addressing specifically ethical issues in your methodological discussion.

On behalf of the Committee may I wish you success in your project.
Yours sincerely


Huzma Kelly
Secretary
University Ethics Committee

cc. Adam
Cunliffe



ACADEMIC REGISTRAR'S DEPARTMENT

Educating for professional life 115 New Cavendish Street London W1W 6UW
www.wmin.ac.uk Tel 020 7911 5000 Fax 020 7911 5118

Appendix 3 -Written Consent Form for L-Histidine study

Title of Investigation: To study the effect of dietary supplementation of an amino acid on energy expenditure and blood sugar levels.

Volunteer's Name: _____

Address: _____

Tel: _____

Email: _____

Date of Birth: _____

Gender: M/ F

I have read the attached information on the research in which I have been asked to participate and have been given a copy to keep. I have had the opportunity to discuss the details and ask questions about this information.

The investigator has explained the nature and purpose of the research and I believe that I understand what is being proposed. I have been informed what the proposed study involves.

I understand that my personal involvement and my personal data from this trial will remain strictly confidential. Only researchers involved in the investigation will have access.

I hereby fully and freely consent to participate in the study, which has been fully explained to me. I understand that I am free to withdraw from testing at any time.

Volunteer's Signature:

Date:.....

As the investigator responsible for this investigation, I confirm that I have explained to the participant named above, the nature and purpose of the research to be undertaken.

Investigator's Name: Varsha Rajashekar **Signature:**

.....

If you have any questions about this, please contact:

Varsha Rajashekar on 020 7911 5000 ext 2830 or

Dr. Adam Cunliffe on 020 7911 5000 ext 3580

Appendix 4 -Medical screening form

UNIVERSITY OF WESTMINSTER



Medical screening questionnaire

Date: _____

This form is used as a pre-participation health and risk factor screening device and should be completed **prior to the commencement of test.**

The information obtained in this medical assessment will be kept as **CONFIDENTIAL**. Only the staff member related to the exercise test may have access to the information.

Surname: _____

Given names: _____

Date of birth: _____

Address: _____

Postcode: _____

Contact Telephone: _____ (Home) _____ (Work)

(1) FAMILY MEDICAL HISTORY

Has any near relative brother (B), sister (S), father (F), mother (M), grandparents (GP) suffered:

Please tick the appropriate column:

	No	Yes	Relation	Age	Details
Apoplexy (stroke)					
Congenital heart trouble					
Rheumatic heart disease					
Heart operation					
Angina					
Heart attack					
Sudden death					
High blood pressure					
High cholesterol					
“Hardening of arteries”					
Asthma					
Lung disorder					
Bronchitis, emphysema					
Hay fever					
Diabetes					
Gout					
Arthritis					
Epilepsy					

(2) PAST MEDICAL HISTORY

Have you suffered any of the following conditions at any time?

Please tick the appropriate column:

	No	Yes	Details
Rheumatic or scarlet fever			
Heart trouble or murmur			
Heart palpitation			
High blood pressure			
Heart attack			
Chest pain/ Angina			
Stroke			
Disease of arteries or veins			
Undue limiting shortness of breath with exercise			
Fainting or blackout			
Loss of consciousness or fainting with exercise			
Epilepsy			
Lung or bronchial disease			
Asthma			
Hay fever			
Anaemia			
Diabetes			
Thyroid disease			
Arthritis, rheumatism or gout spondylitis, disc			

trouble or back injury			
Serious accident or injury			
Surgical operation			
Congenital abnormality			
Other serious illness (or conditions that may affect exercise)			
For female only: normal/ regular periods			

(3) PRESENT MEDICAL CONDITION

Are you currently suffering or have you in the recent past suffered any of the following conditions?

Please tick the appropriate column:

	Immediately prior to the test	
	Yes	No
Cough		
Stuffy nose or sore throat		
Tonsillitis, glandular fever		
Hepatitis		
Diarrhoea/ vomiting		
Headaches		
Shortness of breath		
Pain in chest, left arm or neck at rest, or during physical activities		
Heart palpitations		
Cramp in legs		
Abnormal loss of blood		
Insomnia		
Indigestion or constipation		
Swollen, stiff or painful joints		
Backache		
Sports injury or other injury		
Other symptom or illness, or surgery		
Any deterioration in training or competitive performance		
Any other conditions that may contraindicate to exercise or affect exercise capacity		
For female only: currently in pregnancy		
If yes, provide details:		

(4) CURRENT MEDICATION

This section should also be checked **IMMEDIATELY PRIOR TO THE TEST**. State the name and dosage of any drugs or medicines that you are taking regularly:

Drug	Dose	Time of last dose

VOLUNTEER' SIGNATURE:.....

Thank you for completing this questionnaire.

Appendix 5 - Participant information sheet – Acute L-Histidine study

Title of Investigation: To study the effect of a dietary supplement on energy expenditure and blood sugar concentrations

Dear Participant,

We invite you to participate in an investigation that we believe to be of potential importance. In order to help you to understand what the investigation is about, we are providing you with the following information. Be sure you understand it before you agree to participate. Please do ask any questions you have about the information, which follows. We will do our best to explain and to provide any further information you require.

This study is examining the effects of a particular dietary supplement on blood sugar levels and energy expenditure. The proposed study hopes to address and hopefully clarify the best strategies for use of new non-drug therapies in overweight subjects and those with blood sugar control problems.

We are interested to find out what a particular dietary supplement may have on your energy expenditure (calories burned per day) and blood glucose levels when ingested over a short period of time. In order to do this we will give you a dietary supplement dissolved in 150mls of water to take over a 15 minute period. Some volunteers will be given the ‘active’ supplement and some will be given dummy (placebo) supplement. You will not know which is which. All supplements will contain only food ingredients and there is no known risk involved.

As a subject you will be required to come in to the lab for **2 sessions over a period of 2 weeks**.

Session 1

The first session involves measuring height, weight, your resting metabolic rate (RMR) and skin and core body temperature.

The measurement of RMR will involve you lying on a bed for 45 minute period with breaks of 30 minutes, with a clear perspex hood over your head. You should be in a complete state of rest, but not asleep. To encourage this you will be supplied with a set of headphones with calming music playing, to block out any external stimuli. At the same time, you will be measured for the skin and core temperature. This is done by placing four small sensors in various places on your skin i.e. on the chest bone (sternum), forearm, thigh and calf. You will also be needed to insert a 10 cm flexible rectal probe in order to measure the core temperature.

All equipment is designed to be comfortable during testing and you will be able to stop the testing by request at anytime.

During this preliminary session you will also be made familiar with the various procedures to be followed in the subsequent sessions and this first session will take approximately 4.5 hours of your time.

PLEASE REMEMBER: You should not be under the influence of drugs, HUNGER or FULLNESS for this first session!!!

Session 2:

You will be given a different supplement to take and measurements as in session 1 will be performed.

Risks:

1. Air is drawn into the Perspex hood of the metabolic monitor through a valve in the loop of the hood. Should this valve become blocked- a problem that has NEVER occurred –an alarm will sound. Since a researcher will be in the room at all times whilst you are under the hood, there would be immediate assistance available.

2. To ensure your safety whilst participating in this study we will ask you to complete a health questionnaire, which will give us an indication of whether it is safe for you to participate in such activity.

Note: A qualified first aider will be present during all test sessions.

CONFIDENTIALITY:

Your confidentiality will be protected, both in terms of your personal involvement and your particular data from the study. Your name will not be associated with any of your data that will be used for subsequent analysis.

You are free not to participate and may withdraw from the study at any time without any obligation.

Understand that in the event of injury caused by the product defect of a manufacturer, you will be compensated. In the unlikely event of injury due to negligence, claims must be pursued through legal action.

General information on patient's rights, particularly as regards participation in research studies may also be obtained from your local Community Health Council.

Thank you very much for your interest in the study.

If you have any questions about this, please contact:

Varsha Rajashekar on 020 7911 5000 ext 3895 or

Dr. Adam Cunliffe on 020 7911 5000 ext 3580

Appendix 6 -Participant information sheet – 30 day L-Histidine study

Title of Investigation: To study the effect of a dietary supplement on energy expenditure and blood sugar concentrations

Dear Participant,

We invite you to participate in an investigation that we believe to be of potential importance. In order to help you to understand what the investigation is about, we are providing you with the following information. Be sure you understand it before you agree to participate. Please do ask any questions you have about the information, which follows. We will do our best to explain and to provide any further information you require.

This study is examining the effects of a particular dietary supplement on blood sugar levels and energy expenditure. The proposed study hopes to address and hopefully clarify the best strategies for use of new non-drug therapies in overweight subjects and those with blood sugar control problems.

We are interested to find out what effect a particular dietary supplement may have on your energy expenditure (calories burned per day) and blood glucose levels. In order to do this we will give you some supplement capsules to take over a 10-day period and instructions on how and when to take them. Volunteers will be given both the ‘active’ capsules and dummy (placebo) capsules randomly. You will not know which is which. All the capsules will contain only food supplements and there is no known risk involved.

As a subject you will be required to come in to the lab for **3 sessions over a period of 4 weeks**.

Session 1

The first session involves measuring height and weight, determining your body composition, your resting metabolic rate (RMR), measuring your skin and core temperature, total cholesterol and blood glucose.

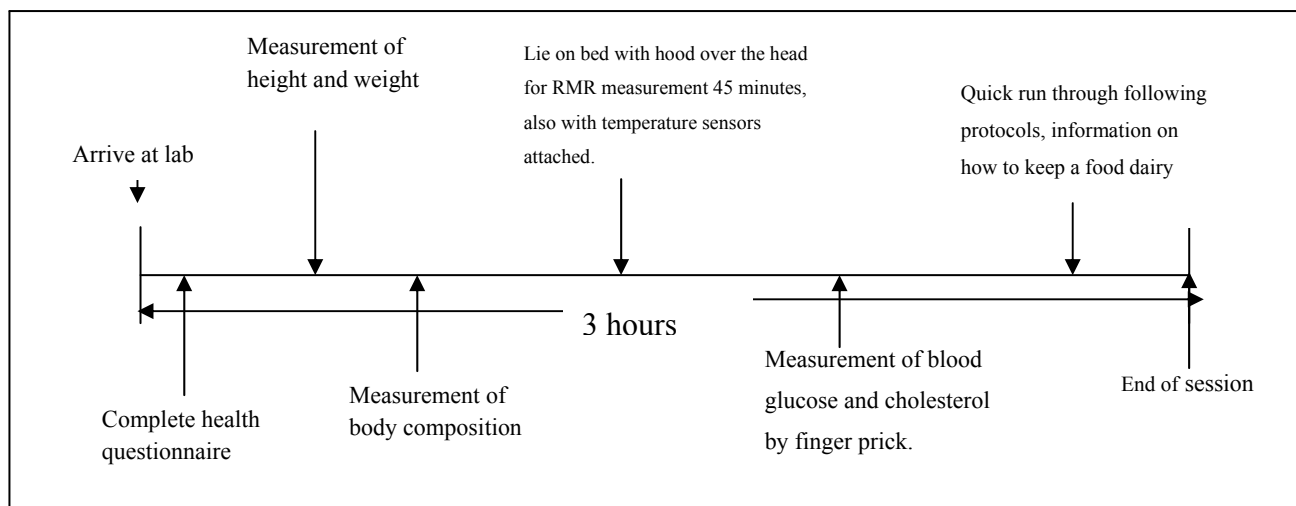
For the measurement of body composition, you will have to sit in a BodPod® (specially designed chair like device, in which the door can be opened from the inside) for about 3 to 5 minutes. To ensure accurate measurement, you should wear closely fitted swimsuit, which you will need to bring along. Body fat composition will be determined by skinfold measurement technique as well. This involves pinching the skin at specific areas such as the upper back, mid upper arm, and waist area. This pinched up skin will be measured with the help of Harpenden callipers and would take less than a minute at each site. These tests and the equipments used are completely safe, comfortable and easy to carry out.

The measurement of RMR will involve you lying on a bed for 45 minutes with a perspex hood over your head. You should be in a complete state of rest, but not asleep. To encourage this you will be supplied with a set of headphones with calming music playing, to block out any external stimuli. At the same time, you will be measured for the skin and core temperature. This is done by placing four small sensors in various places on your skin i.e. on the chest bone (sternum), forearm, thigh and calf. You will also be needed to insert a 10 cm flexible rectal probe in order to measure the core temperature. Following this, analysis of glucose, cholesterol by finger prick blood sampling will be done.

You will be given some supplement capsules to take over a 10 day period and instructions on how and when to take them and directions to maintain a 3 day food diary will be given. All the capsules will contain only food supplements and there is no known risk involved.

All equipments are designed to be comfortable during testing and you will be able to stop the testing by request at anytime. During this preliminary session you will also be made familiar with the various procedures to be followed in the subsequent sessions and this first session will take approximately 3 hours of your time.

PLEASE REMEMBER: You should not be under the influence of alcohol, drugs, HUNGER or FULLNESS for all sessions!!!



Session 2 and 3:

Upon arriving at the lab, blood will be drawn for analysis and your height, weight, body composition, resting metabolic rate and temperature will be measured (Same as session 1), on order to determine the effect of the dietary supplement given.

After completion of testing during the second session, you will be given some more supplement capsules to take over the next 10 day period and instructions on how and when to take them will be given. You also need to maintain a period of ‘wash-out’ for 10 days after the second session and begin taking the second batch of supplements after this and at the end of these 10 days you need to come to the lab for a final testing session.

Risks:

1. Air is drawn into the Perspex hood of the metabolic monitor through a valve in the loop of the hood. Should this valve become blocked- a problem that has NEVER occurred – an alarm will sound. Since a researcher will be in the room at all times whilst you are under the hood, there would be immediate assistance available.
1. To ensure your safety whilst participating in this study we will ask you to complete a health questionnaire, which will give us an indication of whether it is safe for you to participate in such activity.

Note: A qualified first aider will be present during all test sessions.

CONFIDENTIALITY:

Your confidentiality will be protected, both in terms of your personal involvement and your particular data from the study. Your name will not be associated with any of your data that will be used for subsequent analysis.

You are free not to participate and may withdraw from the study at any time without any obligation.

Understand that in the event of injury caused by the product defect of a manufacturer, you will be compensated. In the unlikely event of injury due to negligence, claims must be pursued through legal action.

General information on patient's rights, particularly as regards participation in research studies may also be obtained from your local Community Health Council.

Thank you very much for your interest in the study.

If you have any questions about this, please contact:

Varsha Rajashekar on 020 7911 5000 ext 3895 or

Dr. Adam Cunliffe on 020 7911 5000 ext 3580

Appendix 7 -Participant information sheet – 8 week L-Histidine study

Title of Investigation: To study the effect of a dietary supplement on energy expenditure and blood sugar regulation

Dear Participant,

We invite you to participate in an investigation that we believe to be of potential importance. In order to help you to understand what the investigation is about, we are providing you with the following information. Be sure you understand it before you agree to participate. Please do ask any questions you have about the information, which follows. We will do our best to explain and to provide any further information you require.

This study is examining the effects of a dietary supplement on blood sugar levels and energy expenditure. The proposed study hopes to address and hopefully clarify the best strategies for use of new non-drug therapies in overweight people and those with blood sugar control problems.

We are interested to find out what a particular dietary supplement may have on your energy expenditure (calories burned per day), blood glucose levels, cholesterol, mean skin and core temperature. In order to do this we will give you some supplement capsules to take over an eight week period and instructions on how and when to take them. Some volunteers will be given the ‘active’ capsules and some will be given dummy (placebo) capsules. You will not know which is which. All the capsules will contain only amino acid food supplements and there is no known risk involved.

As a participant, you will be required to come in to the lab for **3 sessions over a period of 8 weeks.**

Session 1

During this preliminary session you will also be made familiar with the various procedures to be followed in the subsequent sessions and this first session will take approximately 2 hours of your time.

The first session involves measuring height and weight, resting metabolic rate (RMR), body fat measurement by skin fold measurements (anthropometry) along with measurements of fasting blood sugar, insulin, leptin, and cholesterol and your mean skin and core temperature.

For the measurement of body composition, you will have to sit in a BodPod® (specially designed chair like device, in which the door can be opened from the inside) for about 3 to 5 minutes. To ensure accurate measurement, you should wear closely fitting swimsuit, which you will need to bring along. This test and the equipment used are completely safe, comfortable and easy to carry out. Your body fat will also be measured by skin fold measurements by the use of equipment called the Harpenden callipers. Skin folds are measured at four different sites in the body i.e. the back (sub scapular), the front of the upper arm (biceps), back of the upper arm (triceps) and the waist area (supraspinale).

The measurement of resting metabolic rate will involve you lying on a bed for 45 minutes with a clear perspex hood over your head. You should be in a complete state of rest, but not asleep. To encourage this you will be supplied with a set of headphones with calming music playing, to block out any distractions. At the same time, you will be measured for the skin and core temperature. This is done by placing four small sensors in various places on your skin i.e. on the chest bone (sternum), forearm, thigh and calf. You will also be needed to insert a 10 cm flexible rectal probe in order to measure the core temperature.

All equipments are designed to be comfortable during testing and you will be able to stop the testing by request at anytime.

After the RMR measurement, blood will be drawn from the vein (venepuncture) as well as from the finger (finger prick) for analysis of blood sugar, cholesterol, insulin, leptin. You will be given the supplement capsules to take over a four-week period and instructions on how and when to take them will be given. All the capsules will contain only food

supplements and there is no known risk involved. During the study, it is necessary for you maintain a three day food diary, once during the start of the study and again during the last three days of the study. You will be provided by a photographic food guide to help you maintain the food diary with ease.

PLEASE REMEMBER: You should not be under the influence of alcohol, drugs, and should be in a fasted state for all the sessions.

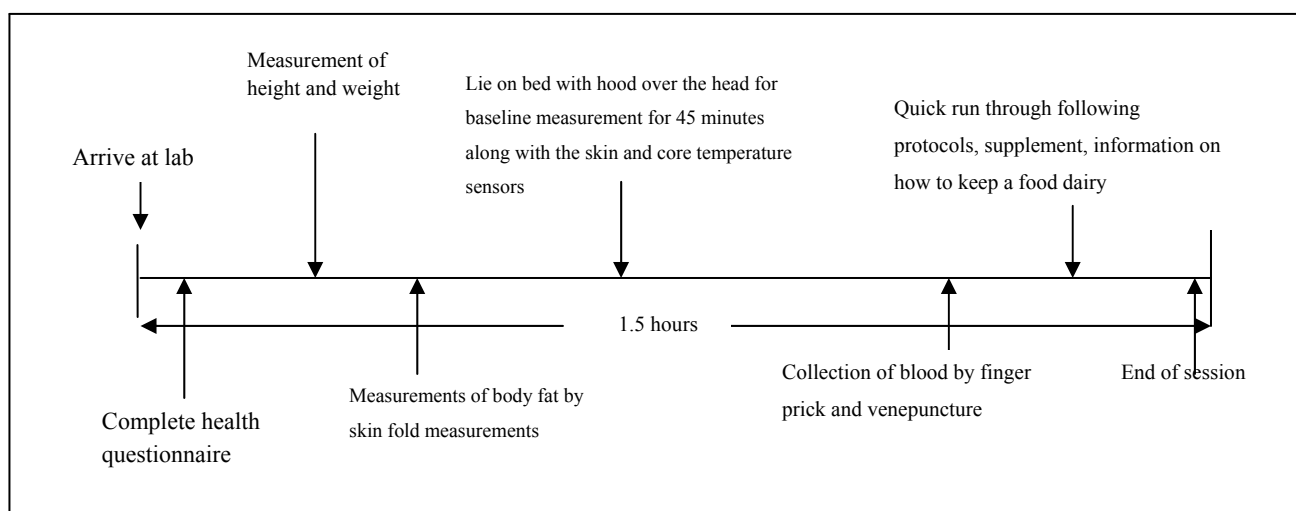
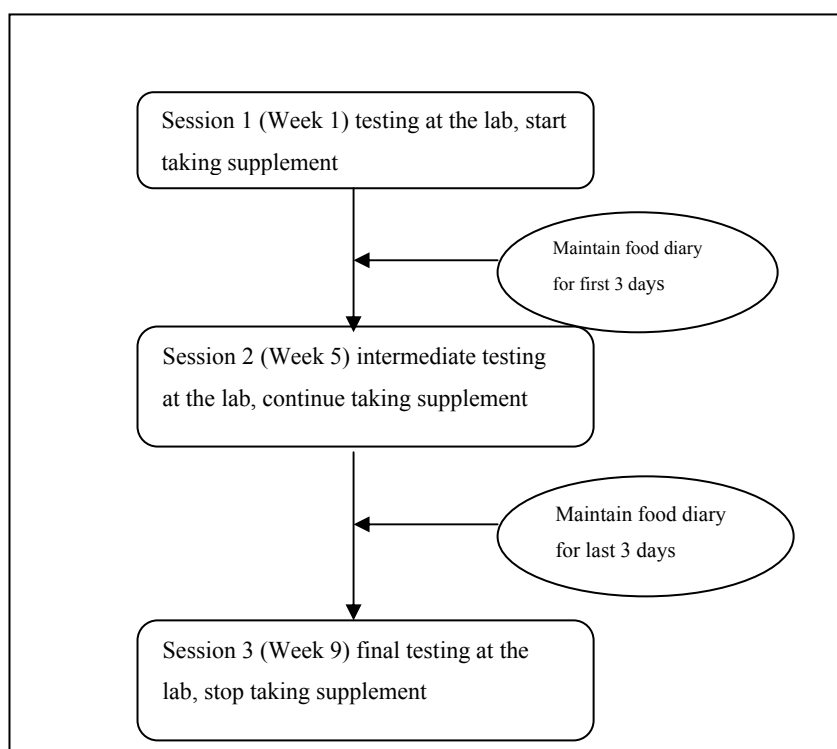


Figure: Summary of session 1.

Session 2 and 3:

Upon arriving at the lab, your weight, body composition, resting metabolic rate and skin fold measurements and skin and core temperature will be measured and blood tests will be performed (Same as in session 1), in order to determine the effect of the dietary supplement given.



Overview of week 1 to 8

Risks:

3. The blood taken during the sessions will be from a vein in the arm. This will be carried out by someone highly experienced in taking blood. In some rare cases, you may have slight bruising for up to 24 hours. A total of 20ml of blood will be taken, which is a very small fraction (approximately 3%) of what an individual would give when donating blood and it is therefore extremely unlikely you would experience any adverse effects.
4. To ensure your safety whilst participating in this study we will ask you to complete a health questionnaire, which will give us an indication of whether it is safe for you to participate in such activity.

Note: A qualified first aider will be present during all test sessions.

CONFIDENTIALITY:

Your confidentiality will be protected, both in terms of your personal involvement and your particular data from the study. Your name will not be associated with any of your data that will be used for subsequent analysis. The data obtained from the study will be accessible only by the principal investigator and the supervisory team.

You are free **not to** participate and may withdraw from the study at any time without any obligation.

CLAIMS:

Understand that in the event of injury caused by the product defect of a manufacturer, you will be compensated. In the unlikely event of injury due to negligence, claims must be pursued through legal action.

FEEDBACK:

Would you like to be aware of the results/outcome of the study? If you wish to receive feedback from the study we are most happy to provide it to you in the form of journal articles or any other publications arising from the data collected during the study.

If you have any questions about this, please contact:

Varsha Rajashekar on 020 7911 5000 ext 2830 or v.rajashekar01@wmin.ac.uk

Dr. Adam Cunliffe on 020 7911 5000 ext 3580 or a.cunliffe@wmin.ac.uk

Thank you very much for your interest in the study.

Appendix 8 - Ethics application form for EGCG study

OFFICE USE: _____ / ____ / _____

University of Westminster
University Ethics Committee

Application for Approval of a Proposed Investigation, Demonstration, Research or Experiment

Section 1 – to be completed by all applicants

1.1 Project Title

Effects of Micronutrient manipulation on energy expenditure and blood glucose regulation

1.2 Applicant Details

Name:

Varsha Rajashekar

Email:

V.Rajashekar01@wmin.ac.uk

Address:

Flat 5, 37, Muswell Hill, London N10
3PN

Telephone Number:

07774516248

Please tick relevant box:

Undergraduate Postgraduate PhD Student Staff

Section 2 – to be completed when applicable

Please note that all applicants with a supervisor(s) must ensure that the supervisor signs the declaration. All staff must obtain the signature of their Dean of School, or School Research Director, as appropriate.

2.1 Supervisor Details

Name:

Dr. Adam Cunliffe

Email:

A.Cunliffe@westminster.ac.uk

School/Department:	Telephone Number:
School of Biosciences/Dept of Human and Health sciences	020-7911-5000 ext 3580
Declaration:	
In accordance with the University's Code of Practice Governing the Ethical Conduct of Investigation, Demonstrations, Research and Experiments, I agree that the applicant named in 1.2 above should submit their proposal to the Ethics Committee for consideration.	
Signed:	
Date:	

Section 3

3.1 Does your work relate to any of the following areas? Please tick box

- Human Participants in Health and Community settings
- Work with prescription drugs
- Work involving foetal tissue
- Drug Studies on human participants
- Pre-marketing drug trials

3.2 Are you proposing work using participants from any of the following categories? Please tick box:

- Prisoners sectioned under the Mental Health Act
- Prisoners or arrestees
- Persons with severe mental illness
- Persons with learning difficulties or brain damage
- Persons with a reduced level of consciousness

3.3 Any work where a qualified clinical person is required to:

- Be responsible for all work carried out
- Be in attendance when certain procedures are carried out
- Ensure that facilities for emergency medical care are at hand

If you have ticked one or more boxes in Section 3, please contact your supervisor and discuss sending your proposal for external approval. More information can be found at:

www.wmin.ac.uk/page-3380

In addition, please complete this form up to Section 3, sign the declaration in Section 11 and send it to:

Carl Hornsey
Assistant Registrar (Student Information)
Academic Registrar's Department
University of Westminster
115 New Cavendish Street
London W1W 6UW

If you have not ticked any boxes in Section 3, please continue to Section 4

Section 4

4.1 Is your work related to any of the following areas? Please tick relevant box:

- Any work involving patients
- Non-clinical work involving bodily fluids
- Administering of a non-food substance
- Work with children
- Deception of participants
- Data not already in the public domain that bears on the issues of criminality
- Work which requires participants to reveal medical history

If you have ticked one or more boxes in Section 4, your proposal will need approval from the University Ethics Committee – please continue to fill out the rest of this form giving as much detail as possible.

If you have not ticked any boxes in Section 3 or Section 4, your work does not require approval by the University Ethics Committee. Please refer to the

University of Westminster 'Code of Practice Governing the Ethical Conduct of Investigations, Demonstrations, Research and Experiments' and consult with your supervisor.

Section 5

5.1 Please provide a brief description of your proposed work below:

Following extensive literature research in the area of drug treatments for weight reduction, hyperglycaemia and hyperlipidaemic states and the ongoing interest in developing non-pharmacological interventions, which, additionally, may have low associated costs to healthcare providers, a potentially efficacious dietary agent has been identified and is introduced below. Review of literature pertaining to the effects of green tea infusion or an extract of its major catechin EGCG, on key metabolic functions, has identified that human studies are limited and many gaps in the knowledge of this area exist. It was therefore selected for further research.

Green tea extract:

Green tea produced from *Camellia sinensis* is a major source of dietary polyphenol, particularly flavonoids. The main flavonoids present include catechins and the four major catechins are epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC) (Cabrera *et al.*, 2006).

Wu and colleagues (Wu *et al.*, 2003), in a study conducted on rats, examined the effects of green tea supplementation on glucose tolerance and insulin sensitivity. It was demonstrated that a 12 weeks supplementation of 0.5g.day⁻¹ green tea lowered fasting plasma glucose, insulin, triglycerides and free fatty acids when compared with a control group. (Dulloo *et al.*, 1999) identified that green tea extract was more effective than an equimolar concentration of caffeine in increasing the 24 hour energy expenditure in humans and also increased lipolysis, thus influencing body weight and composition. A study (Westerterp-Plantenga *et al.*, 2005) conducted on overweight and moderately obese volunteers, both men and women, showed that long term supplementation of EGCG (45mg.day⁻¹) along with caffeine (25mg.day⁻¹) brought about greater weight maintenance after weight loss, when compared to placebo. This supplementation of EGCG along with caffeine also increased thermogenesis, fat oxidation and reduced fasting blood parameters of glucose, insulin, triacyl glycerol and leptin, which are all important risk factors for metabolic disorders.

A study (Choo, 2003) conducted to examine the effects of green tea on rats fed on a high fat diet showed that supplementation with green tea extract not only increased energy expenditure, but also prevented any increase in body weight as a consequence of the high fat diet. The administration of propranolol (a β -adrenoceptor antagonist) inhibited this weight suppressive effect of green tea, indicating that the increased thermogenic effect of green tea exerts its effects through β -adrenoceptor activation.

The effect of green tea supplementation and its mechanisms of increasing energy expenditure and lipolysis are still unclear, but it is being argued that the polyphenols

may inhibit catechol O-methyltransferase (COMT). This enzyme degrades noradrenaline resulting in an increase in noradrenaline release or prolonging its effect on thermogenesis and fat metabolism (Dulloo *et al.*, 1999). To observe the metabolic changes after green tea supplementation test sessions will involve assessing-

- Energy expenditure by indirect calorimetry
- Change in mean skin temperature (MST) and core temperature (CT), heart rate and blood pressure
- Changes in insulin and leptin hormones
- Oral glucose tolerance
- Assessment of appetite by the use of visual analogue scales
- Dietary analysis by the use of diet diary to assess change in food intake
- Measurement of urinary catecholamines and metabolites.

5.2 What are the specific aims of the work you plan to carry out?

To investigate the effects of green tea, future work will involve studies to

- Measure possible dose response relationships in terms of effects of green tea or its principal polyphenol (EGCG) on thermoregulation in lean, healthy men.
- Investigate fasting glycaemic response and thermogenic response following acute (4 hours) supplementation with green tea.
- Effects of chronic supplementation (10 days) of green tea on thermoregulation and energy expenditure in healthy women.

5.3 Please outline the design and methodology of your work

Subject recruitment:

Recruitment of subjects will be achieved via posters placed in all campuses of the University of Westminster, by advertising in daily papers (Evening Standard and Metro - this is a free service for researchers). The criteria for recruitment of healthy volunteers will also include body mass index (BMI) values of 20 and above, 18-55 years of age without any chronic disorders.

In addition, using questionnaires (e.g. medical screening form) and different measurement techniques (e.g. blood pressure, cholesterol), subjects will be assessed for any health related issues, which might make them unsuitable as test subjects (chest pains, respiratory difficulties, heart disease, thyroid disease, orthopedic problems). Participants will be excluded if they are taking medications, which are expected to alter the results of the study (including medication that would affect metabolism or weight loss (psychiatric medication, appetite suppressants, and thyroid medication). Furthermore, subjects who are obese due to a confounding diagnosed disease e.g. Cushings syndrome will not be recruited. Any gross deviation from expected metabolic rate will quickly be identified by the study and appropriate referral made.

The number of subjects required will be determined by sample size calculations once pilot studies have been carried out to determine the size of significant differences in

energy expenditure and metabolic responses that may be observed after ingestion of EGCG and green tea and after differing durations and doses. For the study to have a 5% significance level (α , two sided), and 80% power ($1-\beta$) the sample size will be calculated. The standardised difference (sD) will be calculated as the physiologically significant difference (PSD) divided by the standard deviations of the measures in question, which is energy expenditure.

Study 1 - Effect of EGCG in varying doses (75mg day^{-1} , 150mg day^{-1} , 300mg day^{-1}) on metabolic rate, blood glucose and blood lipid regulation.

Aim:

To study the effects of different doses and arrive at the dose for best potency of EGCG.

Subjects:

Lean and overweight healthy men between the ages of 20-55 years.

Study design:

Chronic intake (7 day), double blinded crossover study with 7-day wash out period between administrations of each dose.

Parameters measured:

- Basal metabolic rate (BMR) by the use of Deltatrac (indirect calorimetry)
- Mean Skin Temperature (MST) by skin thermistors
- Core Temperature (CT) by rectal probe [Volunteers will insert the flexible probe of 10 cm themselves, in a private area. The probe will be thoroughly sterilised after each use. Participants are free not to use the probe in case of any discomfort]
- Heart rate (HR) by Polar heart rate monitor
- Body composition by bodpod
- Diet analysis by the use of 3-day food diary during varied supplementation of EGCG
- Use of visual analogue scales (VAS) for appetite analyses
- Measurement of insulin, leptin by ELISA
- Fasting blood glucose by Hemocue
- Measurement of high density lipoprotein (HDL), low density lipoprotein (LDL), Total cholesterol (TC) by miniphotometer
- Urinalysis to measure bioavailability of catecholamines
- Use of flicker fusion test for assessment of cognitive function.

Study 2 - To study the effects of EGCG in comparison to whole green tea on metabolic rate, blood glucose and lipid regulation.

Aim:

To study and compare the effectiveness of EGCG against whole green tea in regards to metabolism, blood glucose and lipid regulation.

Subjects:

Lean and overweight healthy men between the ages of 20-55 years.

Study design:

Chronic intake (7 day), double blinded, placebo controlled, crossover study with 7-day wash out period between supplementation.

Parameters measured:

- RMR
- Mean Skin Temperature (MST)
- Core Temperature (CT)
- Heart rate (HR)
- Body composition by bodpod
- Diet analysis by the use of 3-day food diary during varied supplementation of EGCG
- Use of visual analogue scales (VAS) for appetite analyses
- Measurement of insulin, leptin by ELISA
- Fasting blood glucose by Hemocue
- Measurement of HDL, LDL, Total cholesterol (TC) by miniphotometer
- Urinalysis to measure bioavailability of catecholamines
- Use of flicker fusion test for assessment of cognitive function.

Study 3 – To study the acute effects of EGCG on metabolic rate and response to glucose load.

Aim:

To observe the acute effects of EGCG consumption on metabolism, blood glucose and lipid regulation.

Subjects:

Lean to overweight healthy men between the ages of 20-55 years.

Study design:

Acute intake (4 hour), double blinded, placebo controlled crossover study with 7-day wash out period between supplementation.

Parameters measured:

- RMR
- Mean Skin Temperature (MST)
- Core Temperature (CT)
- Heart rate (HR)
- Body composition by bodpod
- Diet analysis by the use of 3-day food diary
- Use of visual analogue scales (VAS) for appetite analyses
- Measurement of insulin, leptin by ELISA
- Oral glucose tolerance test and measurement of fasting blood glucose by Hemocue
- Measurement of HDL, LDL, Total cholesterol (TC) by miniphotometer
- Urinalysis to measure bioavailability of catecholamines
- Use of flicker fusion test for assessment of cognitive function.

Data analysis:

Analysis of data obtained will be carried out. It is envisaged that the general linear model of the analysis of variance will be used to assess the effects of supplementation and its duration, macronutrient content of meal on variation in fat oxidation, effect of micronutrient supplementation i.e. green tea extract and EGCG on energy expenditure, substrate oxidation and blood sugar levels. These data will reveal which of the tested combinations of exercise and macro and micronutrient intake are most effective with respect to the parameters tested.

5.4

Start Date:

January 2007

Estimated duration of work:

5 months

5.5 If your work is a multi-centred study, please provide details of any other organisations involved

Contact Name

Contact Name

Address

Address

Telephone Number

Telephone Number

Please provide a copy of any agreement between the organisations

Section 6

6.1 Describe any potential physical/emotional discomforts to participants in the investigation:

- A momentary discomfort whilst taking blood samples (a qualified phlebotomist will be taking the blood samples).
- Possible risk of slight bruising at site of venepuncture for up to 24 hours.

6.2 Aside from 6.1 above, describe potential hazards which may be suffered by the participants? Please give details of any measures taken e.g. COSH, Risk Assessment etc.

COSHH form submitted and risk assessment performed.

6.3 Outline the degree to which these risks are balanced against potential benefits

There should be no risks. Subjects will be monitored at all times during testing. A qualified first aider will be present during all test sessions.

6.4 What criteria will be employed for deciding the end point at which:

- a) the investigation will stop because of unjustifiable further risk?
- b) one method is declared the preferred option and the investigation terminates?

Testing will be stopped immediately on request from subject. No risk should arise from the planned test procedures.

6.5 What monitoring mechanisms will be in place to decide when participants should be withdrawn from the research?

Medical screening forms are used to monitor health and fitness of participants in order to assess health profile and suitability to continue in the study. Also, the principal researcher will follow up the participants closely via email and telephone to monitor progress in between test sessions.

6.6 What procedures and subsequent observations are to be made on participants for the purpose of detecting any complication arising from the investigation?

No complications envisaged which could arise from any equipment or supplementation used during the test sessions.

6.7 Do participants have any previous or existing professional relationship with the investigator?

No

If yes, please explain the circumstances:

Section 7 – Consent of Applicants**7.1 What type of consent will you seek?**

- Written (including email)
- Verbal only
- Not applicable (please give justification below as to why consent is not applicable)

7.2 How and where will you make contact with the participant(s) in order to obtain consent?

Contact with the subjects will be made by advertising the study via posters in all campuses of the University of Westminster, the London area in general, by advertising in daily papers (Evening Standard and Metro (this is a free service for researchers)).

7.3 Is there a subject information sheet?

- Yes (Please enclose a copy with this application)
- No

7.4 Is parental consent required?

- Yes
- No

Section 8 – Confidentiality of Information

8.1 Will the sharing of information be communicated to others working on the project?

- Yes (Please attach a copy of the Participant Confidentiality Code of Practice which will be used)
- No (apart from project supervisors who are full time lectures in the school of Biosciences)

8.2 Will the work include:

- Named participants

Participants whose names have been separately coded

Unnamed participants

8.3 Where will locked files of investigation material be stored?

In a locked cabinet in lab C4.08, the metabolic lab, in new Cavendish building

8.4 If the investigation involves storage of computerised data which might enable the participant it be identified, please name the investigator in charge of Computer System Security for the investigation?

Varsha Rajashekar

8.5 Does the investigation and any planned publication include the use of photographs or videos either of individuals or tissues?

Tissues Yes No

Individuals Yes No

If yes to either of these, please provide a copy of the consent form which participants will be asked to sign for this purpose.

Section 9 - Finance

9.1 Will expenses be paid to participants?

Yes (If yes, how much?)

No

9.2 Will a reward over and above expenses be made to participants?

Yes (If yes, please give more details)

No

9.3 Is this study initiated/sponsored by a pharmaceutical or other industrial company?

Yes (If yes, what is the name of the company?)

No

9.4 Detail any financial or other direct interest to you or to your department arising from this study.

None

9.5 Will this project increase work/cost to any other Department or School?

Yes (If yes, obtain and include the name and signature of the relevant Dean(s) of School(s) concerned:

Name

Signature

No

Section 10 – Insurance

10.1 Are manufacturers providing insurance cover?

Yes (If yes, please enclose a letter confirming insurance cover, including the names of all covered)

No

10.2 Are all of the investigators employees or students of the University of Westminster?

Yes

No

If no, please provide evidence of insurance cover, including:

- list of all people involved in the investigation
- details of the form this cover will take

10.3 Does the investigation involve the use of equipment or medicines?

Yes

No

If yes, please give details of *manufacturer's indemnity*:

All equipments are covered by the indemnity offered by the University of Westminster (see attached letter)

10.4 Does the investigation involve the use of equipment or medicines which are manufactured on site but are not covered by insurance?

Yes

No

If yes, appropriate insurance cover must be arranged and written confirmation of such cover must be attached

Section 11 – Declaration – this Section must be completed by all applicants

Please Read and Sign

The information I have given on this form is true and to the best of my knowledge correct:

Signed:

Date:

Send the completed form to:

Carl Hornsey
 Assistant Registrar (Student Information)
 Academic Registrar's Department
 University of Westminster
 115 New Cavendish Street
 London W1W 6UW



Varsha Rajashekar
85 Shurland Avenue
Barnet
EN4 8DE

24 July 2008

Dear Varsha

App. No. 06/07/27
Varsha Rajashekar: School of Biosciences

"Effects of micronutrient manipulation on energy expenditure and blood glucose regulation"

I am writing to inform you that your application for ethics approval was considered by the University Research Ethics Committee at its meeting of 12 December 2006. Following receipt of the conditions set to you by the University Research Ethics Committee, the application has been approved.

If your protocol changes significantly in the meantime, please contact me immediately, in case of further ethical requirements.

We wish you the best with your study,

Yours sincerely

Huzma Kelly
Senior Research Officer (Policy and Governance)
Secretary, University Research Ethics Committee

cc Dr. John Colwell, (Chair) University Research Ethics Committee

I am advised by the Committee to remind you of the following points:



ACADEMIC REGISTRAR'S DEPARTMENT
Educating for professional life 115 New Cavendish Street London W1W 6UW
www.wmin.ac.uk Tel 020 7911 5000 Fax 020 7911 5118

1. Your responsibility to notify the University Ethics Committee immediately of any information received by you, or of which you become aware, which would cast doubt upon, or alter, any information contained in the original application, or a later amendment, submitted to the University Ethics Committee and/or which would raise questions about the safety and/or continued conduct of the research.
2. The need to comply with the Data Protection Act 1998
3. The need to comply, throughout the conduct of the study, with good research practice standards
4. The need to refer proposed amendments to the protocol to the University Ethics Committee for further review and to obtain University Ethics Committee approval thereto prior to implementation (except only in cases of emergency when the welfare of the subject is paramount).
5. You are authorised to present this University of Westminster Ethics Committee letter of approval to outside bodies, e.g. LRECs, in support of any application for further research clearance.
6. The requirement to furnish the University Ethics Committee with details of the conclusion and outcome of the project, and to inform the University Ethics Committee should the research be discontinued. The Committee would prefer a concise summary of the conclusion and outcome of the project, which would fit no more than one side of A4 paper, please.
7. The desirability of including full details of the consent form in an appendix to your research, and of addressing specifically ethical issues in your methodological discussion.



Appendix 9 - Informed consent form for EGCG studies**Written Consent Form**

Title of Investigation: To study the effect of dietary supplementation of a nutritional compound on energy expenditure and blood sugar levels.

Volunteer's name: _____

Address: _____

Tel: _____

Email: _____

Date of Birth: _____

Gender: M/ F

I have read the attached information on the research in which I have been asked to participate and have been given a copy to keep. I have had the opportunity to discuss the details and ask questions about this information.

The investigator has explained the nature and purpose of the research and I believe that I understand what is being proposed. I have been informed what the proposed study involves.

I understand that my personal involvement and my personal data from this trial will remain strictly confidential. Only researchers involved in the investigation will have access.

I hereby fully and freely consent to participate in the study, which has been fully explained to me. I understand that I am free to withdraw from testing at any time.

Volunteer's Signature:

Date:.....

As the investigator responsible for this investigation, I confirm that I have explained to the participant named above, the nature and purpose of the research to be undertaken.

Investigator's Name: Varsha Rajashekar

Signature:.....

If you have any questions about this, please contact:

Varsha Rajashekar on 020 7911 5000 ext 2830 or

Dr. Adam Cunliffe on 020 7911 5000 ext 3580

Appendix 10 - Participant information sheet – acute EGCG study

Title of Investigation: To study the effect of a dietary supplement on energy expenditure and blood sugar regulation

Dear Participant,

We invite you to participate in an investigation that we believe to be of potential importance. In order to help you to understand what the investigation is about, we are providing you with the following information. Be sure you understand it before you agree to participate. Please do ask any questions you have about the information, which follows. We will do our best to explain and to provide any further information you require.

This study is examining the effects of a dietary supplement on blood sugar levels and energy expenditure. The proposed study hopes to address and hopefully clarify the best strategies for use of new non-drug therapies in overweight people and those with blood glucose control problems.

We are interested to find out what a particular dietary supplement may have on your energy expenditure (calories burned per day), blood glucose levels, cholesterol, mean skin and core temperature. In order to do this we will give you a dose of supplement, in liquid form, to take during the test session. You will be given the ‘active’ supplement and also the dummy (placebo) supplement over the two test sessions. You will not know which is which. This helps us to get unbiased results for the study. The supplement and the placebo will contain only food supplements and there is no known risk involved.

As a participant, you will be required to come in to the lab for **2 sessions over a period of 2 weeks.**

Session 1

During this preliminary session you will be made familiar with the various procedures to be followed in the subsequent sessions and this first session will take approximately 2 ½ hours of your time.

The first session involves measuring height and weight, determining your body composition, your resting metabolic rate (RMR), heart rate, body fat measurement along with measurements of fasting blood sugar, insulin, leptin, and cholesterol and your mean skin and core temperature, urinalysis for metabolites.

For the measurement of body composition, you will have to sit in a BodPod (specially designed chair like device, in which the door can be opened from the inside) for about 3 to 5 minutes. To ensure accurate measurement, you should wear closely fitting swimsuit, which you will need to bring along. This test and the equipment used are completely safe, comfortable and easy to carry out. Your body fat will be measured by skin fold measurements by the use of equipment called the Harpenden callipers. Skin folds are measured at four different sites in the body i.e. the back (sub scapular), the front of the upper arm (biceps), back of the upper arm (triceps) and the waist area (supraspinale).

The measurement of resting metabolic rate will involve you lying on a bed for 45 minutes with a clear perspex hood over your head. You should be in a complete state of rest, but not asleep. To encourage this you will be supplied with a set of headphones with calming music playing, to block out any distractions.

At the same time, you will be measured for the skin and core temperature. This is done by placing four small sensors in various places on your skin i.e. on the chest bone (sternum), forearm, thigh and calf. You will also be needed to insert a 10 cm flexible rectal probe in order to measure the core temperature.

All equipments are designed to be comfortable during testing and you will be able to stop the testing by request at anytime.

When you arrive at the lab, blood will be drawn from the vein (venepuncture) as well as from the finger (finger prick) for analysis of blood sugar, cholesterol, insulin, and leptin. You will need to provide a urine sample.

You will be given the supplement drink along with a glucose drink after which a test called oral glucose tolerance test will be performed during which a finger prick blood sample will be taken every 30 minutes for the two hour test session. The resting metabolic rate will be measured in between the glucose tolerance test. You will be required to fill out a questionnaire to measure appetite at the end of the session.

Prior to the study, it is necessary for you to maintain a three day food diary. You will be provided by a photographic food guide to help you maintain the food diary with ease. You will also need to fill out a questionnaire assessing your appetite.

At the end of the session another blood sample will be taken by venepuncture and you will need to provide a second urine sample.

PLEASE REMEMBER: You should not be under the influence of drugs, and should be in a fasted state for all the sessions

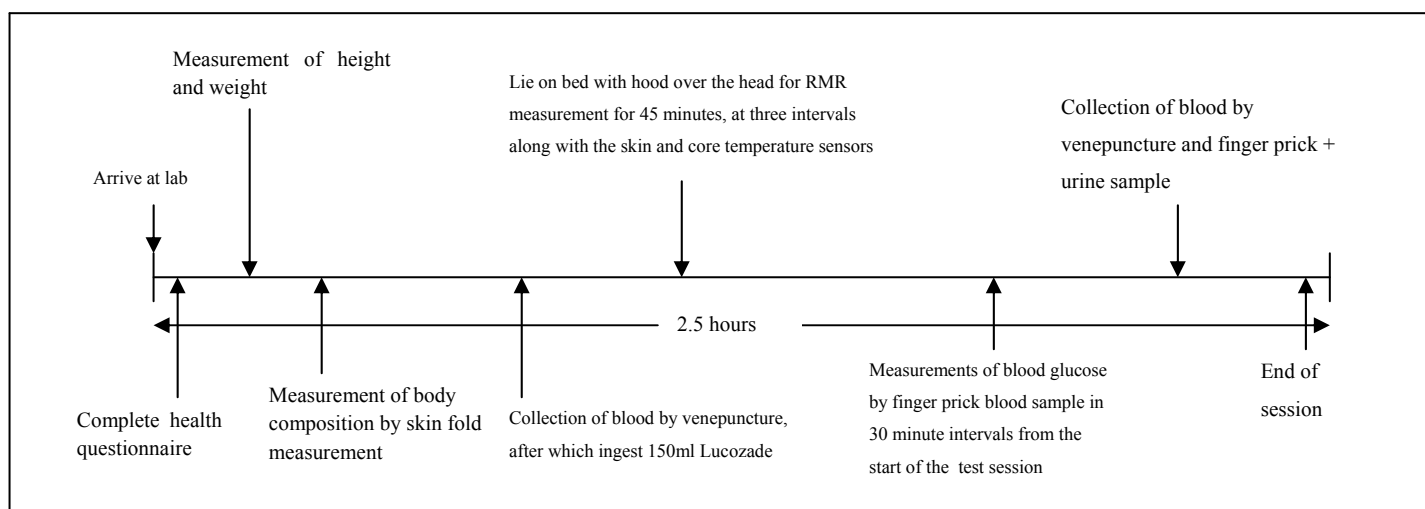


Figure: Summary of session 1.

Session 2:

Upon arriving at the lab, all measurements same as in session 1 will be conducted, in order to determine the effect of the second dietary supplement given.

Risks:

1. The blood taken during the sessions will be from a vein in the arm. This will be carried out by someone highly experienced in taking blood. In some rare cases, you may have slight bruising for up to 24 hours. A total of 20ml of blood will be taken, which is a very small fraction (approximately 3%) of what an individual would give when donating blood and it is therefore extremely unlikely you would experience any adverse effects.
2. To ensure your safety whilst participating in this study we will ask you to complete a health questionnaire, which will give us an indication of whether it is safe for you to participate in such activity.

Note: A qualified first aider will be present during all test sessions.

CONFIDENTIALITY:

Your confidentiality will be protected, both in terms of your personal involvement and your particular data from the study. Your name will not be associated with any of your data that will be used for subsequent analysis. The data obtained from the study will be accessible only by the principal investigator and the supervisory team.

You are free **not to** participate and may withdraw from the study at any time without any obligation.

CLAIMS:

Understand that in the event of injury caused by the product defect of a manufacturer, you will be compensated. In the unlikely event of injury due to negligence, claims must be pursued through legal action.

FEEDBACK:

Would you like to be aware of the results/outcome of the study? If you wish to receive feedback from the study we are most happy to provide it to you in the form of journal articles or any other publications arising from the data collected during the study.

If you have any questions about this, please contact:

Varsha Rajashekar on 020 7911 5000 ext 2830 or v.rajashekar01@wmin.ac.uk

Dr. Adam Cunliffe on 020 7911 5000 ext 3580 or a.cunliffe@wmin.ac.uk

Thank you very much for your interest in the study.

Appendix 11 - Participant information sheet – chronic EGCG study

Title of Investigation: To study the effect of a dietary supplement on energy expenditure and blood sugar regulation

Dear Participant,

We invite you to participate in an investigation that we believe to be of potential importance. In order to help you to understand what the investigation is about, we are providing you with the following information. Be sure you understand it before you agree to participate. Please do ask any questions you have about the information, which follows. We will do our best to explain and to provide any further information you require.

This study is examining the effects of a dietary supplement on blood sugar levels and energy expenditure. The proposed study hopes to address and hopefully clarify the best strategies for use of new non-drug therapies in overweight people and those with blood glucose control problems.

We are interested to find out what a particular dietary supplement may have on your energy expenditure (calories burned per day), blood glucose levels, cholesterol, mean skin and core temperature. In order to do this we will give you three doses of the test supplement, in capsule form, each to be taken for 7 day periods. The supplement will contain only food supplements and there is no known risk involved.

As a participant, you will be required to come in to the lab for **4 sessions over a period of 5 weeks.**

Session 1

During this preliminary session you will be made familiar with the various procedures to be followed in the subsequent sessions and this first session will take approximately 2 ½ hours of your time.

The first session involves measuring height and weight, determining your body composition, your resting metabolic rate (RMR), heart rate, body fat measurement along

with measurements of fasting blood sugar, insulin, leptin, and cholesterol and your mean skin and core temperature, urinalysis for metabolites.

For the measurement of body composition, you will have to sit in a BodPod (specially designed chair like device, in which the door can be opened from the inside) for about 3 to 5 minutes. To ensure accurate measurement, you should wear closely fitting swimsuit, which you will need to bring along. This test and the equipment used are completely safe, comfortable and easy to carry out. Your body fat will also be measured by skin fold measurements by the use of equipment called the Harpenden callipers. Skin folds are measured at four different sites in the body i.e. the back (sub scapular), the front of the upper arm (biceps), back of the upper arm (triceps) and the waist area (supraspinale).

The measurement of resting metabolic rate will involve you lying on a bed for 45 minutes with a perspex hood over your head. You should be in a complete state of rest, but not asleep. To encourage this you will be supplied with a set of headphones with calming music playing, to block out any distractions. You will also be wearing a heart rate monitor to measure resting heart rate. At the same time, you will be measured for the skin and core temperature. This is done by placing four small sensors in various places on your skin i.e. on the chest bone (sternum), forearm, thigh and calf. You will also be needed to insert a 10 cm flexible rectal probe in order to measure the core temperature.

All equipments are designed to be comfortable during testing and you will be able to stop the testing by request at anytime.

After the RMR measurement, blood will be drawn from the vein (venepuncture) as well as from the finger (finger prick) for analysis of blood sugar, cholesterol, insulin, and leptin. You will need to provide a urine sample. Prior to the study and also during the study, it is necessary for you to maintain a three day food diary. You will be provided by a photographic food guide to help you maintain the food diary with ease.

PLEASE REMEMBER: You should not be under the influence of drugs, and should be in a fasted state for all the sessions

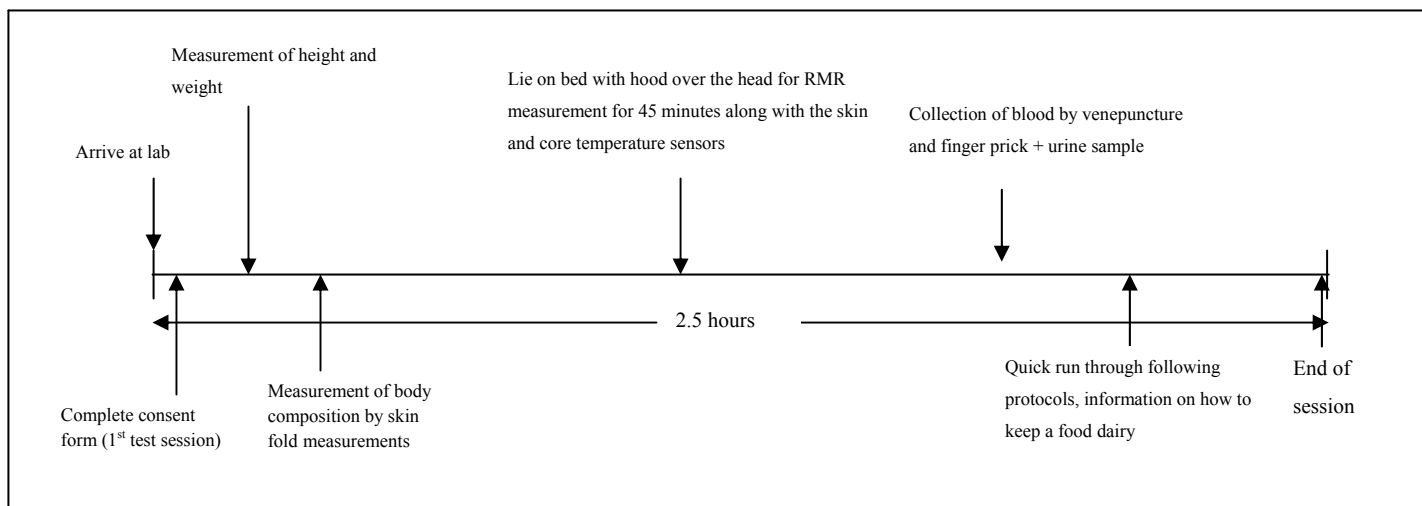
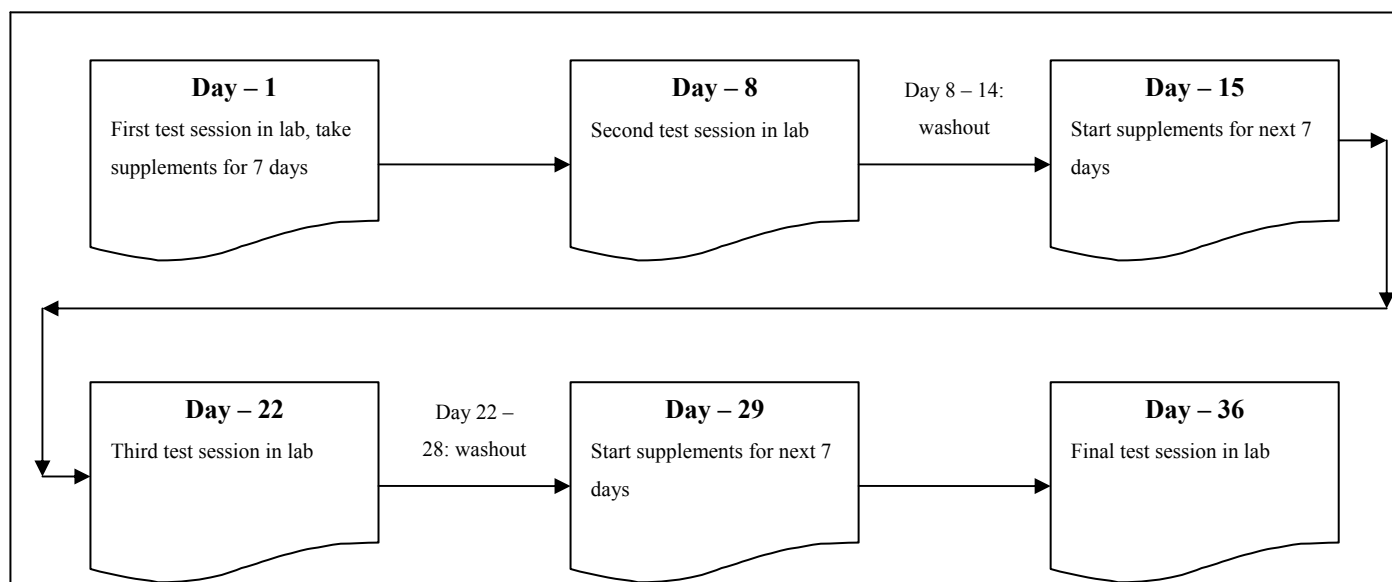


Figure: Summary of session 1.

Session 2 - 4:

Upon arriving at the lab, all measurements same as in session 1 will be conducted, in order to determine the effect of the other doses of supplement given.

Summary of study:



Risks:

1. The blood taken during the sessions will be from a vein in the arm. This will be carried out by someone highly experienced in taking blood. In some rare cases, you may have slight bruising for up to 24 hours. A total of 20ml of blood will be taken, which is a very

small fraction (approximately 3%) of what an individual would give when donating blood and it is therefore extremely unlikely you would experience any adverse effects.

2. To ensure your safety whilst participating in this study we will ask you to complete a health questionnaire, which will give us an indication of whether it is safe for you to participate in such activity.

Note: A qualified first aider will be present during all test sessions.

CONFIDENTIALITY:

Your confidentiality will be protected, both in terms of your personal involvement and your particular data from the study. Your name will not be associated with any of your data that will be used for subsequent analysis. The data obtained from the study will be accessible only by the principal investigator and the supervisory team.

You are free **not to** participate and may withdraw from the study at any time without any obligation.

CLAIMS:

Understand that in the event of injury caused by the product defect of a manufacturer, you will be compensated. In the unlikely event of injury due to negligence, claims must be pursued through legal action.

FEEDBACK:

Would you like to be aware of the results/outcome of the study? If you wish to receive feedback from the study we are most happy to provide it to you in the form of journal articles or any other publications arising from the data collected during the study.

If you have any questions about this, please contact:

Varsha Rajashekar on 020 7911 5000 ext 2830 or v.rajashekar01@wmin.ac.uk

Dr. Adam Cunliffe on 020 7911 5000 ext 3580 or a.cunliffe@wmin.ac.uk

Thank you very much for your interest in the study.

Appendix 12 - Analysis of variation (ANOVA) for data obtained from pilot study investigating L-Histidine ingestion (Chapter 4)

ANOVA for RMR data:

<i>Category</i>	<i>Degrees of freedom (n-1)</i>	<i>Sum of squares</i>	<i>Mean square</i>	<i>f test</i>
Treatment	1	12945.99	12945.99	0.002396941
Subjects	2	168569.34	84284.67	0.01560525
Time	2	22373.74	11186.87	0.002071241
Residual	12	64812549.09	5401045.758	
Total	17	65016438.16		

ANOVA for MST data:

<i>Category</i>	<i>Degrees of freedom (n-1)</i>	<i>Sum of squares</i>	<i>Mean square</i>	<i>f test</i>
Treatment	1	-4115.713617	-4115.71362	2.626215484
Subjects	4	3.416111167	0.854027792	0.000544951
Time	2	1.6511159	0.82555795	0.000526784
Residual	23	36044.8005	1567.165239	
Total	30	31934.15411		

ANOVA for CT data:

<i>Category</i>	<i>Degrees of freedom (n-1)</i>	<i>Sum of squares</i>	<i>Mean square</i>	<i>f test</i>
Treatment	1	0.015	0.015	5.24817E-06
Subjects	4	-24362.63951	-6090.660	-2.15610413
Time	2	0.1210847	0.061	2.14321E-05
Residual	23	64971.434	2824.845	
Total	30	40608.93022		

ANOVA for RER data:

<i>Category</i>	<i>Degrees of freedom (n-1)</i>	<i>Sum of squares</i>	<i>Mean square</i>	<i>f test</i>
Treatment	1	0.002977778	0.002977778	0.002898169
Subjects	2	0.040883333	0.020441667	0.019895176
Time	2	0.001416667	0.000708333	0.000689397
Residual	12	12.32962222	1.027468518	
Total	17	12.3749		

No significant difference was observed in any of the parameters due to treatment, over time and between subjects.

Appendix 13 - Golberg cut-off points to identify validity of reported dietary intake
10 day chronic L-Histidine intake (Chapter 3.3)

Subject	Goldberg's cutoff point after placebo	Goldberg's cutoff point after histidine
1	0.7	0.8
2	0.4	0.3
3	0.5	0.9
4	0.5	0.9
5	0.9	1.3
6	1.1	0.6
7	0.7	0.5
8	0.8	1.3
9	0.9	1.0

The above table shows that 5 participants have underreported dietary intake prior to both the test sessions, and 4 participants seem to have underreported their intake prior to either one of the test sessions.

8 week L-Histidine study (Chapter 3.4)

Subject	Goldberg's cutoff point (Placebo group)		Goldberg's cutoff point (L- Histidine group)	
	Pre-treatment	Post 8 weeks	Pre-treatment	Post 8 weeks
1	1.1	1.3	0.4	0.4
2	0.6	0.7	1.0	0.6
3	0.6	0.9	0.7	1.0
4	1.1	1.3	0.3	0.4
5	0.5	0.7	0.5	0.8
6	0.8	0.8	0.9	1.4
7	1.1	1.0	1.2	1.2
8	0.5	0.7	0.8	0.8
9	0.9	0.7	0.5	0.7

Calculation of the cut-off points for participants in the placebo group showed that 3 participants out of 9 had reported dietary intake during both sampling periods appropriately. All other participants (6) had underreported their dietary intake prior to both sampling periods. In the L-Histidine group, 1 participant had reported adequate dietary intake prior to both sampling periods, 5 participants had underreported dietary intake prior

to both sampling period and 2 participants had underreported dietary intake prior to the last test session and 1 participant underreported dietary intake at pre-treatment.

Acute EGCG study (Chapter 4.2)

Subject	Goldberg's cutoff point prior to placebo ingestion	Goldberg's cutoff point prior to EGCG ingestion
1	0.7	0.4
2	1.3	0.9
3	0.9	0.9
4	0.7	1.6
5	0.7	0.6
6	0.8	1.3
7	0.7	0.6
8	0.8	0.5

Applying the Goldberg cut-off points to the reported dietary intakes during the acute EGCG study, it was found that all participants except one had underreported their dietary intake prior to the placebo ingestion session. Prior to the EGCG ingestion session, 2 participants had accurately reported their dietary intake when 6 participants had under-reported.

Chronic EGCG study (Chapter 4.3)

Subject	Goldberg's cutoff point at baseline	Goldberg's cutoff point after 75mg EGCG	Goldberg's cutoff point after 150mg EGCG	Goldberg's cutoff point after 300mg EGCG
1	0.8	0.9	0.7	1.1
2	0.8	0.7	0.6	0.7
3	0.5	0.6	0.5	1.0
4	0.7	1.5	1.0	1.2
5	1.3	0.9	1.4	1.5
6	0.9	1.0	1.0	0.8
7	0.6	1.0	0.8	0.7
8	0.9	0.8	0.9	0.7

During this study, all participants ingested all 3 doses of EGCG (75, 150 and 300 mg/day), for 7 days each. None of the participants have accurately reported their dietary intake prior to all test sessions.

Discussion:

The application of Goldberg's cut-off points to identify under-reporting of dietary intake showed that majority of the participants underreported their dietary intake prior to/ following one or both supplementations. Having said that, the presumption of the intervention studies as conducted in this thesis is that all aspects influencing energy balance, such as dietary intake and physical activity remain similar to prior, to the commencement of the study. In other words no meaningful change in energy intake from before to during the study was expected, to confound any of the measured variables. The only change expected to participants' daily routine was the intake of the supplementations during the course of the study.

The results of the Goldberg cut-off need to be carefully considered as most of the participants have underreported. However, the use of Goldberg cut-off has been criticised as unreliable to use in studies with small sample sizes. This is due to the assumption that all participants' activity levels are sedentary and for not accounting for medium and high activity patterns (Black, 2000a). Secondly, the Goldberg cut-off only identifies extremely inaccurate reporting (ie, ± 2 SD for the agreement between EI_{rep}/BMR_{est} and physical activity level (PAL) although both underreporting and over reporting can occur to varying degrees (Black, 2000b). It has also been shown that underreporting is not random, but is related to characteristics such as obesity, smoking, dieting, and psychological factors. People with higher BMI and higher body fat percentage underreport (Klingberg *et al.*, 2008; Rasmussen *et al.*, 2007). The reasons for underreporting in all studies could be first, all participants in the 8 week L-Histidine study were overweight and as it is well established that obese subjects commonly underreport (Goris *et al.*, 2000), the study participants similarly may have under-reported. Second, as all participants were regularly weighed, they possibly felt the need to report healthier and low calorie eating pattern as psychological factors such as body shape concerns and mere participation in studies investigating weight loss have been known to influence dietary underreporting (Abbot *et al.*, 2008; Johnson *et al.*, 2005; Maurer *et al.*, 2006). For the purpose of studies presented in the current text, dietary intakes have been underreported to a large extent. Majority of participants have underreported prior to all test sessions and as this is consistent, it is presumed not to affect any other measured parameters of the studies. Participants' RMR and other measured parameters do not seem influenced by underreporting prior to one test session and having reported accurately prior to another. Also, as Goldberg cut-off

calculations seem unsuitable for small sample sizes, the above presented results need to be considered carefully. Further substantiation of reported dietary intakes can be done by measuring 24 hour urinary nitrogen to validate dietary protein intake and and total EE measured by doubly labelled water method (Black *et al.*, 1997); Livingstone and Black., 2003). These validation techniques are above the scope and financial and technical capacity of the current studies and were therefore not performed following the studies proposed herein.

Appendix 14 -Visual Analogue Scales (100mm)

(Questionnaires made as small booklets showing one question at a time)

Questions on appetite and desire for specific food types:

I am not hungry at all hungry _____ How hungry do you feel? _____ I have never been more

I am completely empty _____ How satisfied do you feel? _____ I cannot eat another bite

Not at all full _____ How full do you feel? _____ Totally full

Nothing at all _____ How much do you think you can eat? _____ A lot

Yes, very much _____ Would you like to eat something sweet? _____ No, not at all

Yes, very much _____ Would you like to eat something salty? _____ No, not at all

Yes, very much _____ Would you like to eat something savoury? _____ No, not at all

Yes, very
much

Would you like to eat something fatty?

No, not at all

Appendix 15 - Diet diary

Date:

Day of the week:.....

On rising			
Time	Food/ Drink	Description and Preparation	Amount
Breakfast			
Time	Food/ Drink	Description and Preparation	Amount
Mid morning			
Time	Food/ Drink	Description and Preparation	Amount

Lunch			
Time	Food/ Drink	Description and Preparation	Amount
Afternoon snack / Tea			
Time	Food/ Drink	Description and Preparation	Amount

Evening meal			
Time	Food/ Drink	Description and Preparation	Amount
Later evening-up to last thing at night			
Time	Food/ Drink	Description and Preparation	Amount
Between meals, snacks and drinks (If not mentioned before)			
Food/ Drink	Time	Description and Preparation	Amount
Chocolate			
Toffees, sweets			
Crisps			
Peanuts			

Other snacks			
Beer			
Wine			
Sherry			
Spirits			
Other cold drinks			
Tea			
Coffee			
Other hot drinks			
Ice cream			
Anything else?			

Appendix 16 - Photographic food reference atlas



Food Diary

Name:.....

Human and Health Sciences Department
School of Biosciences
115, New Cavendish Street
London W1W 6UW
Ph: 020-7911-5000 ext 2830
Email: V.Rajashekar01@wmin.ac.uk

We would like you to keep this diary of everything you eat and drink over two weekdays and 1 day over the weekend. This is a very important part of the study and will add greatly to the study. It is important that you do not adjust what you eat and drink just because you are keeping a record. Please continue to eat whatever you wish.

Instructions:

As you will see, each day is marked in sections, beginning with the first thing in the morning and ending with bedtime. For each part of the day write down all food and drink consumed the amounts, and a description if necessary. If nothing is eaten or drunk during a part of the day, draw a line through that section. Record everything at the time of eating, not from memory at the end of the day.

On the next few pages is a list of popular foods and drinks. Next to each item is the sort of thing we need to know so that we can tell what it is made of and how much you had. This list cannot cover all foods and drinks, so try to relate to a similar item if any that you have eaten are missing. Please give as much detail as you can. There is an example on page

For some foods you may find it easier to describe how much you had by comparing it to one of the photographs on pages

Many packet foods have weights printed on them, so please use these to show how much you ate.

At the end of each day there is a list of snacks and drinks that can easily be forgotten. Please write any extra items in here if you have not already recorded them in some other part of the day.

Pictures used in this diary have been reproduced with permission from the author of - A photographic atlas of food portion sizes and Food portion sizes: a user's guide to the photographic atlas.

Food / Drink	Description and Preparation	Amount
Bacon	Lean or streaky; fried or grilled rashers	Number
Baked beans	Standard or reduced sugar	Tablespoons, tin size or picture 1
beef burger (Hamburger)	Homemade, from a packet or take away; fried, microwaved or grilled; well-done or rare; large or small; with or without bread roll	Number
Biscuits	Plain; savoury; cheese, crisp bread, sweet, chocolate, wafer; home-made; include biscuit like kit-kat and penguin; write in the name and brand if you can	Number
Bread (also see sandwiches)	Wholemeal, white or brown; currant, fruit, malt; large or small loaf; thick, medium or thin slices; sliced or unsliced; give brand if possible	Number of slices
Bread rolls	Wholemeal, white or brown; alone or with filling (see sandwiches); crusty or soft; give brand if possible	Number of rolls
Breakfast cereal, bran, wheatgerm	What sort: cornflakes, weetabix, muesli etc.; give brand name if possible	Number of biscuits, tablespoons or picture 2
Bun	What sort: iced, currant, sweet or plain; large or small	Number
Butter for bread	Ordinary or low fat dairy spread, write in the name and brand if you can	Thick, average, thin spread
Cake- small	What sort and brand: cream, iced, sort of filling	Number or picture 3
Cake –large	What sort and brand: cream, iced, sort of filling	Slices or pictures 4
Cheese	What sort: cream, cottage, hard, soft; low fat; write in the name if you can	Tablespoons or picture 5
Chips	Fresh, frozen, oven, microwave or crinkle cut; type of fat for cooking	Picture 6
Chocolate	What sort: plain, milk, white, diabetic; give brand name	Number or bar weight
Chops	What sort: lean or fatty; large or small; fried or baked; well done or rare	Number
Cider	Sweet, dry, vintage, low alcohol	Pints and half pints
Coffee	With milk; half milk/ half water; all milk	Cups or mugs
Condiments	Pepper, salt or substitute	½ or ¼ teaspoon, pinch etc.
Cooking oil	Type; brand name	Teaspoons

Food / Drink	Description and Preparation	Amount
Cream	Half, single, sour, whipping, double, clotted; low fat; fresh or substitute; sweetened or unsweetened	Tablespoons
Crisps	Brand name; low fat; low salt	Packet weight
Egg	How was it cooked: boiled, fried, scrambled, poached, omelette etc	Number
Fish	What sort: fried, boiled, grilled, poached, microwaved; with batter or breadcrumbs; tinned with oil or tomato sauce	Helping or picture 7 and 8
Fish cakes or fingers	What sort: large, medium or small size; fried or grilled	Number
Fruit- fresh	What sort and variety eg. Cox apple; with or without skin	Number or picture 9
Fruit- stewed or canned	What sort and variety eg. Bramley apple; in fruit juice or syrup	Tablespoons
Fruit- juice	What sort; sweetened or unsweetened	Glasses or cups
Gravy	Thick or thin, instant or packet, made with or without dripping or meat juices	Tablespoons
Herbs	Type, fresh or dried	½ or ¼ teaspoons
Honey, jam	Type, specify if low sugar	Teaspoons
Ice cream	Dairy or non dairy; flavour or variety	Tablespoons / quantity
Liver, kidney	Pig, lamb, ox; fried or stewed	Quantity
Margarine	Hard, soft, polyunsaturated, low fat, very low fat; give brand name	Thick, average or thin spread
Marmalade	Type and brand; specify if low sugar	Teaspoons
Mayonnaise	Give name and brand; specify if low fat	Teaspoons
Meat pie, pastie, pastry	What sort; individual or helping, fat used for pastry	Number or picture 10
Meats	What sort; lean or fatty; fried, microwaved, grilled, roast, barbequed; well done or rare; with or without gravy, cut used	Slices, helping
Milk-for drinking on its own or for cereals	Full cream, silvertop, semi skimmed, skimmed, sterilized, UHT, flavoured, powdered, soya	Pints, glasses or cups
Minced beef	On its own, with vegetables, fatty or lean	Tablespoons
Peanuts	Dry roasted or ordinary salted	Packet weight
Pickled, smoked or salted foods	What sort and brand; how eaten; eg. Pastrami, haddock, turkey breast, pate	Tablespoons
Porridge	With sugar or honey; with milk or cream	Bowls
Potatoes	Baked, boiled, mashed, creamed,	Tablespoons

Food / Drink	Description and Preparation	Amount
	fried/ chips, instant, roast; with butter	
Pudding	What sort and brand: eg. Steamed sponge; with fruit; pie (what sort); jelly; blancmange; mousse; instant desserts; milk puddings, give recipe	Tablespoons, slices or pictures-- --
Rice	Brown or white; boiled or fried; rice pudding	Tablespoons or pictures 11
Salad	Describe ingredients, with dressing; what sort of dressing (e.g. oil and vinegar, salad cream, mayonnaise)	Tablespoons or picture 12
Sandwiches and rolls	Wholemeal, white or brown bread; type of filling; butter or margarine: large or small loaf; thick, medium or thin slices	Number of rolls or slices of bread
Sauce –hot	(for vegetables, meat or fish; puddings) what sort; savoury or sweet; thick or thin, give recipe if possible	Tablespoons
Sauce – cold	What sort: e.g. tomato ketchup, brown sauce, soy sauce; salad cream; sweet or savoury	Tablespoons
Sausages	What sort: e.g. pork, beef, pork and beef; low fat; large or small; how cooked	Number
Sausage rolls	Large or small, type of pastry	Number
Scones	What sort: with currants, sweet or plain; cheese	Number
Snacks- in packets	What sort: e.g. cheese straws, twiglets, pretzels (give brand name)	Packet weight
Soft drinks	Squash, undiluted or diluted; fizzy drinks; low calorie; give brand name	Glasses or cans
Soup	What sort; canned, packet, instant or vending machine, homemade; give brand name	Tablespoons, bowl or mug
Soya, Quorn	Mince, burgers or tofu	Number
Spaghetti, other pasta	Canned, boiled; white, wholemeal; in sauce	Tablespoons or picture 13
Spices	Type	½ or ¼ teaspoons
Spreads	On bread, what sort and brand	½ or ¼ teaspoons
Spirits	What sort: e.g. whisky, gin, vodka, rum, at home or in pub	Single measures as in pub
Sugar	Added to cereals, tea, coffee, fruit etc	Heaped or level teaspoons
Sweets	What sort: e.g. toffees or boiled sweets; diabetic; give brand name	Number
Tea	With or without milk; herb, decaffeinated	Cups
Vegetables	What sort and variety; with butter,	Tablespoons or

Food / Drink	Description and Preparation	Amount
	other fat or sauce; how cooked e.g. fried, boiled, microwaved or raw	picture 14
Water	state whether tap, filtered or bottled	Glasses
Wine, sherry, port	White, red; sweet, medium, dry; low alcohol	Glasses
Yoghurt, fromage frais	What sort: e.g. with fruit, natural, plain; flavour; low fat, greek, creamy, soya	Cartons, tablespoons
Homemade dishes	Please say what the dish is called and give recipe or ingredients if possible	Tablespoons or one of the pictures
Ready made meals	What sort; e.g. pizzas, microwave dishes, slimmers' meals etc. please give main ingredients on packet and enclose label if possible	Weight from packet
Meals eaten away from home	What sort: e.g. pizzas, Chinese, Indian dishes, fish and chips, hamburgers, hot dogs etc. please say what the dish is called and give ingredients where possible. Give the name of the restaurant if it a well known chain	Tablespoons or one of the pictures

Weights and measures:

1 ounce = 28.35 grams

1 fluid ounce = 28.41 ml

1 pint = 568.3 ml

1 litre = 1.76 pints

1 pound = 453.6 grams

1 teaspoon = 5 grams

1 gram = 0.0353 oz

1 kilogram = 2.20516 lb

1 tablespoon = 15 grams

1 dessertspoon = 10 grams

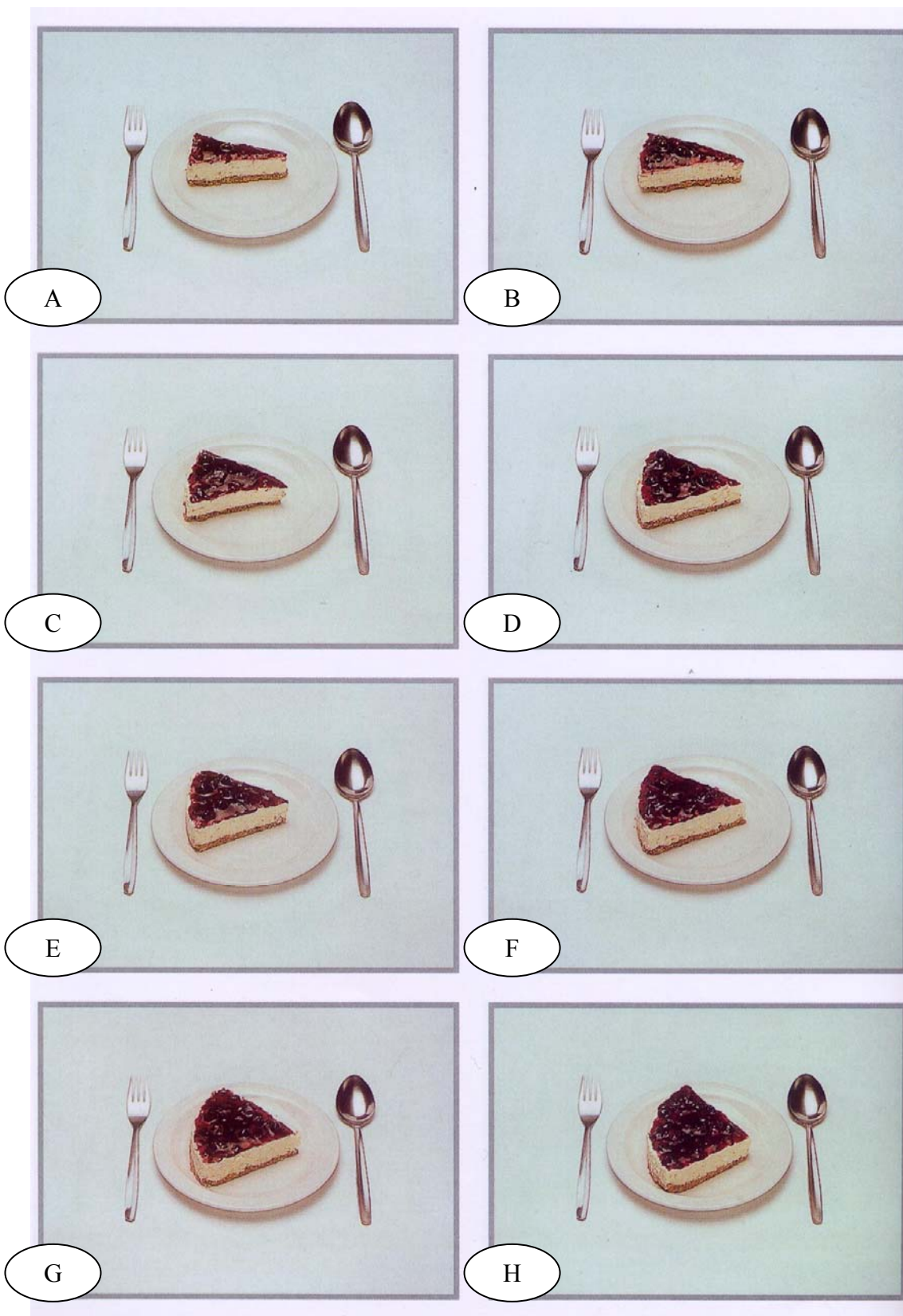
1 cup = 237 ml

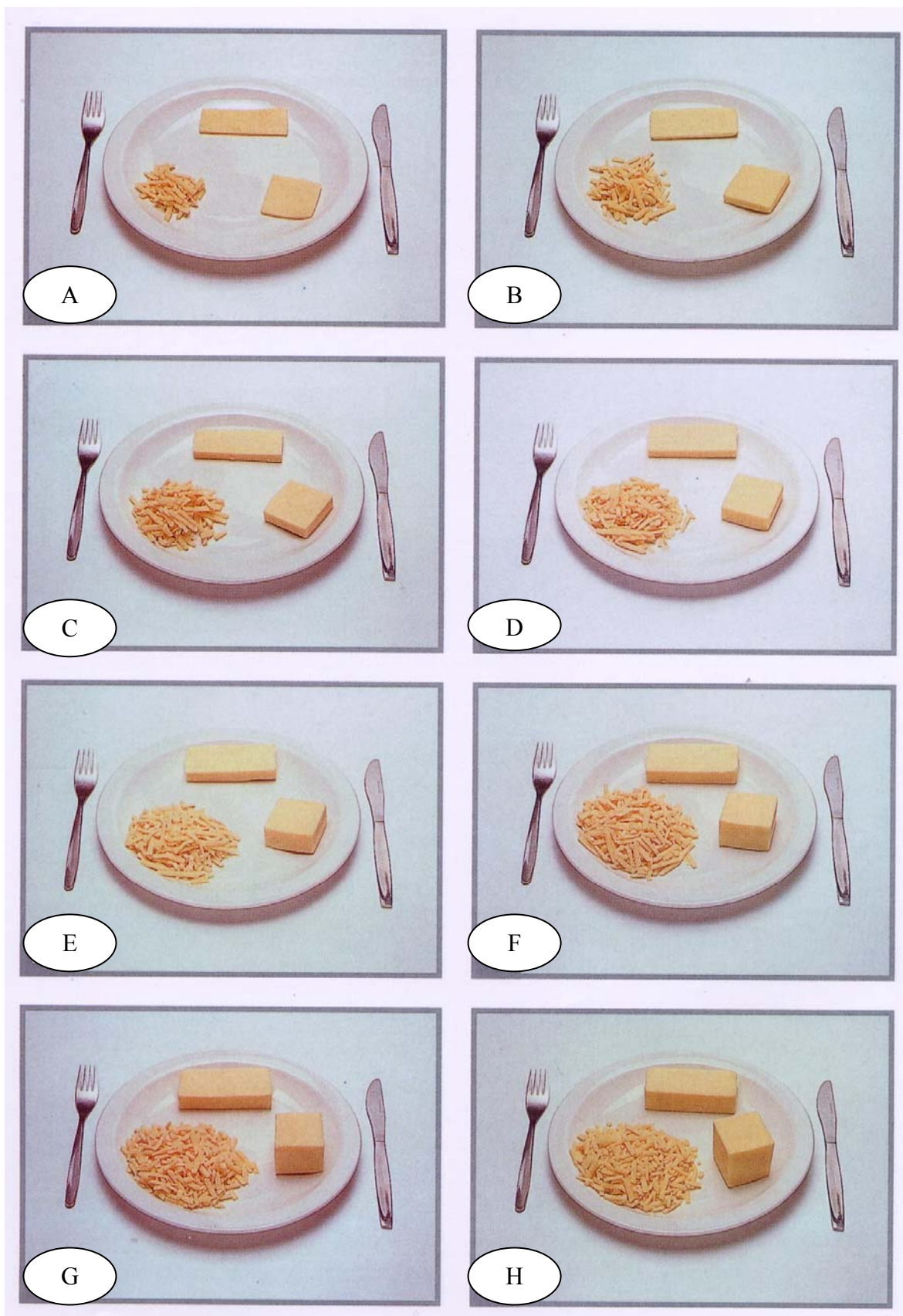
1 quart = 947 ml

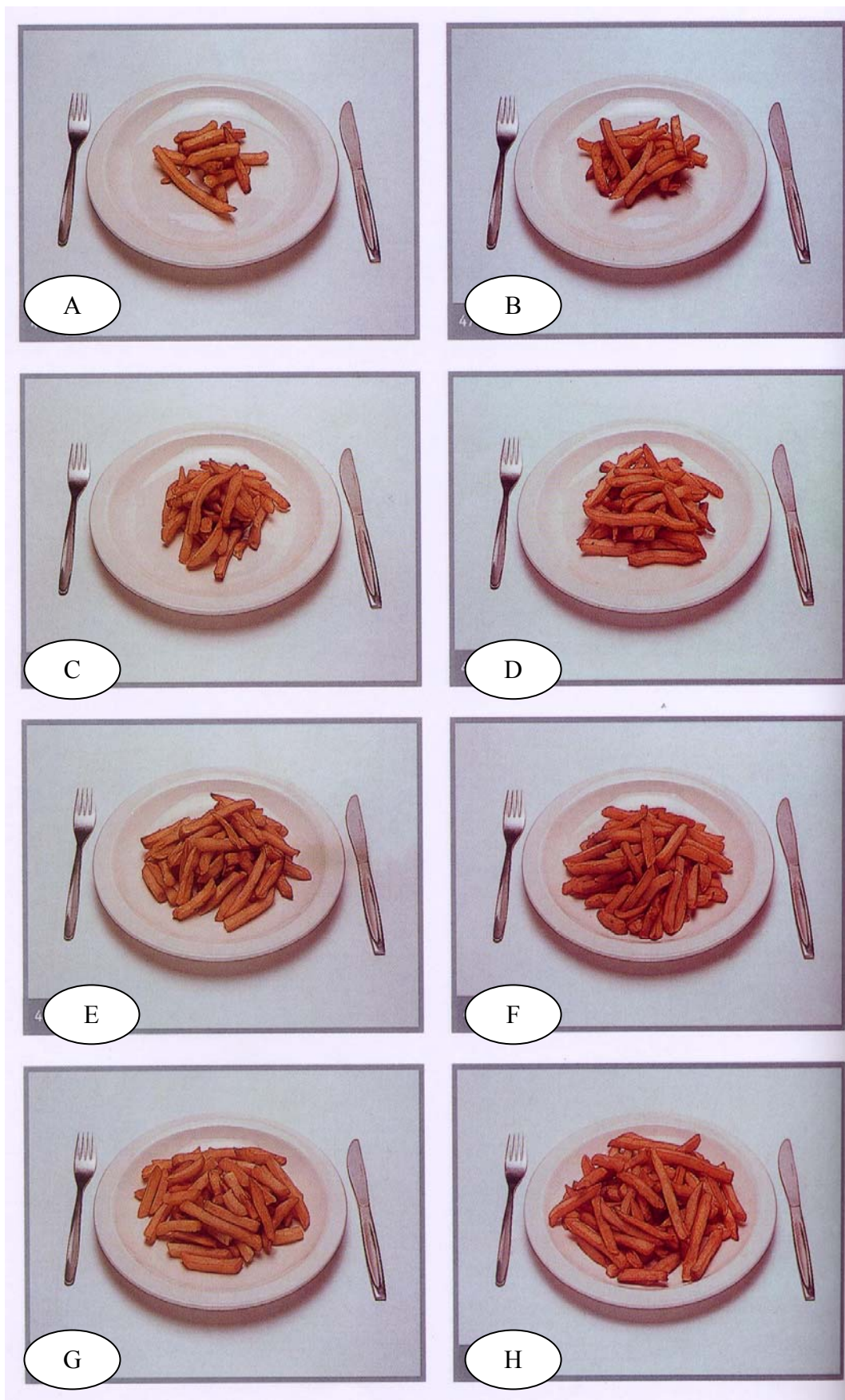
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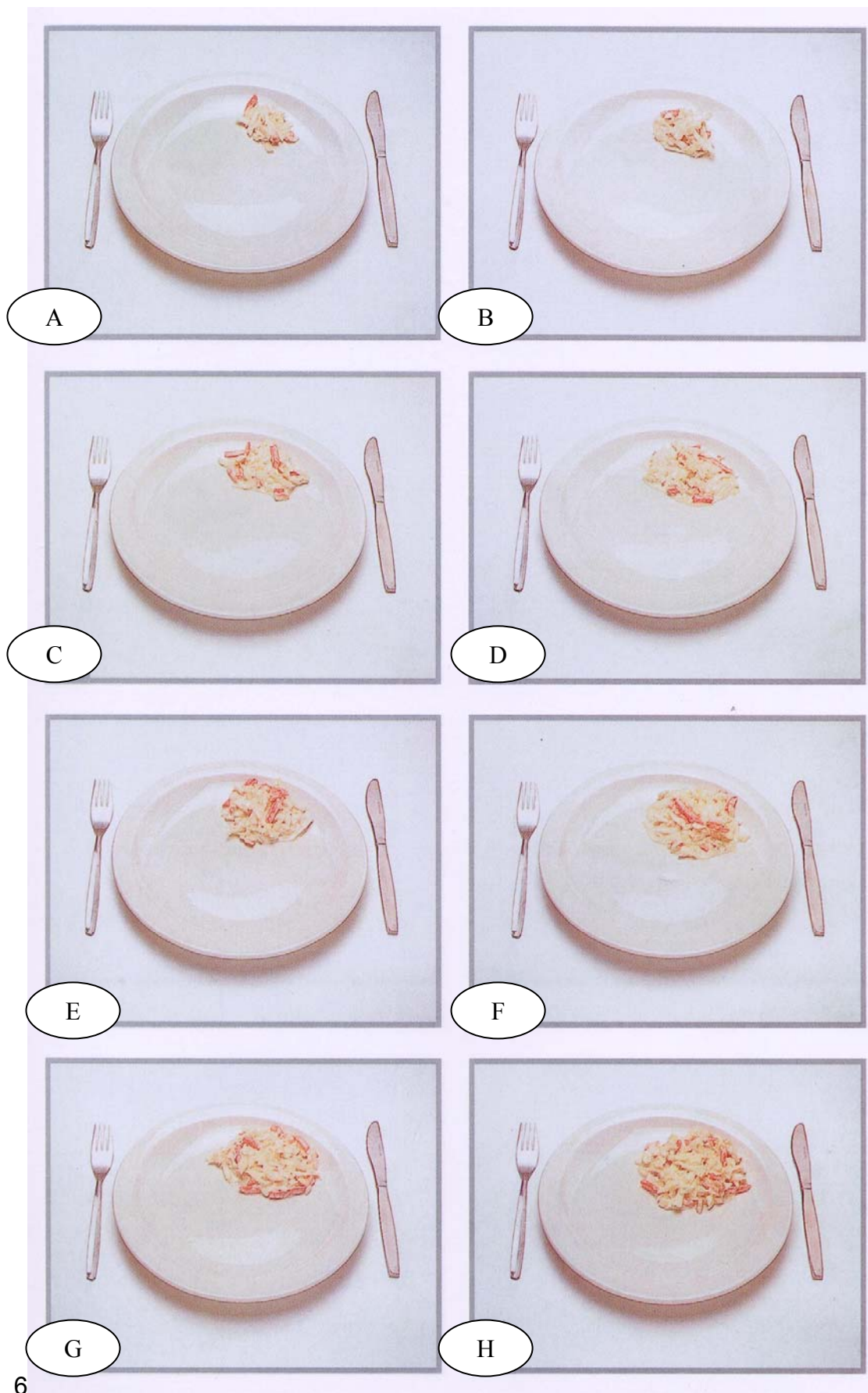
Time	Food/ drink	Description and preparation	Amount
12 pm	Soup	Campbell's condensed cream of mushroom, diluted half ad half with	1 medium bowl

		water	
1 pm	Bread	White, Hovis	2 medium slices
	Butter	Anchor salted	Thick spread
	Biscuits	Jacobs cream crackers	4 biscuits
	Cheese	Tesco matured cheddar	Picture 2A
	Tomatoes	Fresh	2 medium
3 pm	cake	Home made Victoria sponge with jam filling	Picture 15 B
	Tea	Tetley's tea bag	2 cups
	Milk	Full cream, Silver top	Added to each cup
	Sugar	White granulated	2 heaped teaspoons in each

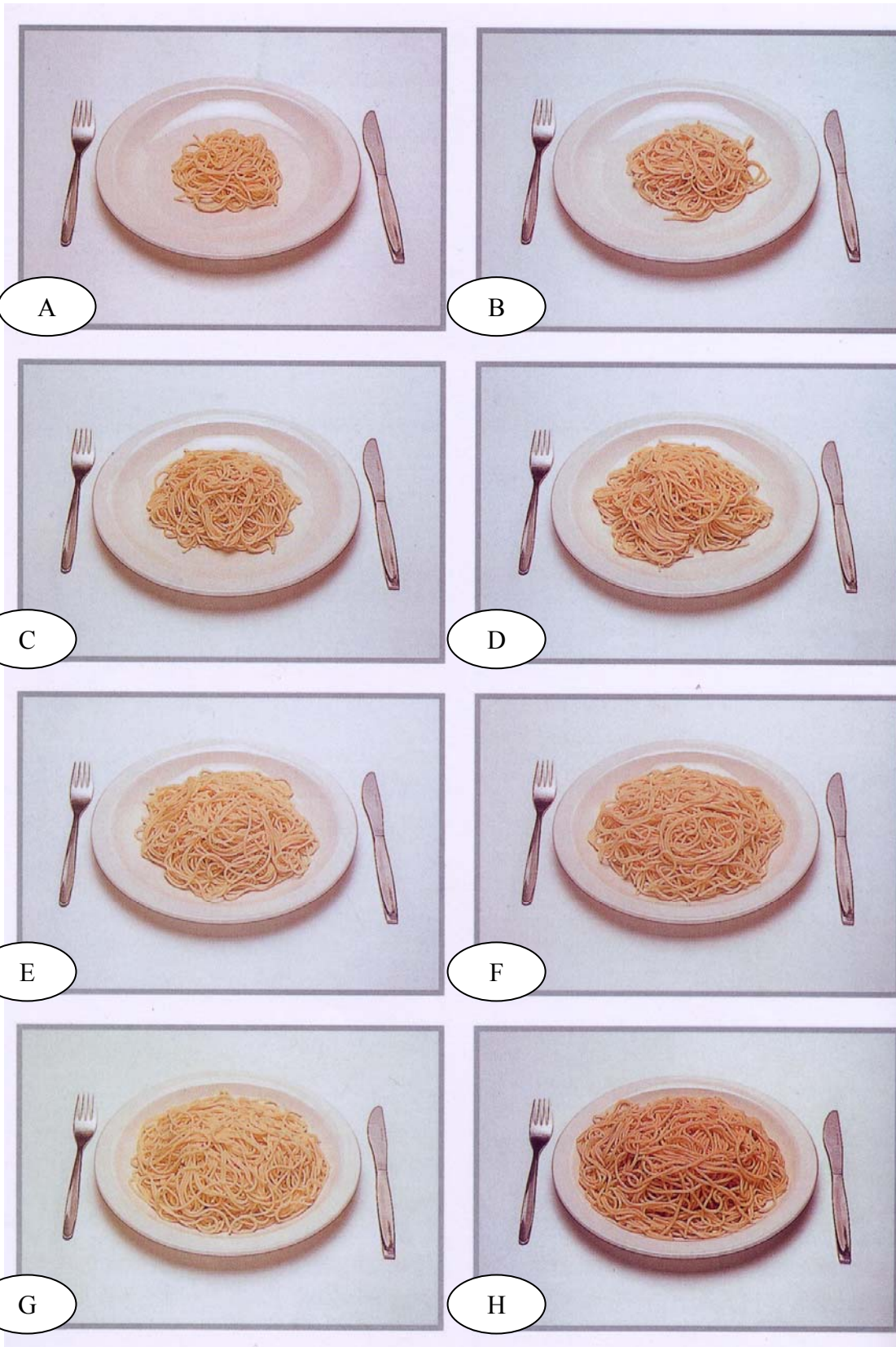


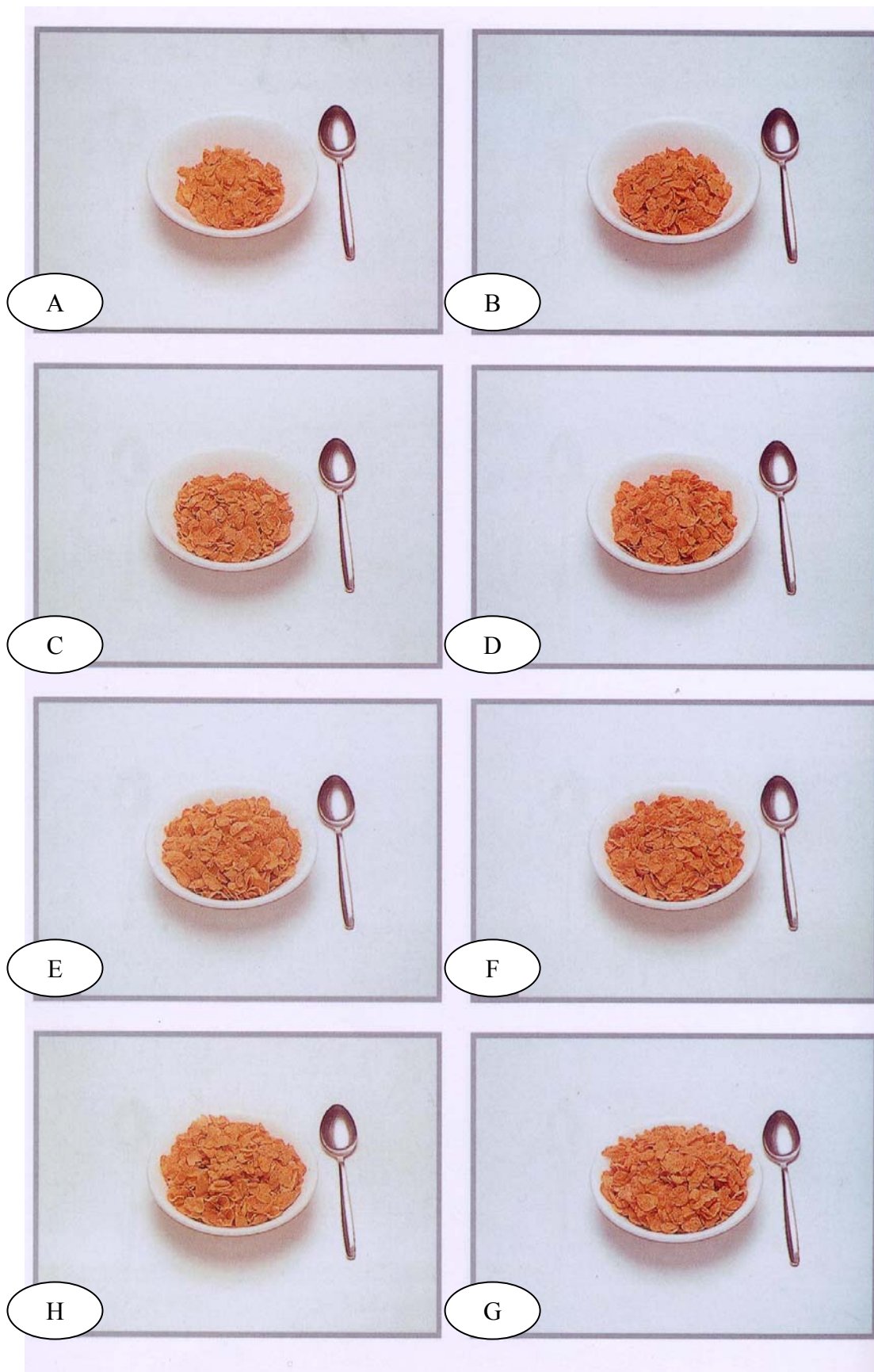


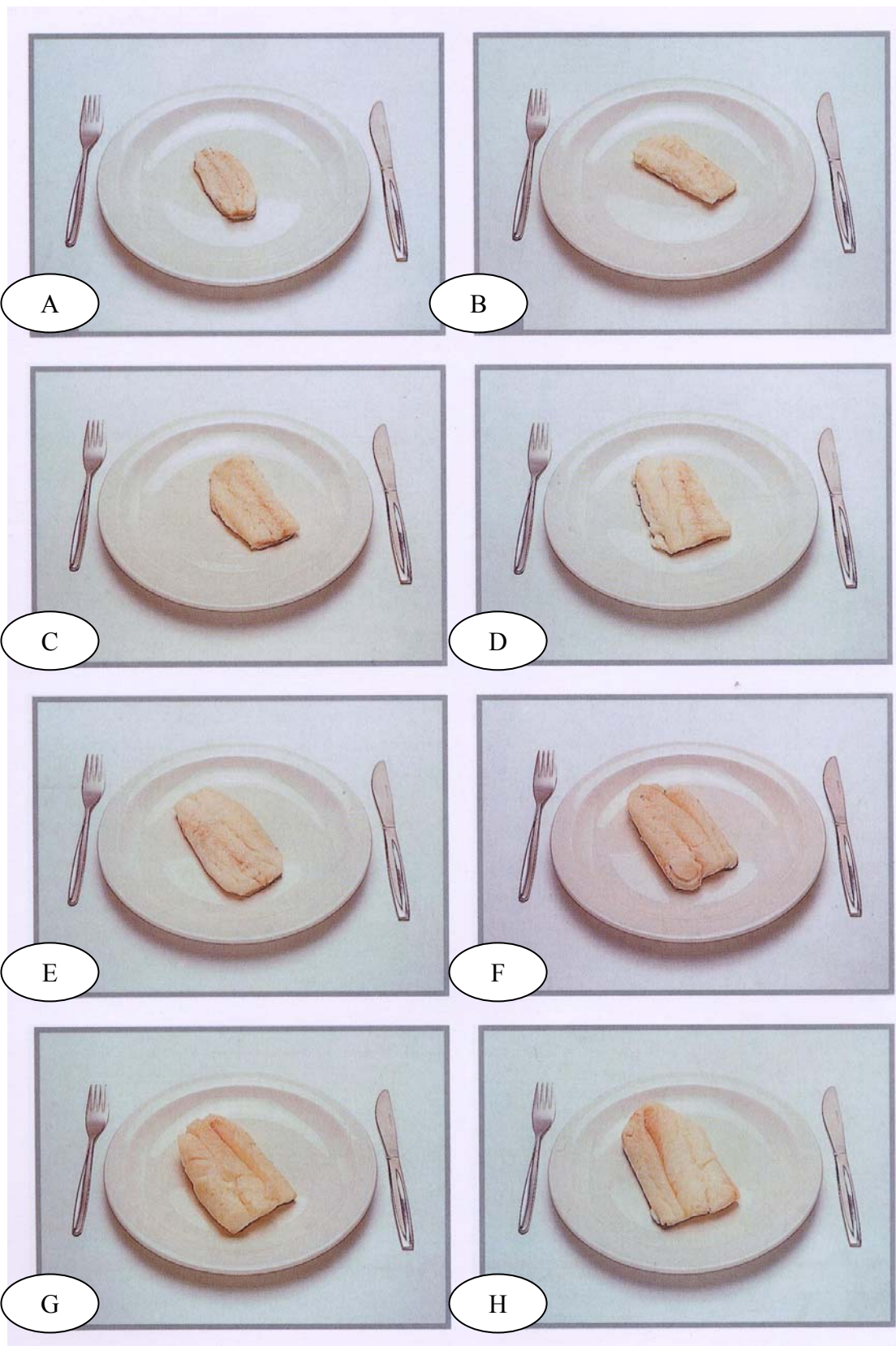


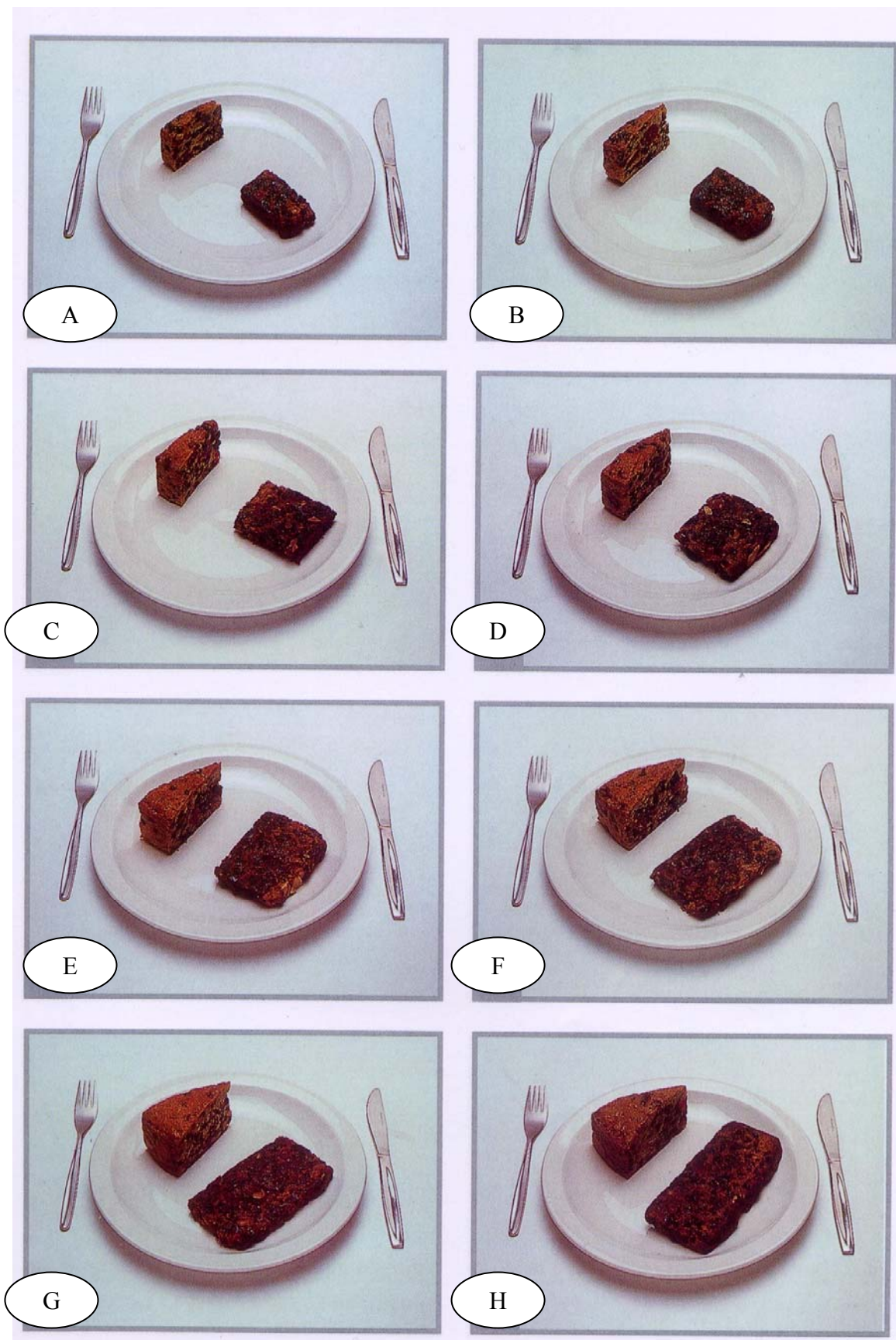


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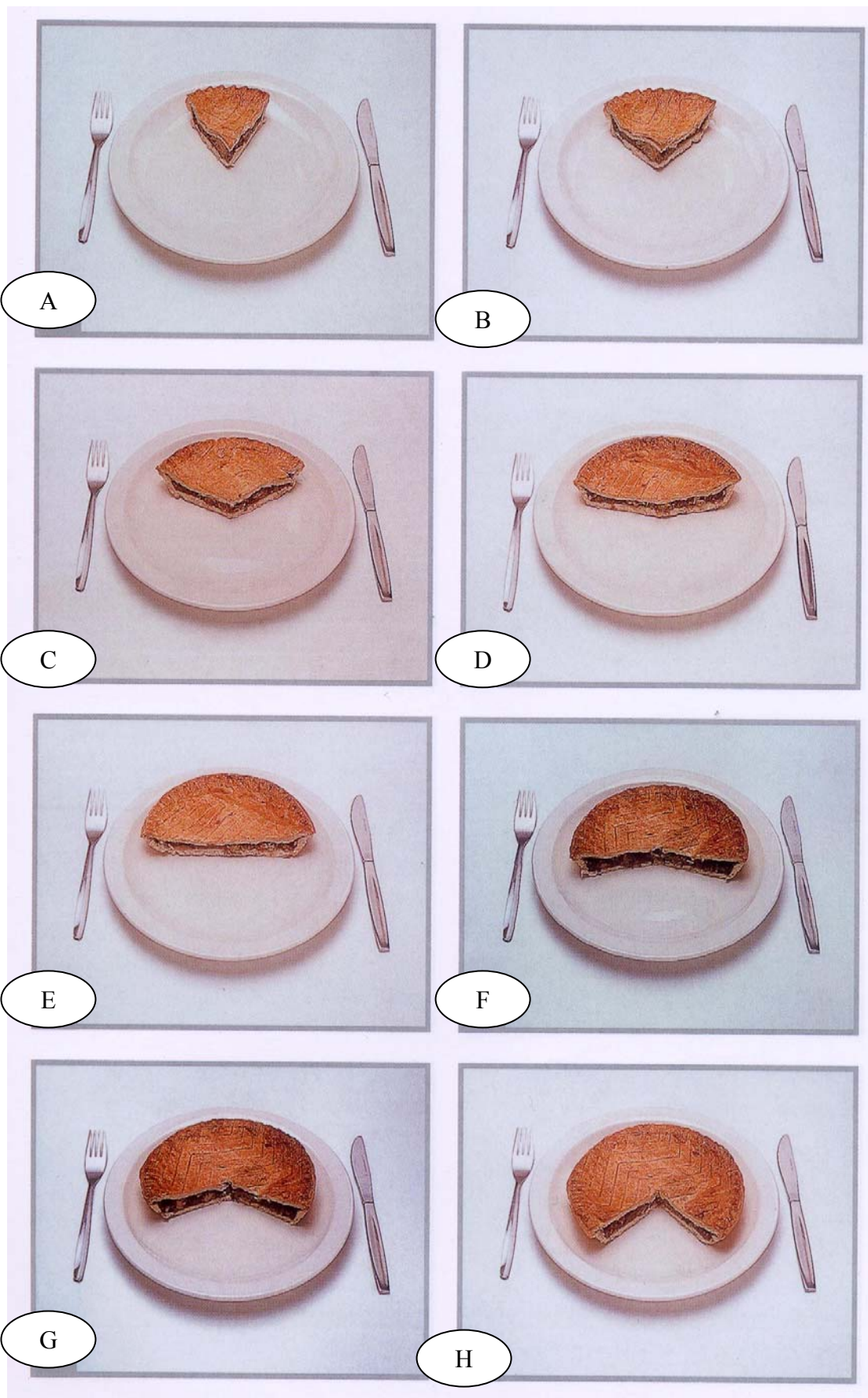


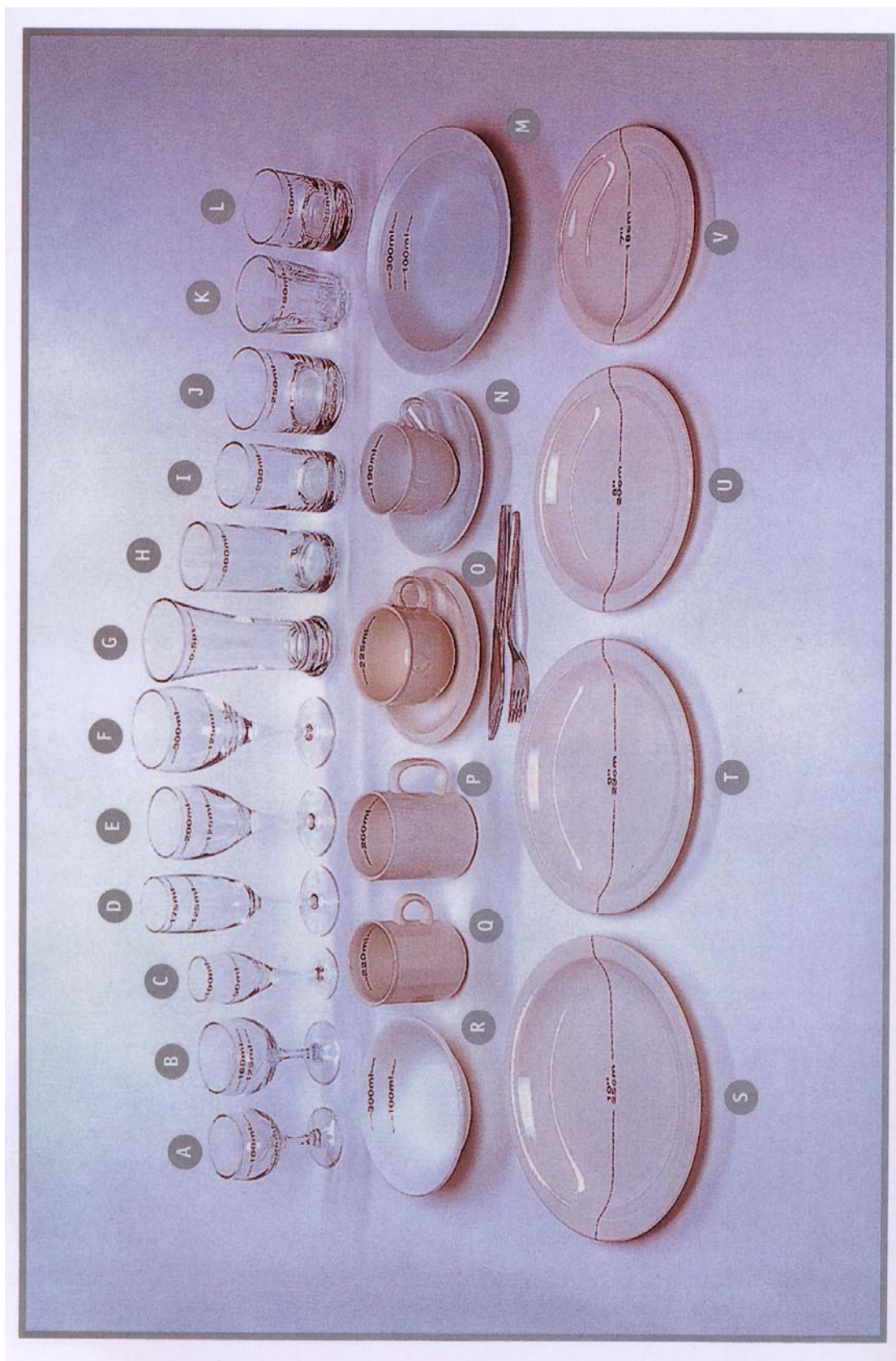


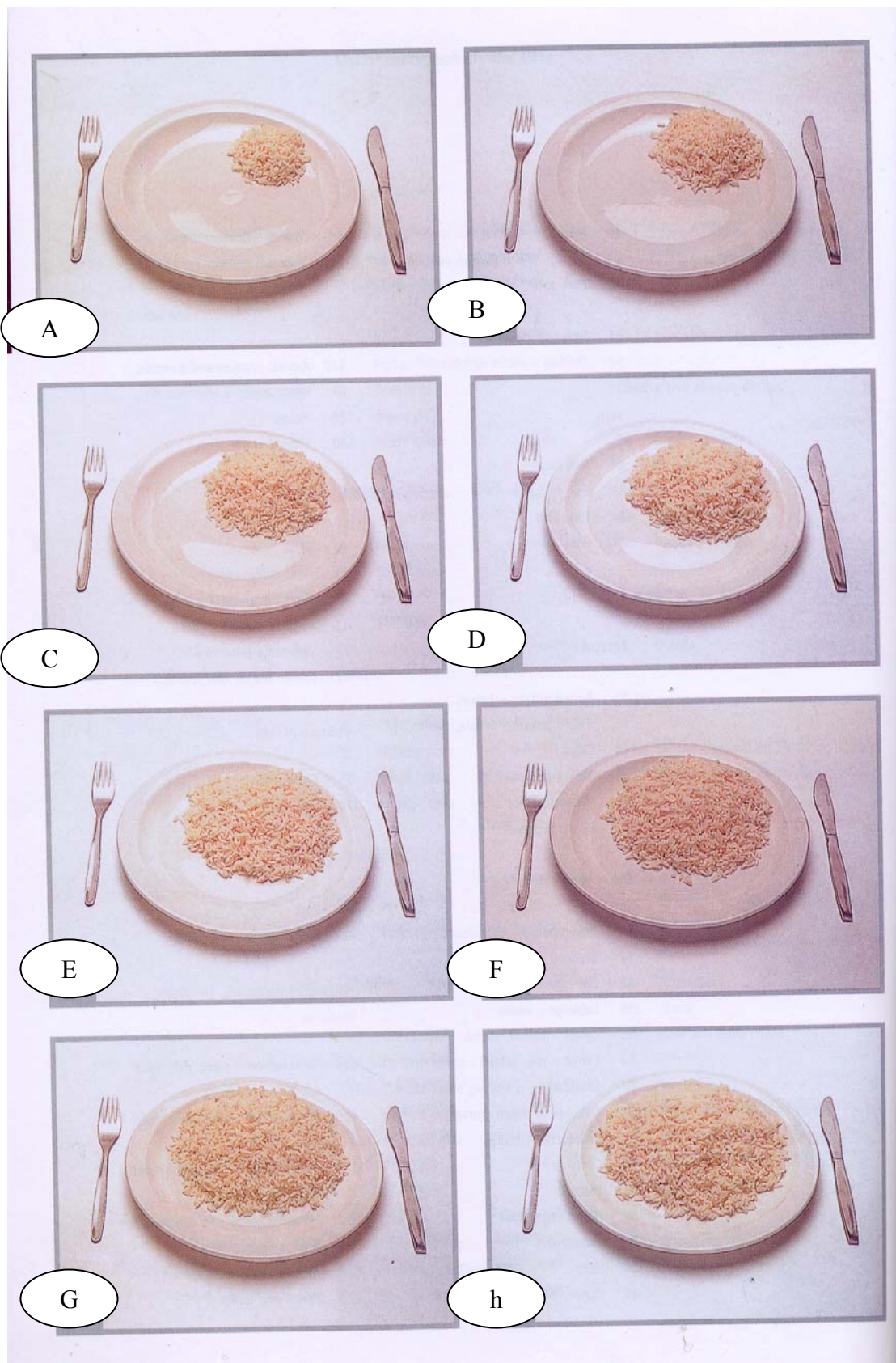


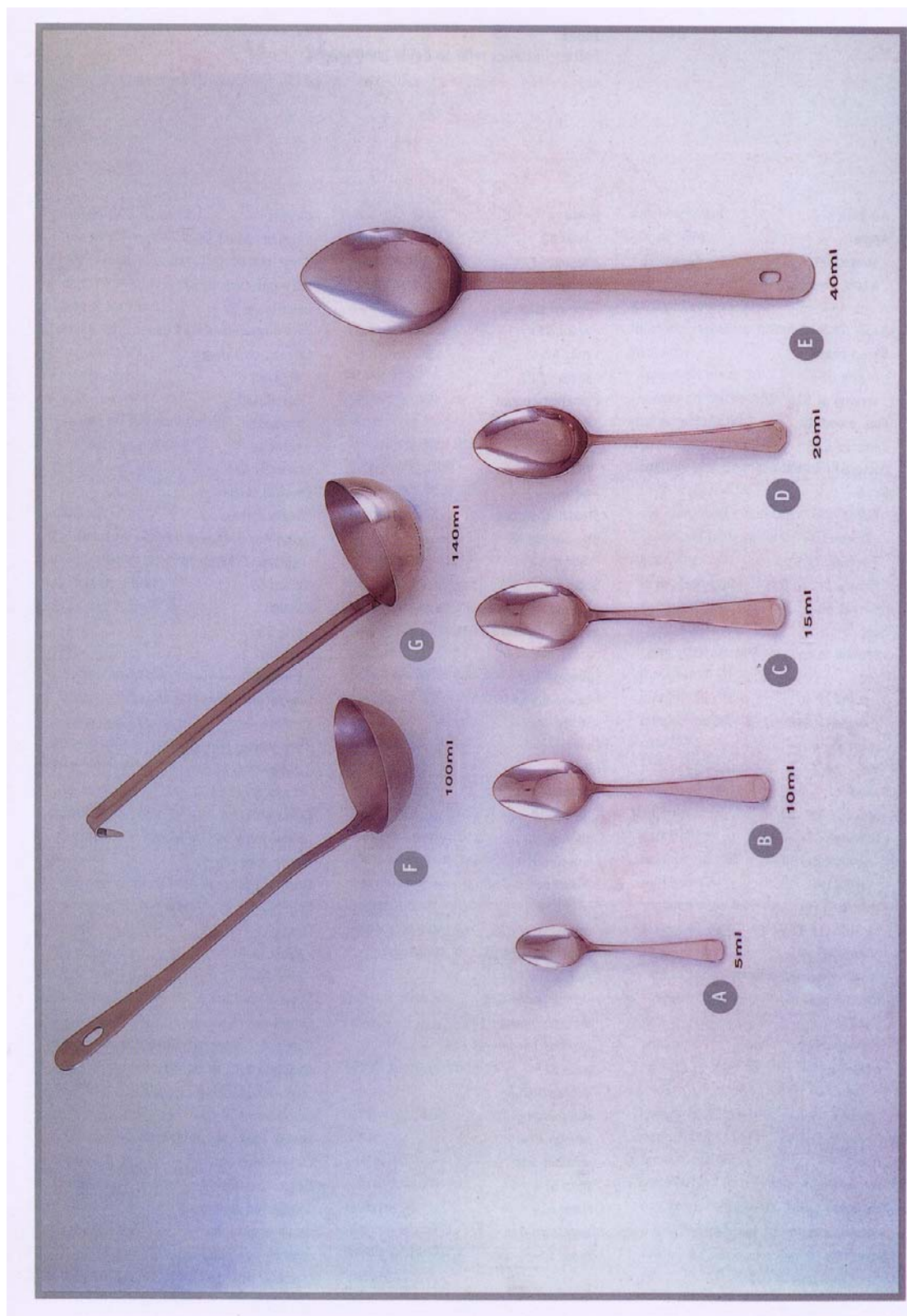


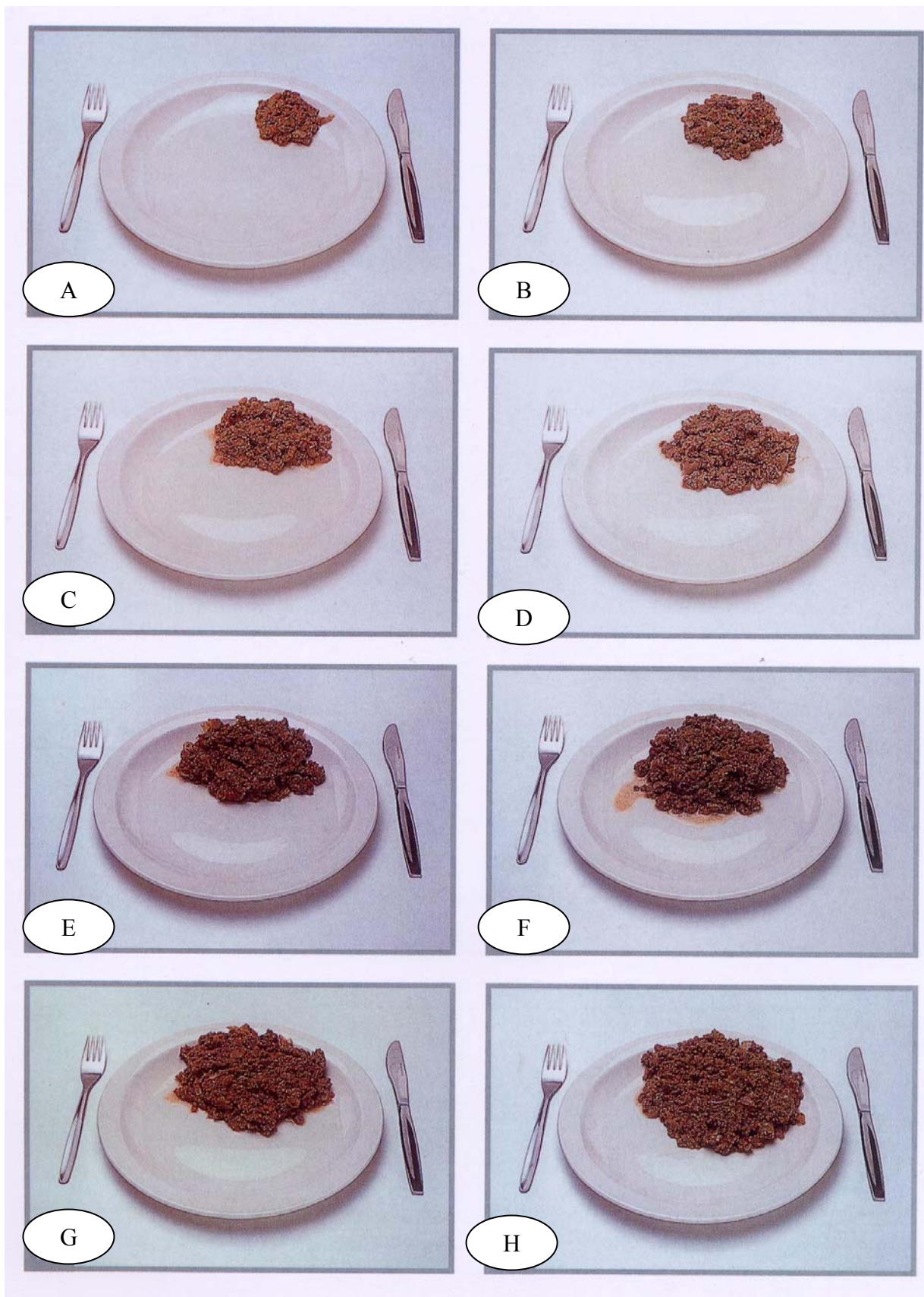


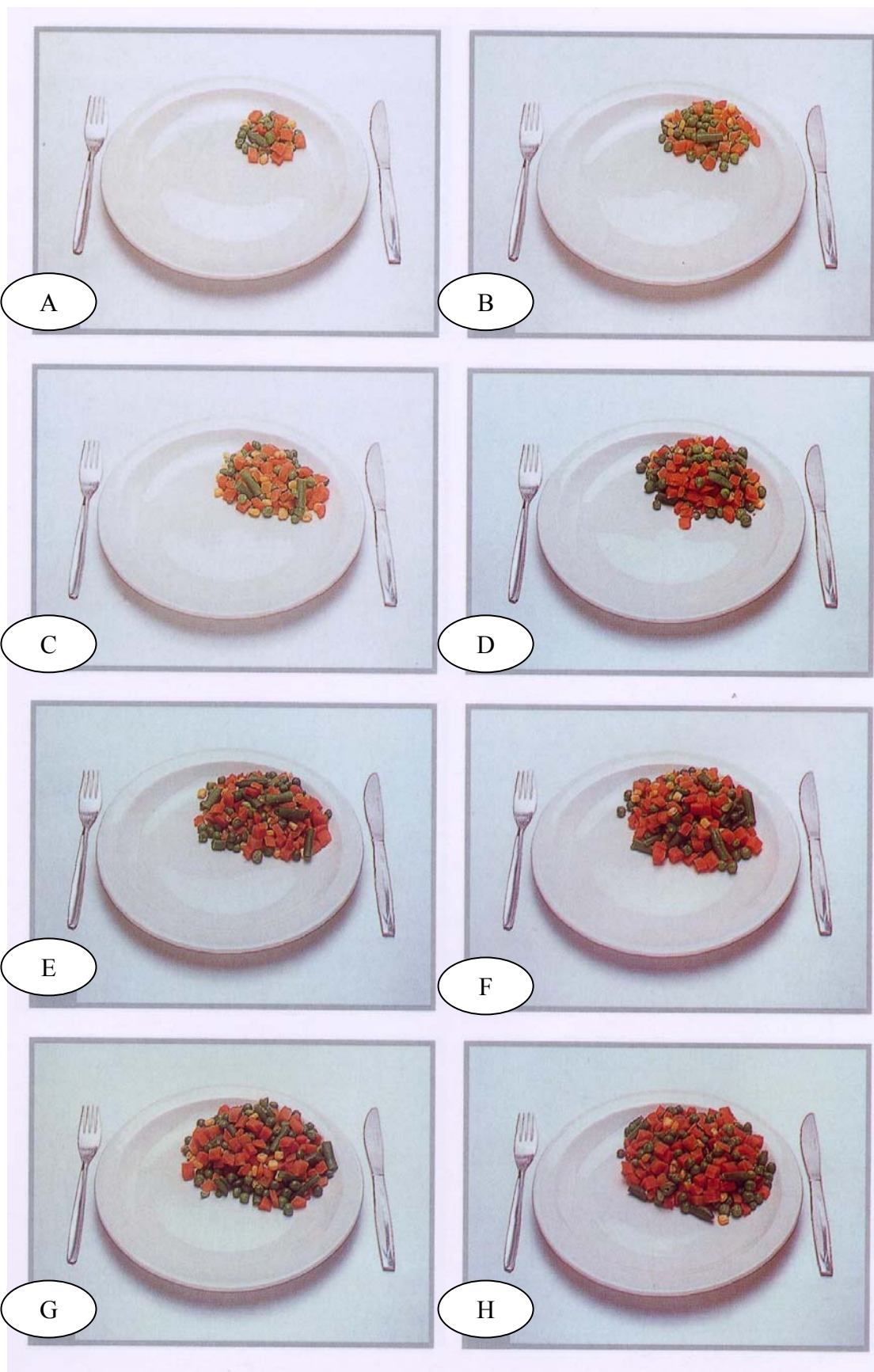












Appendix 17 - Catechin and epicatechin content in raw and processed foods and selected beverages

Product	Catechin content (mg/kg of fresh edible weight) ^{a,b}			Total catechins
	(+)-catechin	(-)-epicatechin	Others	
Apple with skin				
Cox's orange Pippin	12.8 ± 1.67	96.2 ± 18.37	ND ^c	109.0
Elstar	12.4 ± 2.11	81.7 ± 12.41	ND	94.0
Golden delicious	5.3 ± 0.70	74.2 ± 7.44	ND	79.5
Granny smith	15.6 ± 4.33	74.8 ± 15.82	ND	90.3
Apple without skin				
Cox's orange Pippin	12.8	66.5	ND	79.3
Elstar	11.0 ± 3.29	66.4 ± 3.86	ND	77.4
Golden delicious	4.4 ± 0.26	50.7 ± 8.54	ND	55.1
Granny smith	16.5 ± 5.19	65.3 ± 15.24	ND	81.1
Applesauce	6.9	54.1	ND	61.0
Apricot	49.5 ± 43.68	60.6 ± 78.49	ND	110.1
Avocado		5.6 ± 2.91	ND	5.6
Blackberry	6.6 ± 0.58	180.8 ± 21.39	ND	187.4
Blueberry		11.1 ± 1.00	ND	11.1
Broad beans, raw	128.3 ± 160.60	225.1 ± 184.78	EGC: 140.3 ± 127.90	493.7
Prepared	81.6 ± 36.44	78.2 ± 40.93	EGC: 46.5 ± 23.17	206.3
Canned	ND	ND	ND	ND
Cherry, sweet	21.7 ± 9.18	95.3 ± 24.84	ND	117.1
Canned	ND	43.1	ND	43.1
Cranberry	ND	42.0	ND	42.0
Currant, black	7.0	4.7	ND	11.7
Currant, white	3.0	ND	7.0	10.0
Currant, red	12.2 ± 4.35	ND	GC: 12.2 ± 10.85	24.4
Gooseberry	16.7 ± 3.63	ND	GC: 4.4 ± 6.27	21.2
Grape, black	89.4 ± 91.80	86.4 ± 71.20	ECG: 28.1 ± 37.93	203.9
Grape, white	24.7 ± 10.59	10.2 ± 5.18	ECG: 4.3 ± 8.54	39.2
Kidney-bean, canned	16.6	3.5	ND	20.1
Kiwi fruit	ND	4.5 ± 1.05	ND	4.5
Mango	17.2 ± 15.72	ND	ND	17.2
Nectarine	27.5 ± 2.42	ND	ND	27.5
Peach	23.3 ± 5.66	ND	ND	23.3
Peach, canned	18.7	ND	ND	18.7
Pear with skin				
Conference	1.1 ± 0.36	29.5 ± 1.69	ND	30.6
Cooking pear ^d	9.6 ± 4.77	75.4 ± 32.42	ND	85.0
Canned pear	1.8	2.6	ND	4.4
Pear without skin				
Conference	0.4 ± 0.18	8.2 ± 2.72	ND	8.5

Product	Catechin content (mg/kg of fresh edible weight) ^{a,b}			Total catechins
	(+)-catechin	(-)-epicatechin	Others	
Cooking pear ^d	3.6 ± 0.18	29.6 ± 3.25	ND	33.3
Prepared	3.3	21.2	ND	24.5
Plum	33.5 ± 9.13	28.4 ± 31.89	ND	61.9
Raspberry	9.7 ± 2.57	82.6 ± 13.06	ND	92.3
Rhubarb	21.7 ± 11.39	5.1 ± 3.30	ECG: 6.0 ± 3.71	32.8
Prepared	14.8 ± 10.61	3.8 ± 1.31	ECG: 4.9 ± 6.06	23.5
Strawberry	44.7 ± 13.80	ND	ND	44.7
Chocolate, black (Albert Heijn ^e)	132.4	327.4	ND	459.8
Chocolate, milk (Albert Heijn)	38.3	124.9	ND	163.2
Chocolate bar, Mars	21.7	62.5	ND	84.2
Jam, apricot (Albert Heijn)	4.7	5.0	ND	9.7
Jam, cherry (Albert Heijn)	1.6	9.0	ND	10.6
Jam, forest fruit (Albert Heijn)	0.7	15.7	ND	16.4
Jam, strawberry (Albert Heijn)	9.0	ND	ND	9.0
Raisins	29.7	7.1	ND	36.8

a- Data include seasonal and regional variation, ECGG was not detected in any food; no catechins were detected in banana

b- Annual mean ± SD, based on duplicate analyses for each season; food bought in only one season if no SD is given

c- Not detected

d- Different varieties

e- Supermarket chain in Netherlands

Adapted from (Arts *et al.*, 2000a)

Catechin content of selected beverages:

Product	Catechin content (mg/l) ^a		
	(+)- catechin	(-)-epicatechin	Total catechins
Red wine			
Bordeaux (Appellation Controlee, Rineau, 1997)	53.4	42.1	95.5
Cotes-du-Rhone (Appellation Controlee, Les Vendanges, 1997)	47.7	18.8	66.5
Rioja (Siglo, 1996)	22.5	9.2	31.7
Pinotage/ Merlot (Stellenbosch Welmoed Winery, 1997)	25.2	18.9	44.1
White wine			
Bordeaux Sauvignon (Appellation Controlee, Chateau de Nivelle, 1997)	2.1	0.7	2.8

Product	Catechin content (mg/l) ^a		
	(+)- catechin	(-)-epicatechin	Total catechins
Bordeaux (Appellation Controlee, Rineau, 1997)	4.7	1.0	5.7
Other beverages			
Apple juice (Riedel)	ND ^b	ND	ND
Black grape juice (Albert Heijn)	8.0	ND	8.0
White grape juice (Albert Heijn)	2.0	ND	2.0
Iced tea (Lipton ice lemon, non carbonated)	ND	0.8	0.8
Lager beer (Heineken)	ND	ND	ND
Chocolate milk (semi skimmed, Nutricia)	16.1	5.0	21.1

a- Average of duplicate analyses; GC, EGC, ECG, EGCG were not detected in any of the samples

b- Not detected

Adapted from (Arts *et al.*, 2000b)

Appendix 18 -Reference values

- **Percent body fat:**

<u>Body fat rating</u>	<u>Men</u>	<u>Women</u>
Risky (high body fat)	>30%	>40%
Excess fat	21-30%	31-40%
Moderately lean	13-20%	23-30%
Lean	9-12%	19-22%
Ultra lean	5-8%	15-18%
Risky (low body fat)	<5%	<15%

- **Blood glucose:**

Fasting blood glucose ≥ 7 mmol/l

Random blood glucose > 11 mmol/l

- **Total cholesterol:**

Low < 5.2 mmol/l

Average $5.2 - 6.2$ mmol/l

High > 6.2 mmol/l

- **HDL:**

Good: > 0.9 mmol/l

Poor: < 0.9 mmol/l

- **LDL:**

Good: < 3.4 mmol/l

Poor: > 4.1 mmol/l

- **Body mass index (BMI):**

Underweight < 20

Normal $20 - 25$

Overweight $25 - 30$

Obese 30 and above

- **NEFA:**

Men – 0.1 – 0.6 $\mu\text{mol/l}$ (2.8 – 16.9mg/dl)

Women – 0.1 – 0.45 $\mu\text{mol/l}$ (2.8 – 12.7mg/dl)

- **Expected reference values of insulin:**

2 – 25 mU/l with a mean of 9.2 mU/l

- **Expected reference values of leptin:**

Men – $3.84 \pm 1.79\text{ng/ml}$

Women – $7.36 \pm 3.73\text{ng/ml}$

Appendix 19 -Detailed protocol for Insulin ELISA analysis

Insulin, the principal hormone responsible for control of glucose metabolism, was measured using the Mercodia Insulin ELISA 10-1113-01 (Mercodia AB, Sweden). This is a solid phase two-site enzyme immunoassay, based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase conjugated anti-insulin antibodies and anti-insulin antibodies bound to micro-titration well. A simple washing step removes unbound enzyme labelled antibody. The bound conjugate is detected by reaction with 3, 3', 5, 5'- tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450nm.

Sample collection and handling:

Blood was collected by venepuncture into heparin containing tubes (BD Vacutainer® Plasma Tube, Becton Dickinson UK Limited) to prevent coagulation and stored at -80°C until analysis.

Procedure:

All samples and reagents were thawed and brought to room temperature before use. All calibrators and samples were analysed in duplicates as below-

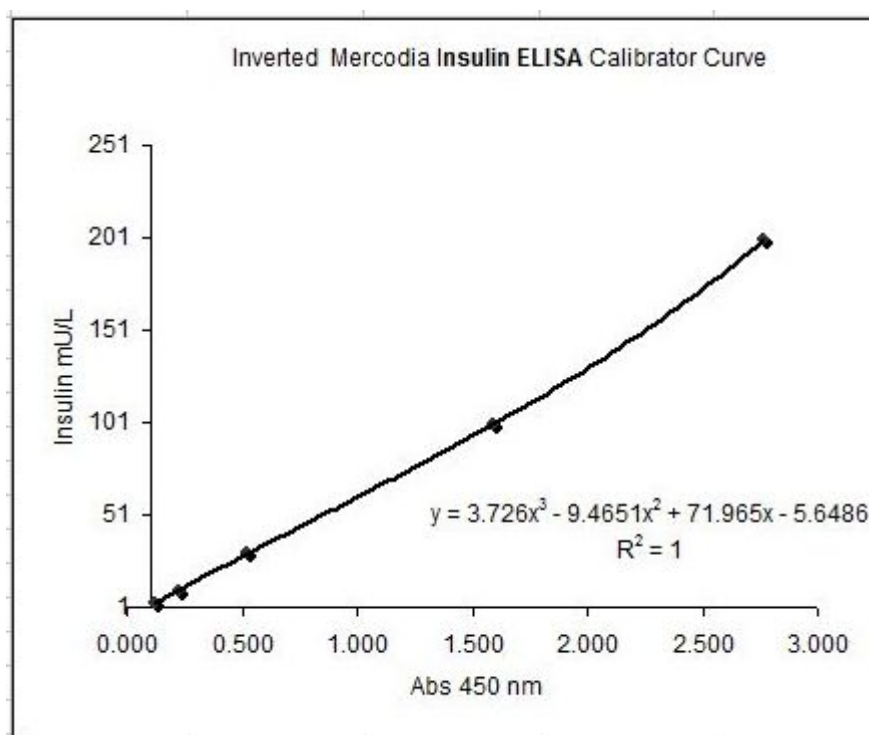
1. Enzyme conjugate solution was prepared by diluting 1ml of Enzyme Conjugate 11X in 10mls of Enzyme Conjugate Buffer and mixed gently.
2. Wash buffer was prepared by diluting 40mls of Wash Buffer 21X solution with 800mls distilled water.
3. 25µl of each calibrator and sample was pipetted out into the appropriate microplate wells.
4. 100µl of enzyme conjugate solution was added to each well.
5. This was then incubated on a plate shaker for 1 hour at room temperature.
6. After incubation, the reaction volume was aspirated and 350µl wash buffer was added to each well which was later aspirated well. This was repeated 5 times and

after the final wash, the plate was inverted and tapped firmly against absorbent paper.

7. To this, 200 μ l Substrate TMB was added into each well.
8. This was left to incubate for a further 15 minutes at room temperature.
9. 50 μ l of Stop Solution was added to each well and placed on a shaker for approximately 5 seconds to ensure mixing.
10. The optical density was then read at 450nm and results calculated with the help of calibration curve.

Calculation of results:

The concentration of insulin was obtained by computerised data reduction of the absorbance for the calibrators, except for calibrator 0, versus the concentration using cubic spline regression.



Example of calibrator curve

Appendix 20 - Detailed protocol for Leptin ELISA analysis

Leptin is a 16 kDa protein hormone that plays a key role in regulating energy intake and energy expenditure, including the regulation (decrease) of appetite and (increase) of metabolism. To measure this key marker of metabolism, Leptin (Sandwich) ELISA EIA-2395 (DRG Instruments GmbH, Germany) was used.

This is a solid phase enzyme-linked immunoabsorbent assay based on the sandwich principle. The microtitre wells are coated with a monoclonal antibody directed towards a unique antigenic site on a leptin molecule. When an aliquot of sample containing endogenous leptin is incubated in the coated well with a specific rabbit anti-leptin antibody, a sandwich complex is formed. After incubation, the unbound material is washed off and an anti-rabbit peroxidase conjugate is added for detection of the bound leptin. After addition of the substrate solution, the intensity of colour developed is proportional to the concentration of leptin in the sample.

Sample collection and handling:

Blood was collected by venepuncture into heparin containing tubes (BD Vacutainer® Plasma Tube, Becton Dickinson UK Limited) to prevent coagulation and stored at -80°C until analysis.

Procedure:

All samples and reagents were thawed and brought to room temperature before use. All standards and samples were analysed in duplicates as below-

Wash solution was prepared by diluting 30 mls of concentrated wash solution with 1170 mls distilled water to a final volume of 1200 mls.

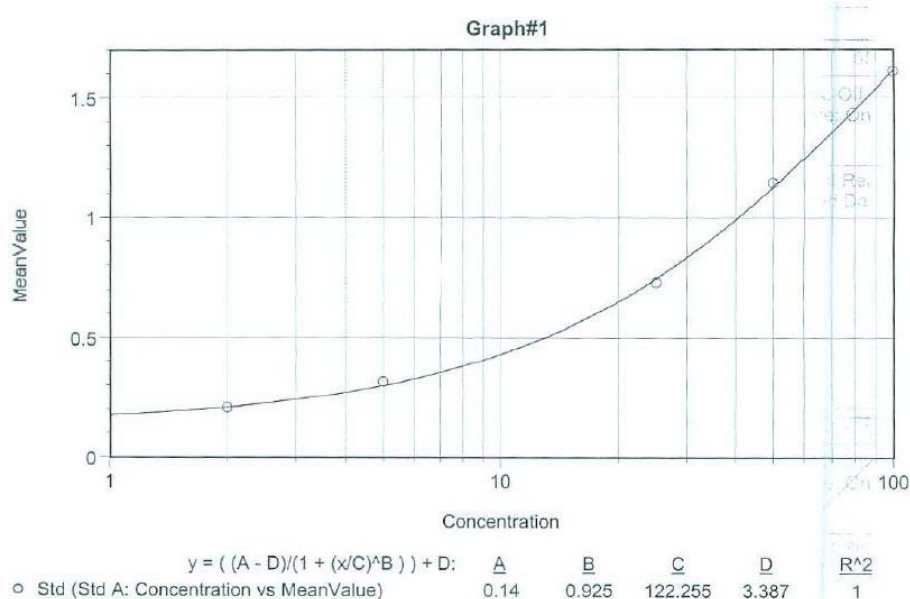
1. 15µl of each standard, control and samples were dispensed into microtitre wells with new disposable pipette tips.
2. To this 100µl of assay buffer was added and thoroughly mixed for 10 seconds by placing on a plate shaker.
3. The above plate was incubated for 120 minutes at room temperature and then washed thoroughly by briskly shaking out the contents of the wells. 300µl of Wash

Solution was added to each well and this was repeated 3 times. This was then tapped sharply on absorbent paper to remove residual droplets.

4. 100µl of Antiserum was then added into each well and left to incubate at room temperature for 30 minutes.
5. The plate was thoroughly washed as explained in point 3.
6. To this, 100µl of Enzyme Complex was added into each well and left to incubate at room temperature for 30 minutes.
7. The above solution was washed thoroughly as mentioned in point 3.
8. 100µl of Substrate Solution was added to each well and left to incubate at room temperature for 15 minutes.
9. To stop the enzymatic reaction, 50µl of Stop Solution was added to each well.
10. Optical density was read at 450nm with a microtitre plate reader within 10 minutes after adding the Stop Solution.

Calculation of results:

Average absorbance values for each standards, controls and study samples were calculated. A standard curve was constructed by plotting mean absorbance obtained from each standard against its concentration with absorbance values on the Y-axis and concentration on the X-axis. Using the mean absorbance value for each sample the corresponding concentration was determined from the standard curve. This was performed using a four parameter logistic function as recommended in the package insert with the help of Softmax Pro version 4.0 (Microplate Data Acquisition and Analysis Software Molecular Devices, USA).



Appendix 21 - Detailed protocol for NEFA analysis

Wako NEFA C test kit (Wako Chemicals GmbH, Germany) was used to measure NEFA concentration in plasma. This kit utilises an *in vitro* enzymatic colorimetric method for NEFA quantification.

Preparation:

- Colour reagent solution A: Dissolve contents of colour reagent A with 10ml of solvent A and mix well.
- Colour reagent solution B: Dissolve contents of colour reagent B with 20ml of solvent B and mix well.

Procedure:

All samples and reagents were thawed and brought to room temperature before use. All standards and samples were analysed in duplicates as below-

1. Pipette into vials 50µl of samples and control solution. To this, add 1000µl of colour reagent solution A and mix well. All samples and control solutions were analysed in duplicate.
2. Incubate for 15 minutes at 25°C.
3. To this add 2000µl of colour reagent solution B and mix well.
4. Accurately incubate for 15 minutes at 25°C.
5. Read absorbance at wavelength 550nm.

Results:

ILAB 600 (Instrumentation Laboratory, Werfen Group IVD, Spain) was used to analyse the samples along with control solutions. The analyser is controlled by an external PC running under Windows XP. The results were displayed onto the excel sheet and later tabulated manually.

Precision:

Within-run CV of 3 repeated assays (n = 20) was 2.7% or less (as reported in the package insert). In the current analysis, 3 control samples were used to maintain quality control.

Results of all 3 control solutions were within expected range and CV was 0.01% at 513 μ mol/l; 0.01% at 617 μ mol/l; 0.005% at 1001 μ mol/l.



ILAB 600, Analysed at the Hugh Sinclair Nutrition Research Group laboratory at the University of Reading.

Appendix 22 - Illustrations of equipment used for methods

Squirrel data logger:



HemoCue® Glucose 201 analyser:



Accutrend GC monitor:



Deltatrac II:



Miniphotometer LP20:

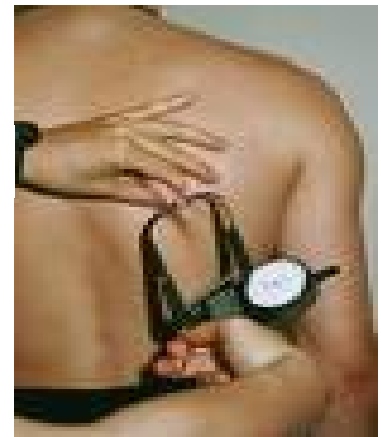


Sphygmomanometer:



Appendix 23 - Skin folds measurement sites

Subscapular



Supraspinale



Biceps



Triceps



Appendix 24 - Commendation by TOAST for obesity research

