THE UNIVERSITY OF HULL

The Effects of Polyphenol Rich Chocolate on Cardiovascular Risk and Glycaemic Control in Type 2 Diabetes Mellitus

Being a Thesis submitted for the Degree of Doctor of Philosophy

in the University of Hull

By

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January 2013

Dedication

I would like to dedicate this thesis to my family, especially my parents for supporting me to start my path in higher education. My children Jack and Lolly, who only have known me while balancing work, research and writing up with family life. Then finally but not least my wholehearted gratitude goes to my wife Jane who has supported me through all the highs and lows of the PhD student.

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Declaration and Acknowledgments

The author of this thesis developed the overarching concept for the work presented in conjunction with Professors Stephen Atkin and Eric Kilpatrick who formed my supervisory team, with initial statistical advice from Mr Alan Rigby, University of Hull.

- I devised the research concept presented in Chapter Three in consultation with Professors Stephen Atkin and Eric Kilpatrick. I undertook all the research and analysis presented in this chapter following advice from Mr Alan Rigby, Statistician, University of Hull.
- The Diabetes Centre Research Fund funded the work in Chapter Four. Dr Stephen Beckett and Nestle PTC, York, UK provided the chocolate as an unrestricted gift. The author carried out the clinical trial work with support from the clinical research team at the Diabetes and Endocrinology Research Team. Thanks goes to Anne Anderson for assisting with the insulin assays and the chemical pathology service of the Hull and East Yorkshire Hospitals NHS Trust for undertaking the biochemical analyses.
- Barry Callebaut BV, (Lebbeke-Wieze, Belgium) funded the work presented in Chapter Five. Primarily, I developed the protocol in collaboration with my supervisory team and advice from Dr Leen Allegaert. I solely conducted the clinical trial. Thanks also go to Anne Anderson, John Shepherd, Dr Karen Smith (Hull and East Yorkshire Hospitals NHS Trust) and Dr Leigh Madden (University of Hull) for advice and support in undertaking biomedical assays.
- Nestlé NRC, (Geneva, Switzerland) funded the work presented in Chapter Six. Again primarily I developed the protocol in collaboration with my supervisory team along with additional support from Dr Karen Cooper and Dr Maurice Beaumont (Nestlé). I conducted the clinical trial with support from the clinical trials team at the Diabetes Research Centre, notably Lee Rollins, Carrie Willby, Anneli James and Lisa Baldwin. Thanks also go to Anne Anderson, John Shepherd, (Hull and East Yorkshire Hospitals NHS Trust) Dr Fraser Courts and Dr Leigh Madden (University of Hull) for advice and support in undertaking biomedical assays. Thanks to Yannis Planoudis (Nestlé) for his diligence in monitoring the conduct of this trial, Lucas Actis-Goretta as project manager for supporting the work and Harrison Macharia for his work on behalf of Nestlé with respect to the statistical analysis.

Overall I would like to thank all the research fellows I spent time with, and for their help cannulating participants, especially Dr Alison Dawson, Dr Ben Ng, Dr Ammar Wakil and Dr Thozhukat Sathyapalan. I would like to thank Nalumon 'Jip' Parnwell for her endless assistance with administration.

Finally my thanks go again to my supervisors Professor Stephen Atkin and Professor Eric Kilpatrick, with an extra note of gratitude to Professor Henry Leese for his support in drafting and finalising this thesis.

Summary Abstract

Background:

Type 2 diabetes mellitus (T2DM) is characterised by increased rates of morbidity and mortality, much of which is related to cardiovascular disease. T2DM is considered to be a non-communicable disease, with its cause and aetiology linked to a number of genetic factors, which are influenced by dietary behaviours and lifestyle. Diet has a significant role to play in the prevention and management of T2DM. Both *in vitro* and *in vivo* studies suggest that plant flavanols may have beneficial effects for people with diabetes, including potentially those found in cocoa and chocolate.

Cesar Fraga suggested in 2005 that perhaps we should eat more chocolate to prevent and manage diabetes, following a study of just 15 healthy individuals, which demonstrated improvements in insulin sensitivity and blood pressure. Despite this remark seven years ago, almost no work has been published outside of that presented in this thesis has investigated the effect of chocolate containing only its native flavanols (predominantly epicatechins) on glycaemic control and markers of cardiovascular risk in T2DM.

Methodology:

An exploratory review of the literature suggested that the effect of chocolate might be greater than that seen with cocoa. This thesis considered the published literature to ascertain whether chocolate provides greater efficacy over cocoa supplementation using a meta-analysis. This led into a series of clinical trials aimed at testing chocolate supplementation at levels which provide an adequate dose of flavanols without leading to excess energy or sugar intake. The first of the three studies consisted of a proof of concept study feeding 45g of chocolate over eight weeks in a double-blinded randomised crossover design (n=12). The second, using 13.5g again used a double-blinded randomised crossover design to assess the acute effects of flavanols rich chocolate over a three-hour period in individuals with T2DM with an induced transient hyperglycaemia caused by a 75g oral glucose load (n=10). The final clinical trial was a three-arm randomised double-blinded parallel study aimed to investigate the effects over a 12-week period of milk chocolate enriched with flavanols in an attempt to maximise palatability.

Results:

The exploratory review with meta-analysis demonstrated that chocolate supplementation resulted in significantly greater reduction in blood pressure (both systolic and diastolic, sub-group analysis, $p<0.05$) compared to cocoa supplementation. Cocoa was significantly more effective at reducing LDL cholesterol ($p=0.02$). The key difference between studies which used chocolate (38.8±13.1%) and those which used cocoa (68.2±13.8%) was the chocolate used in trials appeared to have significantly lower percentage energy from carbohydrate ($p<0.001$).

The pilot study provided 45g of chocolate for 8 weeks, had no adverse effects on weight or glycaemia despite it providing an additional daily energy content of 1.03Mj (246kcal). HDL cholesterol improved following consumption of high flavanols chocolate ($p=0.05$) with HDL cholesterol: total cholesterol significantly falling ($p=0.04$).

The provision of 13.5g of flavanols rich chocolate prior to a 75g oral glucose load, resulted in improved endothelial function, measured by Reactive Hyperaemia-Peripheral Artery Tonography (RH-PAT) as a functional measure and adhesion molecules ($P>0.05$). This effect was accompanied by a simultaneous reduction in urinary 15-F2t-isoprostane (a marker of oxidative stress).

The final study using a milk chocolate and lower doses of epicatechin did not have an adverse effect on weight or glycaemia after 12 weeks, with participants actually gaining weight in the four weeks immediately after the completion of the study ($p=0.002$). No clear beneficial effects of any one chocolate were seen.

Conclusion:

The work in this thesis finally provides evidence to support Fraga's suggestion, that there are benefits in terms of improved lipid profile and endothelial function with chocolate supplementation for individuals with T2DM. The equivocal results from the final study which used a chocolate containing about a third of the epicatechin dose of the first two studies, suggest that the dose required for benefit may be at least that accepted as being beneficial with respect of endothelial function (EFSA, 2012a) at 200mg of flavanols in the general population for individuals with T2DM.

Publications

A number of the chapters in this thesis contain content and data that has been accepted for publication or already published in peer-reviewed journals. These are listed below in reverse chronological order:

Mellor, D.D., Madden, L., Smith, K., Kilpatrick, E. and Atkin, S. (In Press). High polyphenol chocolate reduces endothelial dysfunction and oxidative stress during acute transient hyperglycaemia in type 2 diabetes: a pilot randomised controlled trial. *Diabetic Medicine*. doi: 10.1111/dme.12030

Mellor, D. (2012). A review of the current nutritional guidelines for diabetes. *Practice Nursing*, 23(5), 234-240.

Dyson, P.A., Kelly, T., Deakin, T., Duncan, A., Frost, G., Harrison, Z., Khatri, D., Kunka, D., McArdle, P., Mellor, D., Oliver, L., and Worth, J. (2011). Diabetes UK evidence-based nutrition guidelines for the prevention and management of diabetes. *Diabetic Medicine*, 28(11), 1282-1288.

Mellor, D.D., Sathyapalan, T., Kilpatrick, E.S., Beckett, S. and Atkin, SL. (2010). High-cocoa polyphenol-rich chocolate improves HDL cholesterol in Type 2 diabetes patients. *Diabetic Medicine*, 27(11), 1318-1321.

Additionally material from one of the chapters in this thesis has been already accepted for invited for oral presentation at the American Chemistry Society. This is listed below:

Mellor, D., Kilpatrick, E.S., Sathyapalan, T., Courts, F.L., Madden, L.A., and Atkin, S.L. (2012). Effect of high polyphenol content chocolate on cardiovascular risk markers in subjects with type 2 diabetes after a glucose load. Paper presented at the American Chemistry Society, San Diego.

Finally, of the chapters in this thesis have been already accepted as peer-reviewed abstracts at the following national or international meetings. These are listed below in reverse chronological order:

Mellor, D.D., Kilpatrick, E.S. and Atkin, S.L. (2013) A systematic review and meta-analysis of the effects of high flavanol cocoa and chocolate on markers of diabetes and cardiovascular risk in randomised controlled trials (RCTs): A subgroup analysis, is chocolate better than cocoa? To be presented as a Poster Presentation at Diabetes UK APC (P150)

Mellor, D.D., Kilpatrick, E.S. and Atkin, S.L. (2011). Inter-individual differences in high-density lipoprotein cholesterol in subjects with Type 2 diabetes are related to systolic blood pressure and endothelial function. *Diabetic Medicine,* 28 (Suppl. 1), 46 (P51).

Mellor, D.D., Bolsover, N., Kilpatrick, E.S. and Atkin, S.L. (2011). Attachment style of individuals embarking on an intervention trial: do they differ from the general population? *Diabetic Medicine,* 28 (Suppl. 1), 179 (P486).

Mellor, D.D., Ng, J.M., Aye, M.M., Sathyapalan, T., Kilpatrick, E,S. and Atkin, S.L. (2010). HDL Cholesterol and Reactive Hyperaemia Are Correlated in Type 2 Diabetes Mellitus. *Diabetes*, 59, A290.

Mellor, D., Allegaert, L., Wakil, A., Kilpatrick, E. and Atkin, S. (2009). Acute administration of high polyphenol content chocolate improves endothelial function in type 2 diabetes even in the presence of hyperglycaemia. *Diabetologia*, 52, S498.

Mellor, D.D., Sathyapalan, T., Kilpatrick, E.S., Beckett, S. and Atkin, S.L. (2009). Benefical Effects of Chocolate on Lipid Parameters and Duality of Life in Type 2 Diabetes. *Diabetes,* 58, A570.

Mellor, D.D., Atkin, S.L., Kilpatrick, E.S., Beckett, S., and Sathyapalan, T. (2009). Effect of chocolate on quality of life in people with Type 2 diabetes. *Diabetic Medicine,* 26 (Suppl. 1), 49 (P39).

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Chapter One: Introduction - Metabolic Syndrome in Type 2 Diabetes Mellitus and its Nutritional Management

This thesis considers the potential benefits and risks of consuming chocolate rich in flavanols for individuals with type 2 diabetes mellitus (T2DM). In 2005 Cesar Fraga in the American Journal of Clinical Nutrition suggested that we should consume more cocoa and chocolate to reduce the burdens of hypertension and diabetes (Fraga, 2005). This article was an editorial reporting on a small study of 15 healthy individuals by Grassi et al. (2005a). This represented the emergence of a hypothesis, which fuelled a body of work that has suggested chocolate may reduce blood pressure. This began with a study by Taubert et al. (2003) which suggested a beneficial effect upon blood pressure and culminated with a series of meta-analyses (Taubert, Roesen & Schomig, 2007; Ding, Hutfless, Ding & Girotra, 2006; Ried et al., 2010a; Desch et al., 2010a; Shrime et al., 2011; Hooper, et al., 2012) which have been consistent in their support of the theory that cocoa and chocolate have beneficial effects upon blood pressure, lipid profile and potentially insulin resistance. However, with respect to the effects of chocolate and cocoa in individuals with diabetes, there have been very few studies (Balzer et al., 2008; Curtis et al., 2012).

This chapter will initially describe the condition, of T2DM and then develop the case for the use of chocolate as part of a dietary regimen for this patient group. With the longest intervention including chocolate being one year in duration, there are no data with respect to hard clinical endpoints, including death or myocardial infarctions. Data from a variety of biomarkers or surrogate endpoints will be used to assess the effects of chocolate and cocoa interventions. In addition to the effect of these biomarkers on future risk, in terms of safety, the measures used in the clinical management of diabetes will also be considered.

Overview of Chapter

Aims and Scope of Chapter:

- To consider T2DM as a chronic condition, including its diagnosis and its implications to the individual and in terms of public health.
- To review nutritional management of T2DM.
- To consider the evidence for the potential benefits of cocoa and chocolate as part of the nutritional management of individuals with diabetes mellitus by considering the underlying potential mechanisms, epidemiology and clinical trial data.

To address these aims, this chapter is split into five sections:

1.1 – An introduction to T2DM as a clinical condition, including its definition and the burden it represents on global health.

1.2 – Review of the evidence for the nutritional management of diabetes mellitus, with a particular focus upon the dietary influences on cardiovascular disease risk.

1.3 – The history and potential health benefits of cocoa and chocolate, including a review of the biological effects of polyphenols.

1.4 – A review using systematic methodologies of the evidence from in vitro experiments, epidemiological data, clinical trials in publication and print until the end of June 2012 (an exploratory review with meta-analysis of these data will form Chapter Three).

1.5 – The aims, objectives and hypothesis of the experimental work of the thesis.

1.1– Type 2 Diabetes Mellitus

1.1.1 - Global Burden of Diabetes Mellitus and Metabolic Syndrome

Diabetes Mellitus is the first non-communicable disease to obtain a United Nations (UN) resolution to grant its own global day, akin to communicable diseases including HIV/AIDS (UN Resolution 61/225) (United Nations 2007, Silink 2007). This was in response to the increasing burden and epidemic of diabetes globally. According to the latest International Diabetes Federation (IDF) Diabetes Atlas (IDF, 2011), diabetes was responsible for 4.6 million deaths annually worldwide, or one death every seven seconds. Approximately half of these deaths were also associated with cardiovascular disease, the most common complication of diabetes. This has led to the view that diabetes could potentially lead to a failure to achieve the Millennium Development goals, and could lead to a global failure of public health (Silink, 2007).

In an attempt to reduce the burden of cardiovascular disease in England attributable to diabetes, the National Health Service through its contractual system with primary care clinicians has set a number of cardiovascular associated targets (Information Centre, 2011). Attached to these are financial incentives linked to their implementation (Simon, 2008). The current system is an example of the evolution of care over the last decade. Initially, care objectives were based upon the National Service Framework (NSF) for Diabetes (Department of Health, 2001). These have since evolved based upon best practice recommendations and guidelines developed through the National Institute for Health and Clinical Excellence (NICE) including Clinical Guidance 66 (NICE, 2008). This has latterly been supported by NHS Diabetes, in conjunction with the Yorkshire and Humber Public Health Observatory who are the national lead for diabetes (NHS Diabetes, 2012).

Further support for the development of clinical targets in the management of diabetes mellitus, has been associated with evidence of reduced cardiovascular disease if these targets are achieved (Perk et al., 2012). In practice, targets focus on the assessment of a small number of biomarkers, which are acknowledged as accounting for a large proportion of the risk.

The most commonly assessed markers include hyperglycaemia (raised plasma glucose and glycosylated haemoglobin (HbA1c)), hypertension (raised blood pressure) and the biochemical assessment of lipids (plasma fat levels), especially cholesterol (dyslipidaemia). Chronic hyperglycaemia (UKPDS Group 1998a; 1998b) and hypertension (UKPDS Group 1998b) are associated with increased risk of cardiovascular morbidity and mortality. This relationship is extended to cholesterol, with a level above the 80th centile being associated with a 52% excess risk compared to the bottom quintile (Michael et al., 1986).

The abnormal changes in these risk factors are a common feature of T2DM (Turner et al., 1998), but are not exclusive to diabetes (Alberti, Zimmet, Shaw & Grundy, 2006). When not associated with T2DM, the clustering together of these risk factors has been labelled as the metabolic syndrome (also described as Syndrome X, Cardio-metabolic syndrome, Pre-diabetes and Reaven's Syndrome) (Alberti & Zimmet 1998; Alberti et al. 2006; Alberti et al. 2009; Grundy et al. 2004; Reaven, 1991).

1.1.2 - Diagnosis and Classification of Diabetes Mellitus

A number of classifications of diabetes have been developed, accepted and then revised based upon the emergence of new evidence over the past fifty years (John, 2012). The universal characteristic of all types of diabetes mellitus is persistent hyperglycaemia; the definitions of which have been revised over time in line with emergence of new data. The derivation of the name of the condition comes from the Greek and can literally be

translated as "*honey syphon*"; a reference to the glycosuria often seen as a feature of diabetes, which, in ancient times was diagnosed by tasting the urine of the patient.

The most widely accepted diagnostic criteria were these set out by the World Health Organisation (WHO) and IDF consultation in 2006 (WHO/IDF, 2006). The WHO diagnostic criteria (table 1.1.2), define the stages of glycaemia from normal, through impaired and intermediate states to diabetes although, additionally it is now acknowledged that HbA1c > 48mmol/l (6.5%) is emerging as a new diagnostic criteria for diabetes (WHO, 2011; John, 2012). However HbA1c below this level do not exclude diabetes diagnosed using glucose levels (WHO, 2011), so interpretation needs to be undertaken with caution. Moreover, as the recruitment to the interventional studies presented in this thesis was completed prior to the suggested new diagnostic criteria, elevated glucose will be the focus of this discussion.

Table 1.1.2: WHO/IDF classifications of normoglycaemia, intermediate hyperglycaemia and diabetes mellitus. (WHO/ IDF, 2006).

Definition		Fasting Glucose (mmol/l)	2 hour reading following a 75g glucose load (Oral Glucose Tolerance Test)
Normal/ Normoglycaemia		ADA - <5.5mmol/l WHO - <6.0 mmol/l	ADA and WHO - <7.8 mmo/l
Impaired Fasting	**Intermediate glycaemia**	ADA - 5.5-6.9 mmol/l WHO – 6.0-6.9 mmol/l	ADA and WHO - <11.1mmol/l May also include impaired glucose tolerance
Impaired Glucose Tolerance		ADA and WHO - <7.0 mmol/l May also include impaired fasting glucose	ADA and WHO – 7.8-11.1mmol/l
Diabetes Mellitus		ADA and WHO ≥7.0 mmol/l	ADA and WHO ≥ 11.1 mmol/l

There has been a gradual evolution in the diagnostic criteria of diabetes mellitus, possibly representative of a shift in clinical focus over that time. This has seen a move from the aim of reducing the risk of developing diabetic retinopathy (Kadowaki et al., 1984), towards a focus upon reducing cardiovascular risk (Coutinho, Gerstein, Wang & Yusuf, 1999). The move from microvascular to macrovascular risk has also led to the

shift in diagnostic criteria for diabetes mellitus, from a fasting glucose of ≥7.8mmol/l to ≥7.0mmol/l in the 1997 Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997).

It is apparent that hyperglycaemia is responsible for many of the acute features and symptoms associated with diabetes mellitus, including thirst polyuria and acute complications including infections (e.g. balanitis and thrush). This has led to the suggestion that the principle pathology in T2DM is hyperglycaemia. However, hyperglycaemia is likely to represent the tangible effect of a more fundamental pathology related metabolic incompetence, which in turn is associated with insulin resistance (Taylor, 2012). The insulin resistance results from a range of associated genetic and possibly epigenetic factors which are 'activated by' environmental factors, the most prominent of these being an obese or overweight phenotype (Weyer, Bogardus, Mott & Pratley, 1999) .

1.1.2.1 - Classification of glycaemia

The WHO avoided classifying any level glycaemia as being the normal range (WHO/IDF, 2006). This was based upon the principle that dysglycaemia is merely the routinely measurable manifestation of the underlying metabolic abnormality. Instead the WHO chose to suggest a point at which risk of developing T2DM and cardiovascular risk is considered at its lowest. The alternate perspective may be that this is a reflection of the true lack of consensus from the literature of what a normal level of glycaemia might truly be. This perspective could be considered for the global aspects of the other risk factors for cardiovascular disease. It not only apply to glycaemia, in that the norms are defined for blood pressure, lipids or weight, are those where in epidemiology or intervention trials the rates of cardiovascular disease were seen at there lowest (Perk et al., 2012).

Beyond the upper limit of 'normal', exist states defined as intermediate hyperglycaemia: the level of glycaemia not elevated sufficiently to be considered diabetes mellitus (table 1.1.2). These states can be sub-divided into the classifications of 'impaired fasting glucose' (IFG) and 'impaired glucose tolerance' (IGT). It is highly plausible that 'IFG and IGT are conditions which may be linked, but represent different manifestations of underlying metabolism (Unwin, Shaw, Zimmet & Alberti, 2002).

Abnormal glucose metabolism based upon fasting criteria suggests an inability of the body, either to regulate glucose metabolism in the fasted state and/or a failure to return to fasting state metabolism. Thus, implying that endogenous glucose production predominantly from the liver (with modest contribution from the kidneys) (Gerich, Meyer, Woerle & Stumvoll, 2001) with subsequent exportation into the circulation is not adequately regulated. Raised fasting glucose is often associated with excess central or visceral adiposity (Gautier et al., 2010) leading to the hypothesis that excess visceral adipose tissue has the feature of being metabolically active and contributes to the pathology of insulin resistance (Wajchenberg, Nery, Cunha & Silva, 2009).

The metabolic activity of this adipose tissue may contribute to the futile cycling of nutrients and metabolically active compounds which is acknowledged as primary feature of insulin resistance (Macfarlane, Forbes & Walker, 2008). This may result in ectopic fat deposition in the liver (Taylor, 2008) and pancreas (Lim et al., 2011) adding to the insulin resistance and ultimately the dysfunction and decline in insulin production, thus linking the genetic and environmental risk factors to dysfunction, pathology and ultimately T2DM (Taylor, 2012).

When the insulin resistance cannot be matched by insulin secretion from the beta cells in the Islets of Langerhans's within the pancreas, elevated glucose levels occur. This leads to a states of intermediate hyperglycaemia which then ultimately progresses towards diabetes mellitus (Nathan et al., 2007). It is unclear if the dysfunction of control

of fasting glucose is entirely linked to a relative lack of insulin, or to an excess of counter-regulatory hormones, especially glucagon (Moller, 2001).

Postprandial glucose, which is most typically defined by a 2 hour plasma measurement following an oral glucose load of 75g of anhydrous glucose in 300ml of water (Oral Glucose Tolerance Test (OGTT)) can be used to demonstrate the inability of an individual to handle an exogenous load of carbohydrate. For this reason, the OGTT is the recommended test for impaired fasting glucose and in some cases diabetes (WHO /IDF, 2006). This suggests that in the presence of exogenous glucose, there is an inability of the beta cells to respond adequately to an oral load or meal. This may be the result of insulin resistance, insulin deficiency or a combination of the two leading to a reduction in tissue uptake of glucose by muscle and other tissues. An extension of this view is that the pancreas is unable to secrete either adequate or appropriately timed levels of insulin in response to a glucose load in the circulating plasma (Weyer et al., 1999), resulting in dysregulation of post-prandial glucose metabolism.

1.1.2.2 - Classification of diabetes

Diabetes mellitus falls into 4 broad categories as suggested by WHO /IDF (2006):

Type 1 diabetes mellitus

Type 2 diabetes mellitus

"Other specific types"

Gestational diabetes mellitus (diabetes first presenting in pregnancy).

Type 1 diabetes (T1DM) is typified by an absolute lack of insulin, which therefore needs replacement therapy, although there may be a temporary period when this requirement ceases (known as the "honeymoon period"). Type 1 diabetes mellitus can present with ketosis (high levels of ketone bodies in the urine and blood) due to the

absolute lack of insulin, which may in turn lead to ketoacidosis, a potentially life threatening condition requiring emergency medical intervention.

Type 2 diabetes mellitus is characterised by its insulin resistance as previously described (Section 1.1.2). Despite, two clearly different pathologies, types 1 and 2 diabetes mellitus are associated with macrovascular and microvascular complications (Holman et al. 2008; Nathan 1993).

The predominant macrovascular complications seen in T1DM and T2DM are those of the cardiovascular system. In T1DM these may largely be linked to the degree of hyperglycaemia (Nathan et al., 2005). However with T2DM this association may not be clearly linked with hyperglycaemia, instead being a complex interaction between glycaemia, blood pressure and blood lipids (especially cholesterol and triglycerides). This triad of risk factors in turn appear to be influenced by a range of emerging risk factors (Anderssohn et al., 2010). These include endothelial dysfunction (Heitzer et al., 2001), oxidative stress (Ceriello & Motz, 2004; Brownlee, 2001) and pro-inflammatory markers (Pfützner & Forst, 2006). These risk factors may be categorised as either established or emerging. There has been much debate about whether new risk factors add any additional diagnostic or predictive value beyond that provided by the routine clinical assessments of glycaemia, lipids and blood pressure (Shishehbor et al., 2008).

1.1.3 - Summary of the Classification of Type 2 Diabetes Mellitus

Obesity and particularly central obesity (the excess of body fat around the organs of the abdomen), are associated with metabolic dysfunction including T2DM (Wannamethee & Shaper 1999; Power & Thomas 2011). It is clear that lifestyle factors, especially nutrition have a causative function, through 'over-nutrition', and are crucial to management in the form of dietetic and public health interventions.

1.2- Nutritional Interventions Aimed at Reducing Cardiovascular Risk in Type 2 Diabetes

Type 2 diabetes mellitus, results in a two-fold increased cardiovascular risk in men and three-fold increased risk in women (Wilson, 1998). This may in part be managed via the manipulation of intake of a range of nutritional factors (He et al., 1999; Ness & Powles, 1997). For example, cardiovascular disease risk is thought to decrease with a lower intake of fat and especially saturated fatty acids and trans-fatty acids (Hunter, Zhang & Kris-Etherton, 2010; Mozaffarian et al., 2006).

Epidemiological evidence suggests that a reduction in refined carbohydrates and in particular non-milk extrinsic sugars improves dysglycaemia (Malik et al., 2010). This may be in part by the moderation of weight gain and ideally facilitating weight loss, thus resulting in lower levels of obesity and a reduction in hypertriglyceridaemia (Malik et al., 2010). The nutritional effects of sugar metabolically in individuals with diabetes, has arguably been unduly influenced by the work of Peters, Davidson and Eisenberg (1990) who suggested that an isocalorific substitution of chocolate cake for potato had no effect on glycaemia in individuals with T1DM. This led to a trend within clinical practice, which appears to be based on the unsubstantiated extrapolation of this single study, lacking direct experimental evidence or mechanism as its basis. In its application in T2DM, it ignores the fundamental differences between the underpinning pathologies of type 1 and type 2 diabetes mellitus. Where T1DM, is typified by its absolute deficiency of insulin; and T2DM is an insulin resistant state with increased counter regulation (excess glucagon) leading to increased *de novo* gluconeogenesis (Jiang & Zhang, 2003).

The effect of this misplaced approach to treatment has resulted in a generation of people with diabetes being encouraged to consume foods rich in sugar, such as chocolate, only as part of a meal. This piece of advice was based upon the assumption that the starches

and fibre in the meal were able to help moderate the hyperglycaemic effects of the sugar in the chocolate (Gillespie, 1996). Opinion on the effect of sugars on glycaemia dramatically changed with the introduction of the concept of the glycaemic index, and of glycaemic loads of carbohydrate containing foods (Jenkins et al., 1981). This concept has resulted in the realisation that foods high in carbohydrate and in particular, sugars, may have a more modest effect upon blood glucose than might be expected. With the National Diet and Nutrition Survey (NDNS, 2012), suggesting that British adults consumed 46.5% of the energy intake from carbohydrate, of which 11.4% was in the form of non-milk extrinsic sugar (NMES) or added sugar. This methodology is derived from the area under the curve compared to 'complex carbohydrate' rich foods, which are high in starch and fibre including potatoes and bread, which are also low in sugar with foods high in sugar. The methodology compares 50g of carbohydrate from the test food with 50g of glucose. This is then used to estimate the relative area under the curve compared as a percentage of the area under the curve for glucose. Figure 1.2 shows the glycaemic response curve, the glycaemic index is estimated by calculating the area under this curve using a trapezoid methodology (Brouns et al., 2005).

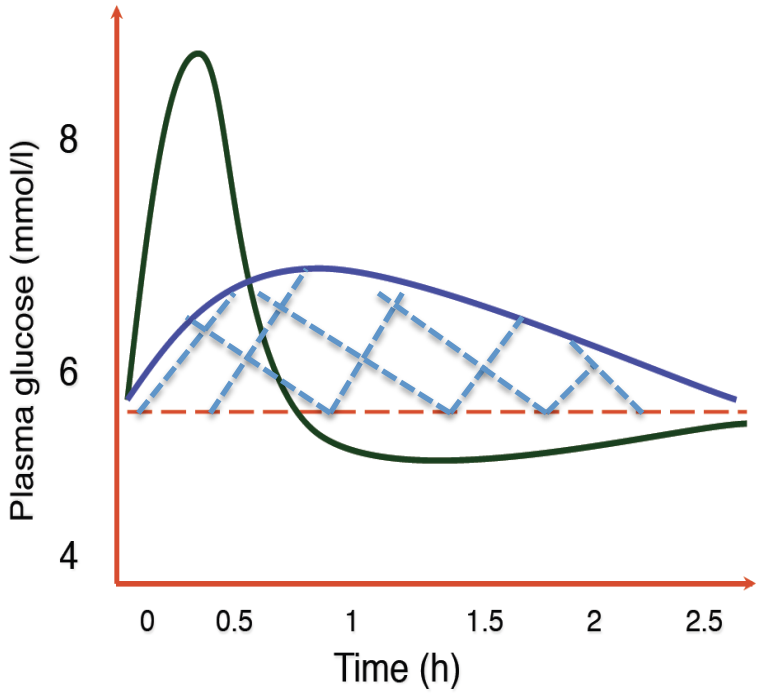


Figure 1.2: The evaluation of the incremental rise of blood glucose (area under curve) after ingestion of a food that contains 50g of carbohydrates, as a percentage of the same amount of carbohydrate from a reference food (50g glucose) and absorbed by the same person. The green line represents a high glycaemic food and the blue a low glycaemic index food (the dashed red line shows the baseline which is taken as the fasting glucose measurement). The area under the blue line indicates the area under the curve, this is represented as a percentage of the area under the curve of 50g glucose, which is calculated using the trapezoid methodology as described by Brouns et al. (2005)

1.2.1 – Recommendations for the Nutritional Management of Type 2 Diabetes Mellitus

A recent review of the nutritional management of both type 1 and type 2 diabetes mellitus in the United Kingdom was undertaken by the Diabetes UK Nutrition Working Group (Dyson et al., 2011), which was in part designed to update the 2003 recommendations (Connor et al., 2003) and move to a systematic approach in line with evidence based practice as is used by the American Dietetic Association (Franz et al., 2008). The Diabetes UK Nutrition Working Group was composed of dietitians and potentially may have led to a bias in their interpretation of the literature. This is highlighted by the emphasis placed on use of structured education as a mode of delivery which may have represented a professional interest (Deakin, Cade, Williams & Greenwood, 2006; Davies et al., 2008). These guidelines included recommendations for

the management of diabetes and the cardiovascular risk in T2DM, of which the primary focus was weight management. It was noticeable that there were no recommendations were made with respect to what the optimal quantities or percentage energy from each of the macronutrients including saturated fatty acids and sugars should be, beyond those recommended to the general population in the Eat Well Plate (Deptartment of Health, 2011) (Figure 1.2.1).

The guidelines also distinguished between the nutritional management of type 1 and type 2 diabetes mellitus, which was a change for UK guidelines. These evidence-based guidelines actually took account of the lack of insulin in T1DM and assumed that hyperglycaemia is the result of abnormalities associated with T2DM. These generally recommend a low fat, high carbohydrate diet, which currently is considered to be the best way for adults to reduce their risk of nutrition related diseases including cardiovascular disease (Department of Health, 1991).

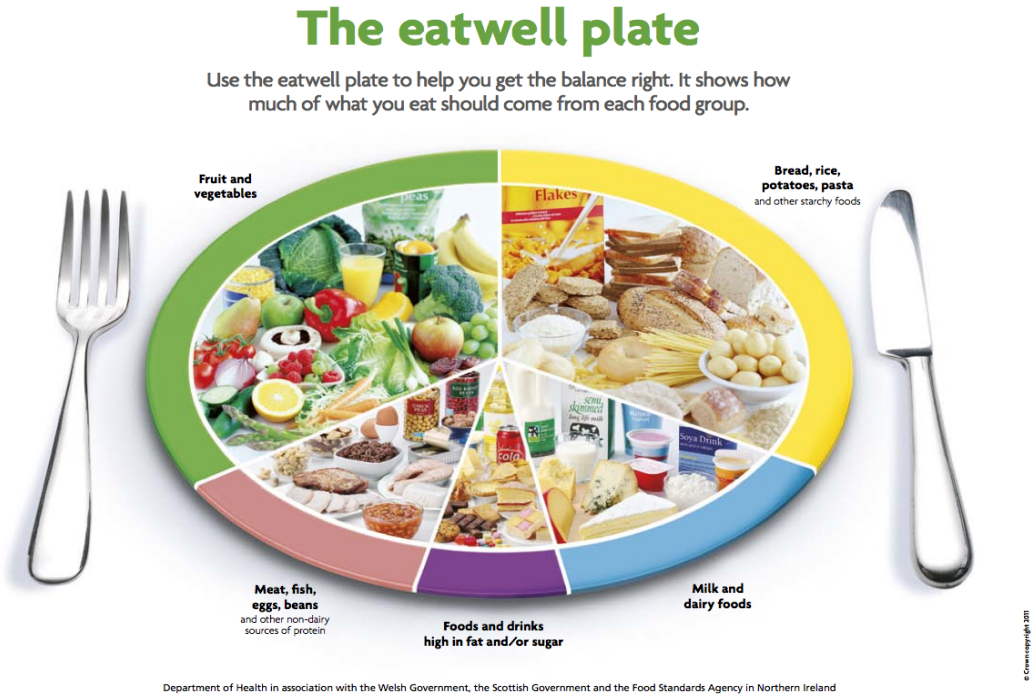


Figure 1.2.1: Eat Well Plate, Reproduced with acknowledgment of Dept. of Health (2011). Reproduced with permission.

Table 1.2.1: Current United Kingdom recommendations for macronutrient intakes (excluding protein which is expressed as grams per kilogram body weight) from the Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy (COMA) (Department of Health, 1991)

Dietary Recommendations	
Total Fat	No more than 35% of food energy
Saturated Fatty Acids	No more than 11% of food energy
Total Carbohydrate	Approximately 50% of food energy
Sugars (Added or NMES)	No more than 11% of food energy
Dietary Fibre (Non Starch Polysaccharides)	Average intake of dietary fibre to 18g per day
Alcohol	Should not provide more than 5% of energy in the diet. Women – should not regularly drink more than 2-3 units of alcohol/day Men – should not regularly drink more than 4-5 units of alcohol/day

Weight loss was proposed to be the strongest predictor of a reduction in the risk of developing T2DM in individuals who had been identified as being at high risk of developing the disorder across a wide range of population groups (Pan et al., 1997; Lindstrom et al., 2003; Ramachandran et al., 2006; Orchard et al., 2005). Weight loss of 5-7% was shown to reduce the risk of progressing to diabetes from impaired glucose tolerance by 57%; this was far effective than treatment with metformin (31% reduction in risk) (Knowler et al., 2002). The benefits of weight management in individuals who are overweight or obese with T2DM (over 90% of cases) are clearly evident.

The new Diabetes UK guidelines recommend weight management as the primary strategy to improve glycaemic control (HbA1c) along with additional improvements in reduction of cardiovascular disease risk (Ray et al., 2009). The approach to weight loss should be via overall energy reduction, rather than restricting any one macronutrient (carbohydrate, fat or protein). However, there were no recommendations for the use of supplements or functional foods, except for stanol and sterol esters, which met the threshold for evidence with respect to reduction in cholesterol with proven effects upon diabetes control or reduction in cardiovascular risk (Dyson et al., 2011). This was likely to be the effect of the substantial amount of clinical trial data reporting the effects of

stanol and sterol esters (Katan et al., 2003; Abumweis, Barake & Jones, 2008; Baumgartner, Messink & Platt, 2011).

1.2.2 - Potential Beneficial Effects of Functional Foods in Type 2 Diabetes

Although there is no evidence for a functional benefit of foods beyond their nutrient composition, this has not stopped a number of foods companies seeking health claims related to functionality of nutraceutical qualities. The term nutraceuticals can be traced back to the 1980s (Kalra, 2003). The originator of the term appears to be Dr Stephen DeFelice, who combined the words 'nutrition' and 'pharmaceutical' in 1989. The word has developed into the accepted international term to describe a food or a food component with potentially health improving properties that go beyond those normally expected from a food or ingredient. These effects can may be associated with nutrients, which have already been defined and accepted as essential for health, or as in most cases a bioactive compound, typically a secondary plant metabolite, which have health improving benefits.

The initial difficulty was that there were no regulations or a legal definition for the meaning of the term nutraceuticals or the effect which is expected to be seen (Zeisel, 1999). Subsequently in many countries across the World a number of regulatory procedures have been put in place. Within the European Union a range of legislation has been passed to create what has come to be known as Article 13, 13.5 and 14 health claims for foods (EFSA, n.d.).

1.2.3 - Methods of Assessing the Effect of Nutritional Interventions upon Cardiovascular Risk

Mann (2006), in a review of the evidence supporting lifestyle management of diabetes, suggested a number of surrogate markers which could be used to evaluate the strength of the evidence reported in nutritional studies for their effect in populations of individuals with diabetes. The effect of this was to produce a more concise and

clinically focused approach compared to the earlier work of the Process for the Assessment of Scientific Support for Claims on Foods (PASSCLAIM) group (Riccardi et al., 2004) formed in Europe by the International Life Sciences Institute (ILSI) and funded by the European Commission. This work reflected upon the wider aspects of body weight regulation and insulin resistance along with diabetes. These recommendations have been further developed and updated by the Dietetic Products, Nutrition and Allergies (NDA) panel of the European Food Safety Agency (EFSA) (EFSA, n.d.). These align with the European Society of Cardiology guidelines (Perk et al., 2012) for cardiovascular risk reduction, United Kingdom Diabetes Prevention Study (UKPDS) risk engine (Mount, 2007) and the IDF standard and platinum definition of metabolic syndrome (Alberti, Zimmet, Shaw & Grundy 2006) (with the addition of oxidative stress (Brownlee, 2001; Ceriello & Motz, 2004) which include assessments of:

- Blood Pressure
- Lipids
- Insulin resistance and glycaemia
- Endothelial dysfunction
- Oxidative Stress
- Inflammation

The challenge of nutritional studies, compared with pharmaceutical studies, is often one of power, duration and population size. This creates a number of potential considerations. Firstly, the surrogate markers, which may be sensitive for larger scale pharmaceutical studies, may not have the level of sensitivity or specificity for smaller scale studies, which is often the case with nutritional intervention studies. The variability of food might also mean that the concept of dose in pharmaceutical studies cannot be easily transcribed across. Therefore this can mean that due to the often-

limited numbers in nutritional interventions, systematic review and meta-analysis methodologies are often required to meet the demand for statistical power and confidence in the generalizability of the findings of studies. However these may not be robust due to the high level of heterogeneity between studies. This can be associated with the design, study population or the intervention used.

Therefore it is clear that there is a need to assess how foods and dietary components affect aspects of diabetes and cardiovascular risk. The following section of this thesis considers the effects of chocolate rich in polyphenols, especially the flavanols, epicatechins, on the six areas of diabetes and cardiovascular risk proposed on the previous page. For the purpose of this thesis, polyphenols will initially be discussed as the class of compounds. However to reduce the risk of mixed terminology; flavanols will be used as the generic term for the polyphenols found in chocolate and cocoa, with epicatechin being used when relevant to the literature and data being discussed.

1.3- Cocoa and Chocolate; History, Composition and Biologically Active Compounds

1.3.1 - Introduction

The Latin name for the cacao tree *(Theobroma cacao)* the source of cocoa, is widely thought to mean 'tree of the Gods'. As such it has been used as a key part of Central American culture for over a millennium. This is highlighted by the Mayans who thought the gods discovered this tree, which led to its use in religious ceremonies. During this time its consumption was highly restrictive, being reserved for the ruling classes and priesthood (Dillinger et al., 2000).

Christopher Columbus first introduced cocoa beans to Europe, where they were thought to be mouldy almonds. It was not until later in the 16th and 17th centuries, with the increasing colonisation of Central America by the Spanish conquistadors that a greater understanding of these practices became apparent. The new colonialists brought monks with them, who conducted rudimentary ethnographic studies of the Native Americans and chronicled their findings. These documents developed into what have become known as codex; they list many potential health benefits of cocoa including an aid to digestion, resisting fatigue, helping reduce malnutrition and weakness along with being a therapy for cough and possible consumption (tuberculosis) (Pucciarelli & Grivetti, 2008). This is described below in the following quotation:

"The divine drink, which builds up resistance and fights fatigue. A cup of this precious drink [cocoa] permits man to walk for a whole day without food".

Aztec Emperor Montezuma II (born c.1480 reigned 1502-1520)

In (Corti, Flammer, Hollenberg & Luscher, 2009)

Despite the reported benefits of cocoa, as a foodstuff, it has developed a somewhat negative profile in Europe. The cocoa houses of London during the reign of Charles II

were seen as places of 'sedition', and equally, in France, attempts were made to ban it as a dangerous drug! These legal attempts are suggestive of a biological activity beyond what might be expected in a normal food (Mehrinfar & Frishman, 2008).

The development of chocolate dramatically altered the desire of the general public to consume cocoa. The effects of industrialisation and its accompanying inventions has allowed the conversion of a bitter, unstable grit containing drink which often had a fatty beer-like 'head', to become the smooth sweet, melt in the mouth, solid chocolate seen today. This has led to the addition of other ingredients including sugar and dried milk, along with varying the quantities of saturated fatty acid rich, cocoa butter. The end result could be considered as a product, which is not compatible with dietary recommendations, being a rich source of saturated fatty acids and sugar.

Table 1.3.1: The typical composition of chocolate (Food Standards Agency, 2002) compared to dietary recommendations, EFSA (2010a; 2010b) and Department of Health (1991)

	Typical 'dark' chocolate McCance and Widdowson 6th Ed. (FSA 2002) (per 100g)	**'Dark' Chocolate Percentage energy**	**UK Recommendations** (Health, 1991) **Male (19-50 years) Total energy and percentage energy**	**EFSA Recommendations** (EFSA, 2010a; 2010b) **Percentage energy**
Energy	2050Kj		10600Kj	
Sugar	59g	49%	<11%*	<10%
Fat	26g	47%	33%	20-35%
Saturated Fatty Acid	16.2g	29%	11%* * Assuming no consumption of alcohol	As low as possible

So, with the potential negative nutritional effects of cocoa in the form of chocolate based on its high energy content (contributed to by the large amounts of saturated fatty acids and sugar), the data available from epidemiological and interventional studies were reviewed to assess the potential effect in humans. A review of PubMed (accessed 30th June 2012) (see Chapter 3 for methodology of literature search and Appendix I for the list of search terms) suggested that pre-1990 the focus of the literature was on assessing potentially harmful aspects of chocolate, with respect to allergies,

theobromine (the caffeine like stimulant), effects of sugar on dental health and negative aspects of its saturated fatty acid content.

From the middle of the 1990s a major change of perspective in the literature is apparent, with the research community becoming increasingly interested in investigating the potential health benefits of cocoa and cocoa containing products. A review following systematic methodologies of the clinical trial data is presented later in this chapter (section 1.5), with the data being incorporated into the exploratory review with meta-analysis, which forms Chapter Three of this thesis.

1.3.2 - *In Vitro* Evidence for the Beneficial Effects of Cocoa and Chocolate

The antioxidant theory as a potential mechanism for the prevention of disease grew in prominence through the late 20th century. This was associated with epidemiological evidence including the World Cancer Research Fund (WCRF) systematic review of the effects of diet on risk of cancer, which suggested high intakes of fruit and vegetables may reduce disease risk (WCRF, 2011). This had the net effect that many foods were investigated in the laboratory to assess their antioxidant capacity. This was often measured using a number of assays, with ORAC (Oxygen Radical Absorbance Capacity) deemed the standard assessment although still with considerable methodological limitations (Ou et al., 2002; Huang et al., 2002). Table 1.4.2 highlights the antioxidant capacity of commonly available foods based on data previously available via the United States Department for Agriculture website (USDA, 2012).

Table 1.3.2: Antioxidant capacity of common foods adapted from (USDA, 2010)

Food	ORAC, Trolox equiv., mcmol per 100 g
Raw unprocessed Cocoa bean	28,000
Red kidney bean	13,259
Blueberry	9,019
Cranberry	8,983
Blackberry	7,701
Raspberry	6,058
Strawberry	5,938
Red Delicious apple	5,900
Granny Smith apple	5,381

The theory appeared to match the clinical study data from *in vivo* investigation*s*. In that many of the observations being apparently mediated via an antioxidant mechanism. These include data suggesting reduced oxidation of low density lipoprotein (LDL) cholesterol (Frankel et al., 1993), reduction in measures of oxidative stress and reactive oxygen species determination (Scalbert, Johnson & Saltmarsh, 2005).

However in 2012, the USDA withdrew publication of ORAC values from its Nutrient Data Laboratory website. This decision was based on mounting data suggesting that there was no link between the antioxidant capacity of foods and how they might reduce chronic disease risk (USDA, 2012). This is in concordance with the review of Hollman et al. (2011) which considered whether the cardiovascular benefits associated with polyphenols could be attributed to their *in vitro* antioxidant properties or whether alternative mechanisms are responsible for any *in vivo* effects. Using the PASSCLAIM criteria, which are the basis for European Food Safety Agency (EFSA) health claims approved by the panel on Dietetic Products, Nutrition and Allergies (NDA), the authors found that any reduction in cardiovascular risk could not be attributed to the antioxidant effects of the polyphenol containing food (Hollman et al., 2011). This publication followed on from the scientific opinion of the NDA panel of EFSA in 2010, which found that a causal relationship could not be demonstrated. The panel also stated that any beneficial, psychological effect (as with physiological effects) could not be linked to the foods antioxidant activity, content or properties (EFSA, 2010c).

Therefore, despite the *in vitro* antioxidant potential, and even though the flavanols in cocoa and chocolate, being predominantly epicatechins are relatively bioavailable compared to many of the other polyphenols (Manach et al., 2005b; Williamson & Manach, 2005), this mechanism appears not to be of clinical relevance. This position is further supported by data comparing the normal concentration of polyphenols in the blood and those following supplementation, with the key physiological antioxidants in

the form of vitamin E, vitamin C and urate (Wayner et al., 1987; Nieto et al., 2000). In this context polyphenols make up less than one percent of circulating antioxidants in plasma supporting the hypothesis that other mechanisms must be at work which may allow the lower concentrations observed to be able to exert biological activity and explain their mode of action in reducing disease risk (Hollman et al., 2011).

1.3.3 - Flavanols and Cardiovascular Disease Risk

Polyphenols (including flavanols) have been described as the most abundant antioxidants in the human diet (Scalbert, Johnson & Saltmarsh, 2005; Manach et al., 2005). It has been suggested that dietary intakes can be as high as 1g per day in a typical Western diet, being approximately 10 times higher than the intake of vitamin C and about 100 times higher than intakes of vitamin E and carotenoids (Scalbert and Williamson, 2000; Manach et al., 2005; Williamson & Manach, 2005). As a result of their abundance this led to phytochemicals being associated with a reduction in risk of a number of diseases over the last decade. It is also possible these compounds may have effects in preserving foods (Li, Henning, Zhang & Zerlin, 2010; Mellor et al., 2010a) or even intestinal roles by acting as prebiotics, with the action of intestinal bacteria upon these compounds resulting in reduced cardiovascular risk (Tzounis et al., 2011).

Despite the lack of evidence for the antioxidant hypothesis *in vivo*, there are large numbers of studies reporting a beneficial effect of polyphenols in reducing cardiovascular disease risk, carcinogenesis and osteoporosis, along with potential to assist in the prevention of neurodegenerative diseases and diabetes mellitus (Scalbert et al., 2005). However global consensus is still being sought, with the World Health Organisation (FAO/WHO, 2003), suggesting that the risk reduction of cardiovascular disease by polyphenols was "possible" but that "insufficient" data were available to support a claim for risk reduction of cancers. This research field has subsequently moved forward rapidly over the last decade, therefore a systematic review of the

literature pertaining to chocolate and cocoa is therefore presented later in Section 1.5 in this chapter.

1.3.3.1 – Polyphenols as a potential nutrient

Polyphenols are a group of the biologically active food components, which appear to have a large and rapidly expanding evidence base for their beneficial effects upon human health. For the purpose of this thesis, polyphenols are not considered as true nutrients as they are not currently deemed to be essential for human health and survival. A review of their bioavailability (Manach et al., 2005) defined polyphenols as an 'abundant dietary micronutrient'. In terms of clear effects upon health it could be argued that polyphenols are a nutrient, through this opinion is yet to be universally accepted. However there is no evidence of harm of dietary intakes, which totally exclude polyphenols, only potential benefits of increased intake. The argument that to be defined as a nutrient, the compound must be essential and *de novo* synthesis cannot occur, does not hold true, as in theory humans could synthesise all the carbohydrate needed. This is also the case for niacin and vitamin D both of which can also be synthesised in humans. Unlike glucose, niacin and vitamin D, polyphenols as secondary plant metabolites are not synthesised by humans, and therefore consumption may provide additional benefits when compared to dietary exclusion. Supporting the initial assertion that polyphenols are a nutrient, originally being described as vitamin P (Mobh, 1939).

There is a growing volume of evidence to support the hypothesis that polyphenols may be beneficial in moderating the risk of developing T2DM, and reducing the risk from the cardiovascular risks associated with T2DM. These effects were categorised by Williamson and Manach (2005) as follows:

- Reduced blood pressure
- Improved lipid profile
- Improved vascular health
- Reduced oxidative stress
- Reduce insulin resistance

These have been associated with cardiovascular risk and morbidity associated with T2DM, and along with inflammation will be the key areas of focus for this thesis.

1.3.4 - Classification of Polyphenols

It is important, prior to considering the wide distribution of polyphenols found in the typical human diet, to consider their chemistry. In terms of their chemical structure polyphenols are phenolic compounds, containing more than one aromatic ring (benzene) in conjunction with one or more alcohol group (hydroxyl). Polyphenols are responsible for many of the organoleptic properties of foods and beverages derived from plants. Along with their proposed health benefits, they are particularly important flavour and colour compounds in many plant-based foodstuffs. It has been proposed that many of the health benefits linked to fruit and vegetable consumption along with tea and wine are an effect of their constituent polyphenols. Although polyphenols have been purported to have beneficial effects upon health they have been poorly described and defined in the literature possibly due to the difficulties in assaying all compounds in a food, with many studies having tended to focus on the small number of characterised molecules. It is additionally plausible that the activity of these polyphenols may be via a

synergistic effect with other components within the carrier food matrix. A further important consideration is that polyphenols also change physically, in terms of their chemical structure, in terms of bioavailability and in concentration within foods post-harvest under the influence of numerous enzymic pathways within the foodstuff, and the effects of food storage and processing. These factors are complex and difficult to define and operate prior to the effects of gut microflora, the ability of the human gastrointestinal tract to absorb polyphenols and their post-absorption metabolism and half-life (Setchell, Brown & Lydeking-Olsen, 2002; Tzounis et al., 2011). Overall, the complex nature of the original molecules, their wide distribution in foods, how they behave prior to consumption, and individual variations in metabolic potential, make it impossible with any degree of certainty to be sure how these molecules have their effects.

The term 'polyphenols' refers to the overarching class of molecules (figure 1.3.4.1.1), which include the flavonoids and several classes of non-flavonoids. The non-flavonoids are considered as having simpler structures and include:

Hydroxybenzoic acids

Hydroxycinnamic acids

Stillbenes

Gallotanins

Lignans

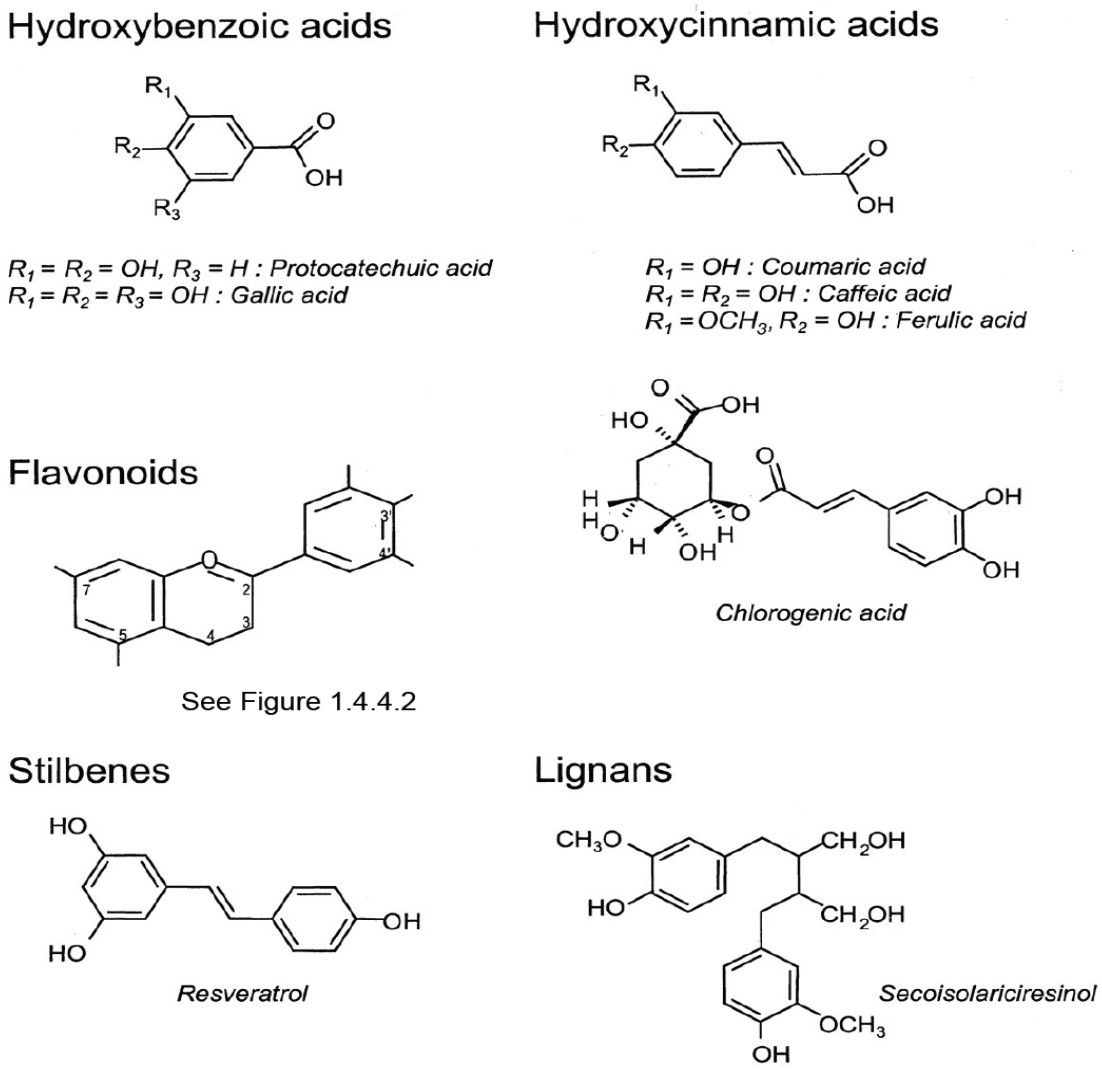


Figure 1.3.4.1.1: Chemical structures of polyphenols showing both flavonoid and non-flavonoid polyphenol structures. Produced with permission from Manach et al. (2005)

These non-flavonoids, are typically less well defined than the flavonoids, and there is some suggestion that they are less bioavailable than the flavonoids (Williamson & Holst, 2008)

The flavonoids can also be classified into a number of different groups:

Flavonols

Flavanols

Isoflavonols

Anthocyanins

Proanthocyanins

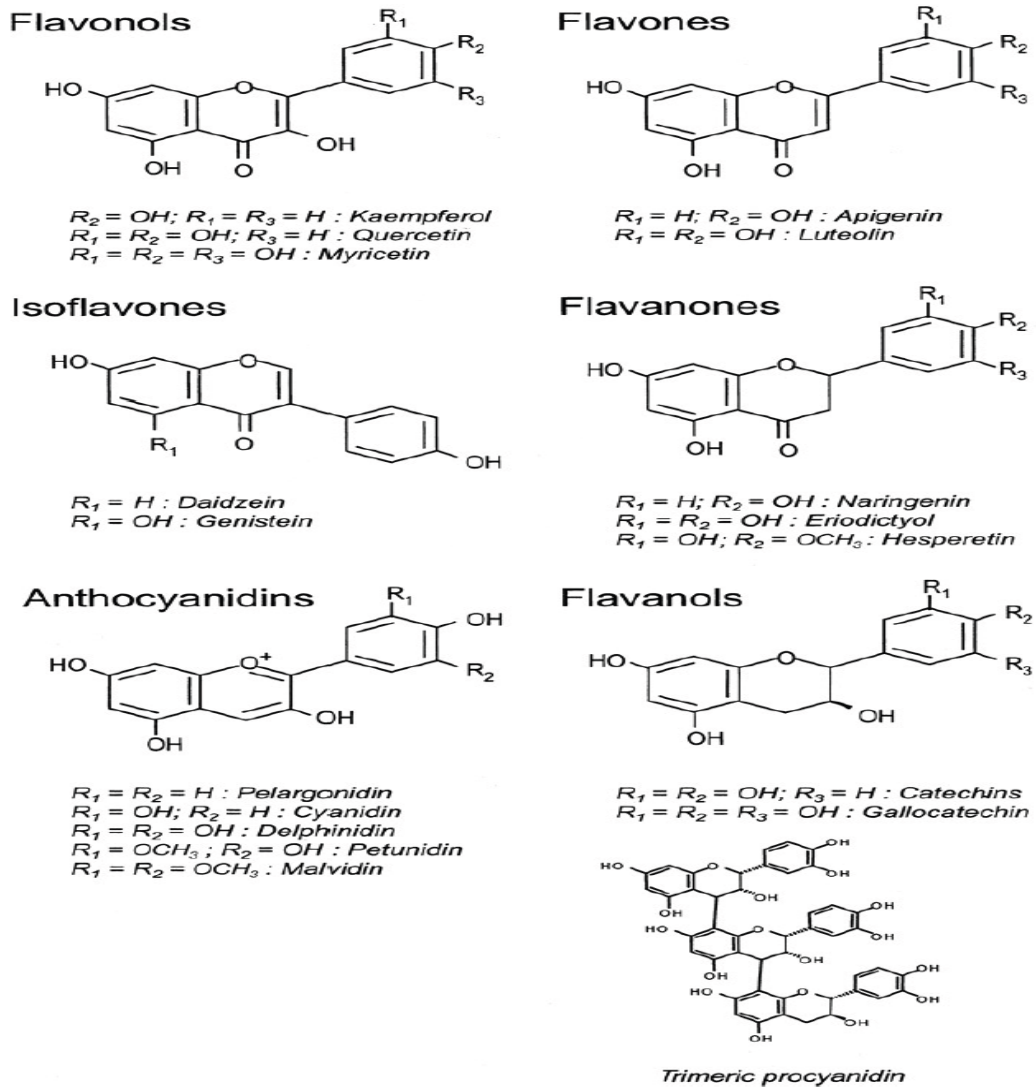


Figure 1.3.4.1.2: Chemical structures of flavonoids showing. Produced with permission from Manach et al. (2005)

In total, more than 4000 flavonoid compounds have been identified in plants, at least in part due to the way the skeleton of the compounds can have groups substituted, e.g. hydroxyl, methoxyl and glycosyl switched or glycosylated (glycone) or aclyated to provide more complex structures.

1.3.4.1 - Flavonols

The major flavonol in the diet is quercetin (figure 1.3.4.1.2), which is found in many foods, and is particularly found in high concentrations in apples, broccoli, onions, red wine and tea. It is also a component of the nutritional supplement Ginkgo biloba. This is significant as the Ginkgo biloba also contains another potential bioactive compounds in

the form of terpenoids. This suggests a number of strategies that can be employed to investigate the effects of polyphenols in foods. The investigator can choose to look at the effects of the pure compound, as a food extract, or the whole food. In the future it is possible that polyphenols with demonstrable bioactivity will be isolated and formulated into compounds, which are then reintroduced into a food matrix to provide a novel functional food.

Demonstrating the effectiveness of flavonols represents a considerable challenge. There is clear evidence of quercetin effects *in vitro*, but *in vivo* studies have not shown consistent effects. The probable reason for the lack of effect of quercetin in clinical trials, is perhaps its poor absorption (Manach et al., 2005).

1.4.4.2 - Flavanols (Flavan-3-ols)

The catechins are the most common group of compounds classified as flavanols (figure 1.3.4.1.2). They are widely distributed in plant-based foods and are further sub classified into five groups:

(+) - Catechin

(-) - Epicatechin

(-) - Epigallocatechin

(-) - Epicatechin gallate

(-) - Epigallocatechin gallate

(+) - Catechins are especially found at highest concentration in broad beans (fava beans), dark skinned grapes, apricots and strawberries. (-) – Epicatechins along with their high abundance in broad beans and dark skinned grapes, are also found in apples, blackberries, cherries, chocolate, cocoa, pears and raspberries. The gallates and gallocatechins are found almost exclusively in tea, especially green tea (Williamson & Manach, 2005).

Proanthocyanidins, is a term often used to describe polymeric chains of other flavonoids; it was initially known as vitamin P by chemists in the 1930s and 40s (Mobh, 1939; Carpenter, 2003) . This further highlights the wide variety of molecules, which make up this group. As well as being antioxidants, these compounds are considered to have the biological effect of being bitter, as a survival mechanism to reduce palatability of the plant to herbivores. As proanthocyanidins are polymeric flavonoids, their distribution is similar to that as described for the flavanols above (also figure 1.3.4.1.2).

1.3.4.3 - Isoflavonols

Much polyphenols research has focused upon isoflavones. These have been studied both in the short term (a period of weeks), often seen as a limitation of the studies investigating isoflavonols, as well as in a small number of studies of up to 1 year in duration. The majority of the studies investigated the effects of isoflavones have been linked to their effects on biomarkers of bone mineral density. It was proposed that the potential to reduce bone mineral loss in women was linked to hormonal changes following the menopause, the mechanism being that isoflavonols have weak effects via their binding to oestrogen receptors, and supplement falling endogenous oestrogen levels in post-menopausal women (Mei, 2001; Atkinson et al., 2004). This effect might help explain changes seen in lipid profiles since increases with High Density Lipoprotein (HDL) cholesterol and decreases in LDL cholesterol levels and the susceptibility of LDL cholesterol to oxidation, are known to be affected adversely by the menopause (Derby et al., 2009).

The effects of isoflavones have been investigated using either soy or red clover as their source (Hamilton-Reeves et al., 2010; Hooper et al., 2009), with most of the literature reporting the effects of soy, either in whole beans, protein extracts (which may have been supplemented with isoflavones) or pure isoflavones. As with flavonols, isoflavones are found in soy mostly in the conjugated glycoside form (glycine), which

can be converted to the free form (aglycone) when the soy is fermented. However the aglycone isoflavones need to be in the glycoside form to be absorbed at the maximum level. This is often considered to be a function of the gut microflora, with some individuals being more efficient than others with respect to this, which in turn results in responders and non-responders to soy supplementation (Rowland et al., 2000). The most abundant isoflavone glycosides in soy are genistin (genistein being the aglycone, which has lower bioavailability) and diadzin (together with it less available aglycone daidzein).

As with the flavonols, isoflavonols have been shown to have activity *in vitro*, some of which is linked to their pseudo-hormonal action, with other observed effects, historically ascribed to an antioxidant mechanism. These effects are potentially difficult to distinguish, as described earlier, from which it may be inferred that these compounds act via a range of pathways, which appear to mimic an antioxidant function, thus making it difficult to be certain of the precise biological effects of these compounds. In postmenopausal women with T2DM, Jayagopal et al. (2002) demonstrated that supplementation of soy protein with isoflavones can reduce insulin resistance along with modest improvements in measures of glycaemic control. Whole soy nuts have also been shown to be beneficial in T2DM (Azadbakht et al., 2003, 2007). However, pure isoflavones in the form of the aglycone isoflavones were shown not to have any effects (González et al., 2007). This might be due to the form in which the isoflavones were provided or to a synergistic relationship between isoflavones and soy proteins.

1.3.4.4 - Anthocyanidins

These are related compounds, and probably the least well studied of the flavanol compounds both chemically and in terms of effects on human health (Williamson & Holst, 2008). Anthocyanidins can exist in glycosylated forms, known as anthocyanins, and produce the deep red and purple pigments in plants. Plants use these properties to

attract pollinators and seed distributors along with their antioxidant role, which includes defending the plant from ultra violet radiation. However it is considered that these compounds have biological activity in foods, both in terms of food preservation (Mellor et al., 2010a), and potentially *in vivo,* following consumption by man.

1.3.5 - Bioavailability

A considerable barrier to polyphenols achieving biological efficacy in humans is their low bioavailability (Williamson & Manach, 2005; Manach et al., 2005). Polyphenols are not all absorbed to the same extent. Bioavailability data assumes, as stated in the reviews of Williamson and Manach (2005) and Manach et al. (2005), that polyphenols must be absorbed and bioavailable in order that beneficial effects can be observed. However in a recent study by an Italian group (Villaño et al., 2010), benefits in terms of a reduction in oxidative stress were reported, but no plasma aspalathin or nothofagin (the dominant polyphenol in the investigational product) were detected. Other groups have detected these polyphenols in plasma, so this might have been a problem with the assay rather than the compounds true bioavailability (Breiter et al., 2011).

Of all the polyphenols, the epicatechins, especially those found in cocoa show the greatest bioavailability with a maximal plasma concentration detected after two hours. Approximately 20% of the total epicatechin consumed is excreted in the urine (Manach et al., 2005). This might explain in part why cocoa polyphenols have been amongst the most studied of this group of compounds.

1.3.5.1 – Inhibitors of polyphenol availability

A range of dietary factors is known to influence the availability of polyphenols. Perhaps the most relevant to dietary intake in the UK, is the influence of milk and dairy produce. This assumption appears to have been derived from epidemiological studies where tea does not appear to have beneficial effects in British populations, the conclusion being that this is a reflection of the British habit of having milk with tea, thus reducing the

availability of the polyphenols (Frei & Higdon, 2003). However data from bioavailability studies using cocoa and milk, suggest that although urinary epicatechins are reduced by the addition of milk, plasma levels are not adversely affected. It is plausible that the fatty acid composition of milk may enhance the absorption of epicatechins. In fact this mechanism is exploited to enhance uptake of a number of pharmaceutical agents (Anderberg, Lindmark & Artursson, 1993; Lindmark, Kimura & Artursson, 1998).

A final potential issue regarding polyphenols is their stability within foods post-harvest. In the case of anthocyanidins, instability can be seen when foods including red cabbage are cooked, when a drop in pH alters its structure, and hence the colour from red to blue. Bąkowska, Kucharska and Oszmiański, (2003), also demonstrated the lack of stability of polyphenols when exposed to ultra violet light. However this is not the case for all polyphenols or food matrices. For example, the polyphenols in chocolate have been shown to be stable for up to two years (Hurst, Payne, Miller & Stuart, 2009).

1.3.6 - Observed Health Effects of Polyphenols

1.3.6.1 - Reduction in blood pressure

A number of polyphenolic compounds have been reported to lower blood pressure, both in animal models and in clinical trials in human participants. A study using wine polyphenols in rats, (Diebolt, Bucher & Andriantsitohaina, 2001), was the first to propose not only a reduction in blood pressure, but increase in the expression of a number of genes suggesting the potential for an epigenetic effect. The genes whose expression is enhanced appear to be associated with endothelial function via increased nitric oxide and prostaglandin production, which result in reduced blood pressure. More recently, studies supplementation with cocoa in humans has been shown to lead to an inhibition of angiotensin converting enzyme, a key step in the pathway which leads to a

reduction in the synthesis of angiotensin, which could lead to a reduction in blood pressure (Persson, Persson, Hägg & Andersson, 2011).

1.3.6.2 - Improved lipid profile

Polyphenols have been associated with the beneficial effects of dyslipidaemias. This might also explain why diets rich in plant-derived foods and polyphenols have been associated with a reduction in cardiovascular risk (Jenkins et al., 2003). Data from mice models of diabetes, suggest that polyphenols might act via hepatocellular AMP-activated protein kinase (AMPK) along with its downstream target, acetyl-CoA carboxylase. The effect of polyphenols also appeared to be approximately 200 times more potent than that of metformin in their effect upon lipid metabolism (Zang et al., 2006). This work also linked AMPK to the pathogenesis of the dyslipidaemia in an animal model of diabetes. As with the case of blood pressure there are a number of alternate mechanisms, with a further possibility for lipid modification being via modulation of the ATP Cassette Binding protein, which may also improve lipid profile (Noe et al., 2004). In data from human studies improvements in lipid profiles have been seen in populations with diabetes (Mursu et al., 2004), with increased HDL cholesterol levels. Other research in populations without diabetes have suggested that polyphenols may reduce the oxidation of LDL cholesterol (Hudthagosol et al., 2011).

1.3.6.3 - Reduction in insulin resistance

The mechanism, by which insulin resistance may be modified by polyphenols, has been suggested to be via a similar mechanism to the improvement in lipid profiles, the AMPK pathway, although in the case of insulin resistance, the AMPK pathway needs to be activated in both skeletal muscle and liver (Hegarty, Turner, Cooney & Kraegen, 2009; Zang et al., 2006).

1.3.6.4 - Improved endothelial function

In many of the studies which demonstrated a reduction in blood pressure the effect of polyphenols has been attributed to endothelial function (Diebolt, Bucher & Andriantsitohaina, 2001). It has been suggested that the improvement in endothelial function derives from an increase in redox sensitive activation of the phosphatidylinositol 3-kinase/Akt pathway, which results in activation of endothelial nitric oxide synthase (eNOS). This is thought to be via an increase in intracellular free calcium concentration and activation of oestrogen receptors (Schini-Kerth et al., 2010). These changes appear to go beyond just nitric oxide, as polyphenols may act on other endothelium factors.

1.3.6.5 - Reduced oxidative stress

Although *in vitro* studies suggest powerful effects of polyphenols in terms of their abilities to scavenge free radicals, the data from *in vivo* participants are considered to be 'confusing and equivocal' (Halliwell, Rafter & Jenner, 2005; Halliwell, 2007). However it can be challenging to assess oxidative stress *in vivo*, as a number of assays measuring damage to genetic material can be insensitive to dietary manipulation (Collins, 2005). Also the potential difficulties presented by poor absorption of polyphenols, suggest that any effects are likely to go beyond just their antioxidant capabilities and likely act via multiple mechanisms (Williamson, 2009). It is highly plausible that other aspects of polyphenol action might be supported by their antioxidant capabilities, but this is not their primary beneficial effect. It is a logical hypothesis that the beneficial effect of polyphenols upon endothelium by increasing the synthesis of endothelium derived factors such as nitric oxide and prostaglandins, which have additional free radical scavenging capabilities, and would enhance the pool of these beneficial compounds.

1.3.6.6 - Other potential effects of polyphenols

The effects on endothelial function may extend into other aspects of vascular health. Polyphenols have been demonstrated to inhibit platelet aggregation (Demrow, Slane & Folts, 1995; Freedman et al., 2001), the potential mechanism which may be by a decrease in the production of superoxide anions and an increase in nitric oxide synthesis (Freedman et al., 2001). A further potential effect, which polyphenols may have on health, is as 'an anti-nutrient' or chelating agent. This apparently 'negative effect' could potentially have beneficial effects in the gastrointestinal tract, and potentially *in vivo* by binding metal ions, which could otherwise be pro-oxidants. Although such an effect has been hypothesised as beneficial, it could as a potentially increase the risk of mineral deficiencies, especially iron deficiency anaemia (Samman et al., 2001). It appears likely that polyphenols have a range of effects. It is likely that the comment of Williamson (2009) is correct, in that polyphenols have a 'multitude of methods of action, with the mechanisms discussed above often interacting and overlapping in their action'.

1.3.7 - Summary of the Polyphenols and their Potential Effects

Polyphenols are a diverse group of plant-derived compounds which have been shown to have dramatic antioxidant actions in vitro, but which it is difficult to replicate in humans. The effects of polyphenols potentially in enhancing health are hampered by their lack of bioavailability and stability/poor shelf life. Although polyphenols are abundant in many foods, and widely consumed in Western diets, it is a challenge to select a potential source for food supplementation, as the foods need to be palatable, provide a rich source of polyphenols, and be stable and well absorbed. The polyphenol most likely to fulfil these requirements are epicatechins from cocoa, with the caveat for individuals with diabetes, cocoa and chocolate may present other nutritional challenges, where potential benefit of polyphenols might be negated by other nutritional factors (especially the high energy, saturated fat and sugar content) within the final food product.

1.4 Systematic Review of the Effects of Cocoa and Chocolate on Cardiovascular Risk

1.4.1 - Epidemiological Evidence for Beneficial Effects of Cocoa and Chocolate

The case for potential benefit has been described in section 1.3. The purpose of this section is to consider critically the evidence and need for further work based on the published data. The approach will be using the structure of an inverted pyramid of evidence. The review aims to critique the literature, with Chapter Three summarising the data and presenting an exploratory review with meta-analysis comparing chocolate to cocoa in its effects on the six biomarkers of cardiovascular risk and glycaemia control defined previously in Section 1.2.3.

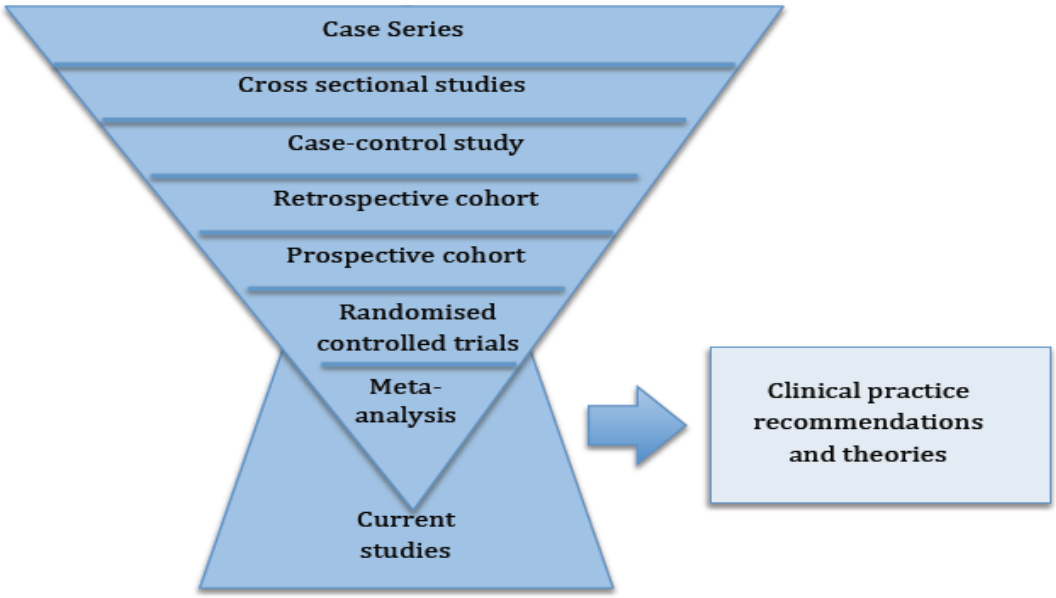


Figure 1.4.1: Inverted pyramid of evidence, highlighting the hierarchy of clinical evidence in reverse, with registered clinical studies currently as adding to the data. The position of meta-analyses is cited as having greater power of showing the effect than randomised controlled trials, but also linking into the design of current studies.

Work on the Kuna Indians, an indigenous population who inhabit islands near Panama, provided initial insight into the health effects of cocoa (Hollenberg et al., 1997;

Hollenberg, 2006; McCullough et al., 2006). This ethnic group have little age-related increase in blood pressure or associated hypertension. However, when members of this ethnic group move to the urban environment of Panama City, they lose this protection and revert to the expected levels of hypertension. This could be said to represent the classic 'migration type' study suggesting a lack of a genetic effect, and that the increase in hypertension must be attributed solely to environmental influences. The island-dwelling Kuna consume large quantities of cocoa daily, both as drinks (up to 5 cups a day) and as an ingredient in cooking. Those living in Panama City have a very low consumption of cocoa. Moreover, the island dwelling Kuna have a 3-fold higher level of nitric oxide metabolites in their urine compared to the city dwellers (Hollenberg et al., 1997; McCullough et al., 2006). This epidemiological study clearly suggested an association between cocoa flavanols and improved vascular function as suggested in section 1.4.2.4 and warrants investigation via clinical trials to quantify the effect and assess causality.

Epidemiological evidence on the effect of chocolate have largely been derived from studies in which the data collection was initially undertaken as part of other studies, and the effect of cocoa or chocolate consumption examined as a *post hoc* analysis. The majority of the data were derived from European prospective study data sets (Buijsse, Feskens, Kok & Kromhout, 2006; Buijsse et al., 2010; Janszky et al., 2009) with contributions from studies undertaken in the USA (Djoussé, Hopkins & Arnett, 2011) and Spain (Recio-Rodríguez et al., 2012). These later two studies were cross-sectional in design, and lack the predictive power of the European cohort studies. The European studies also had the strength of reporting the hard endpoints of death or myocardial infarction, suggesting that chocolate consumption is associated with a reduction in rates of mortality.

1.4.1.1 – European epidemiological data

Data from the Zutphen Elderly study (Buijsse et al., 2006) reported the effects of the consumption of foods containing cocoa in 470 elderly men. The initial data was collected in 1985 and the outcome data analysed 15 years later. Buijsse et al. (2006) found no association between sugar confectionary consumption and mortality (p=0.54), but did demonstrate a significant inverse association between habitual cocoa containing food intake and cardiovascular mortality rates (as well as all-cause mortality rates). This was in agreement with the cross-sectional data collected in 1985, which suggested a significant inverse association between blood pressure and habitual intake of foods containing cocoa. These data were subsequently confirmed by an analysis of the Potsdam cohort from the European Prospective Investigation into Cancer (EPIC). Chocolate consumption and blood pressure were assessed at baseline and were found to be inversely associated in 19357 participants aged 35-65. The follow up data suggested that the highest intake of chocolate had a significantly lowered risk of myocardial infarction and stroke than the lowest consumers (RR=0.61 (95% confidence interval 0.44-0.87) p=0.014 for trend), with 12% (95% confidence interval 3-36%) of this effect explained by the effect of chocolate on blood pressure after a mean follow up of eight years (Buijsse et al., 2010).

This inverse relationship gained further support from the data presented in the Stockholm Heart Epidemiology Program (Janszky et al., 2009), which reported the outcomes of a population of 1169 patients who had survived their first acute myocardial infarction. At the point of diagnosis, patients reported their intake of chocolate over the preceding 12 months, and were then followed up for eight years. As previously seen, there was a strong inverse association between chocolate consumption and cardiac mortality rates. The highest quartile of consumers had an adjusted hazard ratio of 0.34 (95% confidence interval 0.17-0.70) when compared to non-consumers. Also in keeping

with the Zutphen cohort (Buijsse et al., 2006) there was a weak inverse association with total mortality and chocolate consumption.

These three studies have been included in the systematic reviews of Buitrago-Lopez et al. (2011) and Khawaja, Gaziano and Djoussé (2011) who both acknowledged that the evidence from prospective cohort studies suggests a strong association between cardiovascular mortality and cocoa/chocolate consumption. However, this type of data does not equate to 'evidence of causality' and it is necessary to undertake experimental work, such as randomised controlled trials in order to elucidate a cause and effect. Khawaja, Gaziano and Djoussé (2011) also suggested that further work is required to elucidate the underlying mechanisms of action, which in view of the widespread abandonment of the antioxidant hypothesis, due to a lack of evidence and low plasma concentrations (Hollman et al., 2011; EFSA, 2010c; USDA, 2012) is logical and compatible with the evidence supporting mechanisms linked to nitric oxide synthase, ATP binding cassette proteins, AMP kinase or Angiotensin Converting Enzyme.

Cross-sectional studies, which can only suggest an association with incidence, have suggested an inverse link between chocolate consumption and calcified atherosclerotic plaque (Djoussé, Hopkins & Arnett, 2011) but not with cocoa consumption. Recio-Rodríguez, et al. (2012) undertook a smaller cross-sectional study in Spain, finding no association between cocoa consumption and arterial stiffness. The study by Djoussé et al., (2011) considered data from 2217 individuals with increased risk of cardiovascular disease from the NHLBI Family Heart Study cohort, and later, 351 middle aged Spanish adults. These mixed results might have been an artefact of the study design and the population selected for the Spanish study (Recio-Rodríguez, et al., 2012), where by its nature the work of Djoussé, Hopkins and Arnett (2011) with a larger cohort, was potentially sensitive enough to detect an effect and by its nature behaving akin to a retrospective cohort study.

A final series of data worthy of consideration were presented in three studies from the USA; one cross-sectional study of a statin cohort (Golomb, Koperski & White, 2012) and two analyses of the NHANES data collected between 1999-2004. Golomb, Koperski and White (2012), found that chocolate consumption was negatively related to body weight. This perspective was given further support by the NHANES data in adults, teenagers and children (O'Neil, Fulgoni & Nicklas, 2011a; 2011b), which considered the relationship between chocolate and confectionary intake and body weight (body mass intake), cholesterol, blood pressure and C-reactive protein (O'Neil, Fulgoni & Nicklas, 2011a). This study found a significant inverse association between chocolate intake and body weight ($p=0.0096$) and waist circumference ($p=0.0067$). For the biomarkers assessed, the data suggested a significant positive association between chocolate intake and HDL cholesterol ($p=0.0187$) and a significant negative association with C-reactive protein ($p=0.0174$). Thus, in a population of 15023 adults (over 19 years old) it suggested that higher chocolate consumption is associated with a lower risk of metabolic syndrome (O'Neil, Fulgoni & Nicklas, 2011a), potentially supporting the case for chocolate intake being beneficial in T2DM. This study together with the data from other epidemiological studies, excluding that of Recio-Rodríguez et al. (2012) suggest there is a reduced cardiovascular mortality and risk associated with chocolate consumption.

Overall it is clear from the epidemiological data that there is a very strong association between chocolate consumption and improved cardiovascular risk which warrants interventional clinical trials to elucidate this effect further and assess the potential causal beneficial effects of chocolate.

1.4.2 - Systematic Review of Clinical Trials

The systematic review of the data was undertaken following Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Liberati et al., 2009)

with a review of the literature being undertaken at the end of June 2012. The review will focus upon studies reporting primary outcomes related to the effects of chocolate and cocoa supplementation in the six areas set out below, and described in Section 1.2.3 and 1.3.3.

Blood pressure

Lipids

Insulin resistance and diabetes control

Endothelial function

Oxidative Stress

Inflammation

In addition, weight change was also included, based upon the findings of Golomb, Koperski and White (2012) and O'Neil, Fulgoni and Nicklas (2011a; 2011b) to see if similar neutral or postive effects on weight are reported in clinical trials.

1.4.2.1 - Clinical trials data: blood pressure

Many trials investigating the effects of cocoa or chocolate consumption on health have included blood pressure as either a primary or secondary outcome. The first report to suggest beneficial health effects of chocolate consumption was published as a research letter in the Journal of the American Medical Association (Taubert et al., 2003). This small study selected thirteen elderly participants with isolated systolic hypertension, and in a crossover design, gave them either 100g of dark chocolate following seven days without chocolate for 14 days or 90g of white chocolate, then after a seven day wash out participants were crossed over. The dark chocolate was said to contain 500mg of polyphenols and the white chocolate was said to be polyphenol free. There are no details of how the polyphenols were analysed. Daily blood pressures were measured, and from day nine to fourteen there was a significant reduction in systolic blood pressure (for diastolic blood pressure, this was seen from day ten onwards). The

reduction in blood pressure was 5.4± 2.4/1.8±2.0 mmHg, which was clinically significant and equivalent to the effect seen when increasing habitual physical activity to the recommended levels (Arroll & Beaglehole, 1992).

This protocol was repeated by Grassi et al. (2005a), using healthy participants with the additional assessment of insulin resistance (determined by Homeostatic Model Assessment (HOMA)). This was based upon the hypothesis that improved insulin sensitivity mediates an increase in nitric oxide, which has the additional benefit of improving insulin-mediated glucose uptake. This study produced a reduction in systolic blood pressure after 15 days supplementation (107.5±8.6 compared with 113.9±8.4 mmHg, $P<0.05$) with no effect on diastolic blood pressure and a significant reduction in insulin resistance as measured by both the HOMA and the quantitative insulin assessment of insulin resistance (QUICKI) methodologies. Both these assessments are based on measurements of insulin and glucose levels, both assessed in the fasting state (Matthews et al.,1985; Wallace & Matthews, 2002; Matsuda & DeFronzo, 1999). Although of potential clinical benefit, this effect does not appear to fit with the hypothesis of insulin-mediated glucose uptake. The study reported the assessments of insulin sensitivity only and demonstrated just an improvement in the fasting state. These data would perhaps be better expressed as an area under the curve analysis to assess glucose removal effectively. However, the reduction in HOMA, which may be partially through nitric oxide, may be of relevance when considering feeding chocolate to participants with T2DM.

The potential disadvantage of these initial studies, apart from design considerations, is the large quantity of chocolate used. At 100g this represents an energy intake of 480kcal (2020Kj), which represents almost 25% of the recommended requirements for an adult (SACN, 2011). In an attempt to address this, (Taubert et al., 2007) investigated the effects of 6.3g of chocolate, providing only 30kcal (126kj) of energy, using a parallel

study design, but over a longer timescale of 18 weeks. This study as with this group's earlier work demonstrated a significant improvement in this healthy middle-aged (56-73 years) population with prehypertension. Systolic blood pressure was reduced by 2.9±1.6mmHg ($p<0.001$) and diastolic blood pressure by 1.9±1.0 mmHg. This study suggested there were no other effects on weight or glucose; the blood pressure reduction was associated with an increase in S-nitroglutathione and the appearance of cocoa polyphenols in plasma and implied that in prehypertension, a small dose of chocolate (providing 5.1mg epicatechin) may still impart an improvement in blood pressure, in individuals with this condition but no other risk factors of cardiovascular disease.

Despite the data suggesting potential benefits of relatively small amounts of chocolate (approximately ½ a square), it should be noted that these data were from relatively healthy participants, where blood pressure was only moderately elevated. Grassi et al. (2008) published data demonstrating the effects of 100g of dark chocolate in glucose intolerant hypertensive participants. This study employed the same dose of chocolate and similar protocol as Taubert et al. (2003) and Grassi et al. (2005a; 2005b), The findings also concurred with those of this group in healthy participants; an improvement in fasting measures of insulin resistance and blood pressure (systolic blood pressure reducing by 3.82±2.40 mmHg and diastolic blood pressure by 3.92±1.9 mmHg). In this study data for the 120 minutes time point was reported, showing a significant improvement following 15 days of dark chocolate ($p=0.035$), but again no dynamic assessment of area under the curve for glucose or insulin was reported.

Although early clinical trials showed a common effect: improvements in systolic blood pressure, further questions remain unanswered. Ried, Frank and Stocks (2009) attempted to compare high polyphenol dark chocolate supplementation with capsules of lycopene. This study did not show any blood pressure lowering effect over an eight-week period, but did suggest that participants found it difficult to comply with

consuming 50g of dark chocolate at the time of the study. This supports the model that smaller quantities of chocolate or alternative formulations may be warranted.

A criticism of much of these data is the nature of the placebo or control used (often white chocolate), in that it is clearly different visibly but also in terms of other biologically active compounds, namely caffeine and theobromine. To try and address this, van den Bogaard et al. (2010) using a cocoa supplement, matched the flavanol content but varied the dose of theobromine and the results, suggested that higher doses of theobromine could increase systolic blood pressure, but acutely reduce central blood pressure (assessed by the measurement of brachial pulse wave form). However no improvement in blood pressure was seen with the natural theobromine cocoa, which still provided 305 mg flavanols compared to the placebo in this group of healthy individuals participating in this crossover study.

In other words, the dose and form of the supplement appear not to have been adequately defined. In studies which had primary outcomes associated with blood pressure, those using chocolate as the intervention had a consistent blood pressure lowering effect Desch et al. (2010b) described a two-dose study using 6g per day of dark chocolate (based on Taubert et al. (2007)) compared to 25g per day. This study did not have a control group, but did suggest a significant reduction in blood pressure in both arms. The higher dose of chocolate did lead to slightly more weight gain (0.8kg), but the lack of control limited the value of this study. A more systematic range of flavanol doses was tested by Davison et al. (2010); using a control with 33mg flavanols compared with cocoa drinks with 372, 712 and 1052mg in a parallel designed study, using healthy participants over a 6 week period. This study showed no effect on blood pressure when measured in the clinic room, but 24-hour ambulatory measurements showed mean improvement in systolic (5.1±5.1mmHg; p=0.001) and diastolic (3.0±3.2mmHg; p=0.002) at the highest dose only. No dose response effect was seen, suggesting that

more research is needed to explore the apparent difference in results between cocoa and chocolate, since the matrix or nutrient composition of the supplemental formula may have an influence.

Further support for the hypothesis that chocolate may have a superior effect on blood pressure, was reported by Sudarma, Sukmaniah and Siregar (2011) who compared the effect of 30g dark chocolate with 25g white chocolate. This study only reported an approximate energy content of the chocolates and no information about the carbohydrate content or the flavanol content or type was given. Despite these limitations, after 15 days, this population of prehypertensive participants had a significant reduction in systolic blood pressure (difference between groups not reported; $p=0.001$), which was accompanied by an increase in serum nitric oxide levels (assessed by measuring the sum of nitrites and nitrates) (difference between groups not reported; $p=0.001$). This study supports the hypothesis that reduction in blood pressure is mediated via nitric oxide and that improved endothelial function might accompany this change.

1.4.2.2 - Clinical trials data: lipid profile

One of the first clinical trials of the effect of cocoa and dark chocolate investigated the effects of the addition of both of these compounds upon LDL cholesterol. This study, by Wan et al. (2001), found an increase in total serum antioxidant capacity as measured by Oxygen Radical Absorbance Capacity (ORAC), a measure which subsequently has been rejected as a marker of relevance to human health (USDA, 2012; Lotito & Frei, 2006; Hollman et al., 2011). Potentially of greater interest was the apparent protection by chocolate and cocoa supplementation of LDL cholesterol from oxidation, despite a reduction in HDL cholesterol. This trial was a parallel design in healthy participants lasting for four weeks, but may be criticised because the two arms of the study were combined together at baseline, making critical assessment of the data difficult.

However, the findings were potentially confirmed by a trial which lacked a control arm by Osakabe et al. (2001) who fed healthy participants 36g of cocoa, and after two weeks found similar changes in plasma antioxidant status and increased resistance in LDL cholesterol to oxidation. Mathur, Devaraj, Grundy and Jialal (2002) used a similar protocol, with a six week feeding intervention and a slightly larger dose of cocoa and chocolate (36.9g dark chocolate and 20.95g of cocoa powder, compared to 16g dark chocolate and 22g cocoa (Wan et al., 2001). However instead of a control group, Mathur et al. (2002) utilised a washout period. This could be argued to be a source of potential bias, along with the lack of any restrictions regarding other dietary flavanols in the protocol. However, a reduction in LDL cholesterol was still seen with the data of Mathur et al. (2002) without seeing a change in plasma antioxidant capacity.

The first clinical trial to investigate the effects of chocolate alone (rather than in conjunction with cocoa) upon lipid profile was by Mursu et al. (2004), which investigated healthy individuals, found that supplementation led to a reduction in LDL cholesterol. Unlike Mathur et al. (2002) and Wan et al. (2001), Mursu et al. (2004) who used a parallel study design in their population of 45 participants in response to eating either 75g white chocolate, dark chocolate or high polyphenol chocolate, all groups demonstrated an increased resistance to LDL cholesterol. Mursu et al. (2004) then suggested that this might be a property of the fatty acids in cocoa. However they did report an increase in HDL cholesterol with the dark (11.4%) and high polyphenol dark chocolates (13.7%), implying a potential role for flavanols too. It was also of note that the white chocolate group lost -1.1 ±2.7kg bodyweight over the three-week intervention period of the study. This was the first study to report a change in a routinely measured marker of lipid metabolism, which is inversely related to cardiovascular mortality (Wilson, Abbott & Castelli, 1988).

Subsequently an increase in HDL cholesterol has been reported in healthy participants (Nanetti et al., 2008) given 50g of flavanol rich chocolate, but the value of this work was limited by the lack of a control group. Hamed et al. (2008) demonstrated an increase in HDL cholesterol of 9% and a reduction of LDL cholesterol of 6%, but again this study, which used 70g of 70% cocoa dark chocolate and was limited by the lack of a control arm.

The three studies that used chocolate alone (Mursu et al., 2004; Nanetti et al., 2008; Hamed et al., 2008) all suggested an increase in HDL cholesterol in healthy participants. Studies that utilised cocoa, however only reported increased resistance to oxidation of LDL cholesterol. This position was partly challenged by the data of Baba et al. (2007) who claimed in a parallel study of 160 participants fed either placebo or one of three doses of cocoa (providing trace, 64.5, 96.7 or 129mg per day epicatechin) but HDL cholesterol was increased, along with a reduction in oxidation of LDL cholesterol. However, the analysis of variance did not reach significance ($p=0.055$) for HDL cholesterol. This was partially the result of all of the chocolate arms significantly demonstrating an increase in HDL cholesterol and suggesting that these findings may not be as valid as those of Baba et al. (2007) who felt there was a high risk of type 1 error. Therefore, it appears as with blood pressure the effect of the matrix or formulation of the chocolate or cocoa warrants further investigation. This was considered partially by Khan et al. (2011) who in a population with increased cardiovascular risk, demonstrated that cocoa in milk when compared to milk alone in a crossover design of only four weeks in length can increase HDL cholesterol ($p=0.008$). This effect was accompanied by the appearance of urinary cocoa metabolites. It is clear that a crossover study, that the study would not be blinded and this might have been a source of confounding. Moreover, the population was not well defined; it included individuals with diabetes and/or three cardiovascular risk factors and did not control for medication.

The exploration of varied matrices or formulations of cocoa and chocolate, along with the effect of matrix on the action of polyphenols was investigated in a study which used a base control of cocoa cream with added cocoa polyphenols, hazelnut cream and plant sterols in one of four combinations (Solà et al., 2012). This study, in hypertensive participants, demonstrated a reduction in blood pressure with the cocoa cream alone, which was meant to be the control ($P<0.05$) and the effect was further enhanced for diastolic blood pressure by the addition of cocoa polyphenols. It was claimed that LDL cholesterol was reduced by the combination of cocoa with hazelnut cream, but this was estimated using the Friedewald equation (Friedewald, Levy & Fredrickson, 1972), so care needs to be taken with its interpretation, as this can be adversely influenced by changes in triglyceride levels.

It therefore appears that cocoa polyphenols may help to protect lipids from oxidation, and that chocolate may perhaps increase HDL cholesterol. However many of these studies were lacking with respect to their design, with either no control, or one which was not matched in terms of appearance, taste, energy content or carbohydrate load.

1.4.2.3 - Clinical trials data: insulin resistance and glycaemia

In three studies Grassi et al. (2005a; 2005b; 2008) investigated the effects of administration of 100g of dark chocolate for 15 days, and found that measures of fasting insulin and glucose metabolism improved in healthy individuals, those with hypertension and those with impaired glucose tolerance. However these measures were secondary to effects on blood pressure. Only two further studies both by Almoosawi (Almoosawi, Fyfe, Ho & Al-Dujaili, 2010; Almoosawi et al., 2012) investigated the effects of chocolate on glycaemia as one of their primary outcomes. Their first study compared the effect of a 500mg polyphenol content chocolate with a 1000mg polyphenol content chocolate on fasting plasma glucose (Almoosawi et al., 2010), but did not measure insulin and the plasma glucose measurement was made using a near

patient testing meter (Accutrend GC, Roche Diagnostics, Burgess Hill, West Sussex, UK), a method which lacks the validity to be acceptable for diagnostic purposes due to a high coefficient of variance. Despite the limitations of a lack of a control, fasting glucose was seen to decrease in this population of healthy overweight and obese individuals along with a significant reduction in blood pressure after two weeks of supplementation. These reductions were seen in both the 500mg and 1000mg polyphenol chocolates. In their follow up to this study, Almoosawi et al., (2012), compared the 500mg polyphenol chocolate with a placebo chocolate with negligible polyphenol content over a four-week period in a crossover design with two-week washout. This study also stratified the women into overweight and lean based on their BMI being above or below 25kgm^{-2}. Of note, no effects on lipid profile were seen, but blood pressure was significantly decreased in both lean and overweight participants ($p<0.001$), and plasma glucose significantly decreased in overweight participants ($p=0.007$) but not in lean participants. The difference in fasting insulin and HOMA (Homeostatic Model Assessment, to assess insulin resistance) appeared to be linked to an increase in fasting insulin in the placebo group ($p=0.001$). In this study, the glucose was measured in a laboratory using a hexokinase assay, which is considered to be more robust (Slein, 1963).

It is therefore possible that modest effects on glucose and insulin metabolism may occur with chocolate consumption. However, it is unclear if this extends beyond healthy, overweight and impaired glucose tolerant participants, to individuals with T2DM.

1.4.2.4 - Clinical Trials Data: endothelial function

Endothelial function or dysfunction has been a major area of interest as a primary outcome of cocoa research. To the end of June 2012 (the cut off point for the data collection for the exploratory review with meta-analysis) there have been twenty-three publications reporting effects of cocoa on endothelial function. Since this may be one of

the earliest manifestations of cardiovascular disease (Quyyumi, 1998; Vita & Keaney, 2002) it is a biomarker of potential interest and significance for nutritional intervention studies. When considering the literature, four aspects warrant consideration. Firstly the methodology, the two predominate methodologies reported in the literature are Flow Mediated Dilation (FMD) and Reactive Hyperaemia-Peripheral Artery Tonometry (RH-PAT). Using sildenafil (an inhibitor of cGMP-phosphodiesterase type 5, which enhances the effects of nitric oxide) to induce an improvement in these two functional assessments of endothelial function, Aversa et al. (2008) concluded that although related, these two functional measurements might represent different aspects of endothelial function. Secondly, along with the functional measures, biomarkers can be used, commonly in the form of adhesion molecules found on endothelial cells and circulating platelets. Thirdly, there are aspects of endothelial mechanism linked to inhibition or assessment of nitric oxide metabolism. Finally, the experimental design is open to question; whether the study protocol tended to assess change following acute ingestion (2-6 hours), after several days of feeding or both, as it is plausible that the short-term mechanisms of endothelial function improvement may differ to any chronic effects.

The first clinical trial used RH-PAT to assess the role of nitric oxide on the potential effect of cocoa in 27 healthy volunteers (Fisher et al., 2003). This study fed 821mg of flavanols in a cocoa beverage for five days (only seven individuals underwent the placebo). This non-randomised trial demonstrated that a flavanol rich cocoa beverage could improve endothelial function acutely, and to greater effect, after 4 days. These findings appeared to be associated with nitric oxide, as this effect could be completely reversed by injecting N^G-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase. This association was also demonstrated using FMD and nitric oxide metabolites following a single dose of 176mg flavanol containing cocoa beverage

(Heiss et al., 2003) in 20 participants in a crossover study. The study included two participants with diabetes amongst a population with and without cardiovascular disease risk but who were not particularly obese. Heiss et al. (2005) extended these findings with similar cocoa formulations to a small sample of eleven smokers and found that a single dose could acutely reverse the endothelial dysfunction often seen with smoking. This effect in smokers was further supported by a chocolate supplementation study, where increases in serum nitrates and nitrites were observed along with improved flow-mediation dilation 2 hours after ingestion (Loffredo et al., 2011).

The benefits seen in endothelial function measured by FMD were not limited to young and middle aged participants. Monahan et al. (2011), in a crossover design study, acutely tested a range of five energy matched doses of cocoa from 0g to 26g (flavanol doses 0.0, 9.3, 25.8, 66.6 and 146 mg) in 23 healthy older adults (63±2 years old). Monahan et al. (2011) additionally demonstrated a dose dependent increase in FMD.

Engler et al. (2004) undertook the first study to investigate the effects of chocolate rather than the cocoa drinks used in earlier work on endothelial function, and were also the first to measure fasting endothelial function following two weeks of chocolate consumption. In a parallel-randomised study, healthy participants were fed 46g of chocolate with either 46mg epicatechin (213mg flavanols) or only a trace of these compounds. Engler et al. (2004) concluded that in their study population, a significant improvement of endothelial function was seen which coincided with an increase in serum epicatechins. These authors also suggested that this was independent of any improvement in oxidative stress or lipid profile, which does not concur with the conclusions of other researchers. To elucidate the potential mechanisms, Schroeter et al. (2006) reported data from five investigation which suggested that the significant improvements in FMD seen with cocoa can be produced by supplementing with the

flavanol epicatechin. Both of these supplements can also increase nitric oxide metabolites, which can be demonstrated in *ex vivo* models.

Grassi et al. (2005b), studied individuals with untreated hypertension who were fed 100g of dark chocolate using largely the same protocol as in their previous study with healthy participants (Grassi et al., 2005a) and then later in individuals with impaired glucose tolerance (Grassi et al., 2008). The dark chocolate was stated as containing 65.97 mg epicatechin by their determination, but had no reference to the 500mg claimed in their previous work. In this crossover design study, twenty participants showed an 11.9±7.7/8.5±5.0 mmHg reduction in blood pressure, which is a large change and comparable to many pharmaceutical agents! Along with this, improved insulin resistance, insulin sensitivity index and endothelial function, were also seen suggesting, that despite the caveat that this was a large amount of dark chocolate (100g) eaten over a short period of time (15 days) the effects on multiple aspects of cardiovascular risks are dramatic and of potential clinical significance. Before incorporating regular consumption of dark chocolate into public health messages, long term the effects of its high energy density and saturated fatty acid content need to be investigated and considered. These data were developed by Vlachopoulos et al. (2005), who showed that three hours after consuming 100g of chocolate, 17 healthy volunteers demonstrated improved endothelial function without altering aortic stiffness. This was despite the potential sources of confounding created by the control being sham feeding, which did not match the participant experience or energy and macronutrients intake. This effect was further supported by the data of Hermann et al. (2006) in a parallel design study, with 20 participants, where the intervention was a lower dose of 40g of dark chocolate. In addition to improved endothelial function after two hours this showed an improvement in shear stress dependent platelet function.

In a complex four arm study design (Kurlandsky & Stote, 2006), which combined interventions including chocolate with almonds in a population of healthy women over six weeks in a parallel design it was suggested, that it is also possible to show a significant improvement in adhesion molecules with 41g of chocolate alone ($p=0.03$), together with a decrease in circulating intercellular adhesion molecule-1 (ICAM-1). This observation was not replicated in the groups where chocolate was in combination with almonds. Although ICAM-1 did not change, in a study supplementing 32 postmenopausal women with a cocoa beverage containing 446mg of flavanols over 6 weeks, an improvement in flow mediated dilation and vascular capillary adhesion molecule (VCAM) were seen (Wang-Polagruto et al., 2006). These data suggest that individual assays of biomarkers are not perhaps valid in isolation. It may be preferable for trials to consider using multiple markers of endothelial function to enhance the validity of any findings. Further evidence was presented by Monagas et al. (2009) despite what could be suggested as an inadequate control, in the form of milk, with the active ingredient being cocoa in milk, which would therefore mean participants, would not be blinded. In a study of 42 high risk participants using a parallel study following 4 weeks of cocoa supplementation, Monagas et al. (2009) reported a significant improvement in biomarkers of endothelial function, including a reduction in expression of markers from monocytes (very-late antigen (VLA-4), CD40 and CD36 all $p<0.05$) and reductions in p-selectin and ICAM-1 ($p=0.007$ for both markers). In addition to improvements in adhesion molecules, Vázquez-Agell et al. (2011) in an acute cocoa supplementation study in healthy participants (n=18) in milk or water, compared with milk alone, found improvements in ICAM-1 and e-selectin, but not VCAM which accompanied a decrease in NF-κB activation in mononuclear cells. These data of Vázquez-Agell et al. (2011) further support the position that to be assured of an effect

of cocoa or chocolate upon endothelial function, more than one marker should be reported as improving following supplementation.

As regards the mechanisms for improvements in endothelial function, although acute responses are well described being mostly associated with nitric oxide metabolism, but the beneficial effects seen in longer-term studies might be different. Heiss et al. (2010), in a study using a 30-day supplementation period in a crossover study with 16 participants with coronary artery disease found that cocoa rich in flavanols can mobilize early endothelial progenitor cells. These cells have been linked to endothelial repair, thus providing a plausible mechanism for acute and prolonged improvements in endothelial function (Shantsila, Watson & Lip, 2007).

The first study which failed to find an effect of either acute or chronic ingestion of chocolate and cocoa upon markers of endothelial function was by Farouque et al. (2006). A parallel study of 40 individuals with cardiovascular disease also showed no effect of supplementation (Farouque et al., 2006). This lack of effect might be an artefact of significantly higher total and LDL cholesterol values in the high flavanol group, which may have acted as a source of confounding, with the populations not appearing to be adequately matched.

The first study to investigate the effects of cocoa supplementation in individuals with T2DM was reported by Balzer et al. (2008). These data considered the effect of cocoa beverages following both acute (after 2 hours) and 30 days supplementation. This study used a 963mg flavanols per day dose (split into 3 doses) in a parallel designed study of 41 medicated participants with T2DM to produce a 30% ($p<0.0001$) increase in fasting flow-mediated dilation. This was seen despite the additional 159 kcal (668Kj) and 15 grams of sugar consumed suggesting that there is potential for cocoa/chocolate supplementation in T2DM. This study represents one of only two studies reporting the

effects of cocoa flavanols in populations defined by their T2DM prior to my work, which is reported in this thesis.

The effect of the sugar and carbohydrate content given acutely was partially explored by Faridi et al. (2008) with further data from a six week feeding study reported by Njike et al. (2011). Both studies emphasised the comparison of cocoa rich in polyphenols with and without sugar and its effect upon endothelial function and both concluded that flavanol rich cocoa improved endothelial function, which maybe attenuated by sugar, and that sugar-free preparations may augment these effects. This was despite Faridi et al. (2008) reporting the first phase of their work, which included an equivalent dose of flavanols from chocolate as that given in the cocoa phase. No statistical comparisons of these data were reported. It is plausible that high sugar might inhibit the benefits of cocoa flavanols, however whether cocoa or chocolate is the optimal delivery vehicle was not investigated, despite the potential of having the data to address this question.

The favoured mechanism for improved endothelial function appears well elucidated. Current consensus is that this is via the up regulation of nitric oxide; however potentially the data of Grassi et al. (2005b) suggests that insulin-mediated pathways may have a role in addition to nitric oxide. This mechanism is likely also to be influenced by the carbohydrate/sugar content of the cocoa or chocolate, which via a hyperinsulinaemic effect may inhibit the size of the bioavailable pool of nitric oxide (Dworakowski et al., 2008; Munzel et al., 2008).

Muniyappa et al. (2008) in a 2-week supplementation trial using cocoa, demonstrated an improvement in insulin-mediated vasodilation, using the gold standard insulin clamp methodology (Matsuda & DeFronzo, 1999), instead of the simpler and cheaper HOMA or QUICKI reported by other groups who have reported improvements in insulin resistance with cocoa or chocolate. Muniyappa et al. (2008) did not observe an improvement in insulin resistance, as seen by Grassi et al. (2005b; 2008). This suggests

that the more robust methodology used in this cohort of twenty individuals taking part in a crossover study for a 2 week supplementation study with a one week washout period, means that changes in insulin resistance may not be a primary effect of cocoa flavanols, whilst maintaining support for an insulin mediated effect on the bioavailability of nitric oxide. This has significant implications for cocoa flavanols intervention trials, as formulations need to potentially minimise their carbohydrate content, whilst maintaining palatability to achieve maximal efficacy with respect to improved endothelial function.

The effects of cocoa supplementation do not appear to be limited to the rested or inactive state. Berry et al. (2010), found that consumption of 701mg of cocoa flavanols (compared with 22mg of cocoa flavanols) reduced the rise in blood pressure in response to exercise associated with improved endothelial function. This suggests a beneficial effect of combining cocoa polyphenols with physical activity. The potentially adverse effects of night shift working on endothelial function and cardiovascular risk is known (Amir et al., 2004; Su et al., 2008), and has been shown to be reduced by supplementation with flavanol rich chocolate, however these data are potentially confounded by the lack of a control group (Kim et al., 2012).

Westphal and Luley (2011) investigated the effect of feeding fat in combination with cocoa flavanols in a placebo controlled, double blind crossover study of 18 healthy participants. They combined cocoa rich in flavanols (918mg) or cocoa poor in flavanols with a meal consisting of whipping cream (3ml per kg, consisting of 33% fat). The data from this study, suggested that cocoa flavanols have the potential to reduce the metabolic stress, which results in endothelial dysfunction in the post-prandial state. Despite these data the effects of cocoa polyphenols on a carbohydrate load have not been tested.

1.4.2.5 - Clinical trials data: oxidative stress and inflammation

A few studies have focused on inflammation as a primary outcome while some have co-reported markers including C-reactive protein (CRP) (Hamed et al., 2008). 'Oxidative stress' is often seen as the biologically relevant equivalent of antioxidant capacity measured in food. This is however a controversial area, as many potential biomarker compounds have been suggested; but as in the case of ORAC, a number of these including malondialdehyde (MDA) also referred to as 2-thiobarbituric acid reactive substances (TBARS). Both of which have been challenged in terms of their biological validity. Although widely used in studies and therefore the published literature, TBARS have been rejected as a valid marker of nutritional influence upon oxidative stress (Mensink et al., 2003). Despite these limitations, this review will consider all markers reported; but these will not included as part of the exploratory review with meta-analysis, where only isoprostanes will be considered is reported in Chapter Three, as this has been acknowledged as a valid marker of oxidative stress (Mensink et al., 2003).

Much of the initial data in this field was published at a time when the scientific community favoured the antioxidant hypothesis and the biological relevance of ORAC. Rein et al. (2000a) reported work linking a rise in plasma epicatechin following ingestion of 80g of dark chocolate, with an increase in plasma antioxidant capacity and TBARS. These data also demonstrated a non-significant increase in uric acid after giving flavanol rich chocolate. Uric acid is the primary antioxidant in plasma and was still present in greater than a thousand fold concentration than the peak epicatechin level. Perhaps of more relevance are markers of oxidative damage, in the form of damaged in vivo molecules. The PASSCLAIM group (Mensink et al., 2003) proposed isoprostanes, as a suitable surrogate measure of oxidative stress. These molecules are the free radical catalysed product of peroxidation of arachidonic acid and other long chain polyunsaturated fatty acids. These are relatively stable and are solely formed by *in*

vivo synthesis (Lawson et al., 1998), unlike MDA and they have become preferred measures of oxidative stress. Wang, Schramm and Holt (2000) demonstrated a rise in plasma levels of flavanols results in decreased levels of 8-isoprostane, along with the increase in antioxidant capacity and TBARS. The acute beneficial effects of cocoa upon F_2-isoprostanes were also reported in healthy volunteers by Wiswedel et al. (2004) and in smokers (Carnevale et al., 2012). Few chocolate supplementing studies have focused on oxidative stress as a primary outcome. A study by Fraga et al. (2005) examined oxidative stress and demonstrated that high polyphenol milk chocolate (105g, 168 mg flavanols) consumption in young footballers for 14 days reduced MDA and uric acid, along with improvements in lipid profile and blood pressure.

The value of isoprostane levels as a reflection of, cocoa flavanols, might relate flavanol chocolate increasing plasma prostacyclin (32% $p<0.05$) and decreasing plasma leukotrienes (29% $p<0.04$), both in an in *in vivo* crossover study in humans and in vitro work (Schramm et al., 2001), with data suggesting an effect upon eicosanoid metabolism as a result of the supplementation with chocolate. The eicosanoids have a role as endothelial derived factors, and thus an inter-relationship with nitric oxide, which reduces endothelial dysfunction. It is also plausible, that this mechanism might explain, in part, the effect of cocoa flavanols on isoprostanes (a breakdown product of eicosanoids). This hypothesis might suggest reductions in isoprostanes may not be via improved antioxidant status or reduced oxidative stress.

Additionally cocoa flavanols have been shown to reduce erythrocyte susceptibility to damage by peroxide acutely (Zhu et al., 2005), and increase the resistance of DNA to damage by oxidative stress despite no change in total antioxidant activity (Spadafranca et al., 2010). These data further support the hypothesis of an alternative mechanism to the antioxidant preposition. The protective effects on reducing markers of oxidative

stress have also been demonstrated in exercise, which is known to increase circulating free radicals (Davison et al., 2012; Allgrove et al., 2011).

1.4.2.6 - Clinical trials data: cardiovascular risk

Composite or combined cardiovascular risk estimates have been the focus of a number of studies, the rationale being the exploratory nature of these studies or that since data on individual markers may not demonstrate the necessary power to reach significance. In addition reducing cardiovascular risk, dark chocolate has been shown to inhibit collagen-induced platelet aggregation (Innes et al., 2003). These effects may relate to interactions in eicosanoid metabolism or pathways associated with adhesion molecules. This theory was partly explored by Flammer et al. (2007), in an acute feeding study of 22 heart transplant patients who were fed 40g of dark (70% cocoa) chocolate in a double blind protocol. These data demonstrated an increase in endothelium-dependent coronary vasomotion and reduced platelet adhesion. In addition the isoprostanes and antioxidant capacity of plasma were improved (Flammer et al., 2007). More recently these effects were also demonstrated in healthy participants (Shiina, Funabashi & Lee, 2009).

Davison, Coates, Buckley and Howe (2008) reported an improvement in the composite measure of improved endothelial function, insulin resistance and blood pressure in overweight and obese participants following supplementation with 902mg flavanols in cocoa. These benefits were not further enhanced when combined with exercise. When combined with sterol esters, cocoa flavanols in chocolate appeared to demonstrate a positive effect upon cholesterol and blood pressure reduction (Allen et al., 2008; Polagruto et al., 2006; Solà et al., 2012). However, these studies lacked the ability to attribute those effects to specifically the flavanols or sterol ester component of the intervention.

Cardiovascular effects of flavanol rich chocolate alone were investigated by Flammer et al. (2011) in patients with heart failure. In 20 patients, the acute (over 2-hours) and four week effects were assessed. No effects on blood pressure were seen, perhaps a reflection of the high degree of antihypertensive poly-pharmacy in the study population. Endothelial function improved and platelet adhesion both decreased (Flammer et al., 2011). In a study of 93 post-menopausal women with T2DM all treated with HMG-co-ase inhibitors or 'statins', were given 1 years supplementation with a combination of flavanols and isoflavones given in chocolate (Curtis et al., 2012). There was a reduction in the level of LDL cholesterol (calculated from Friedewald (Friedewald, Levy & Fredrickson, 1972)), total cholesterol: HDL cholesterol: total cholesterol ratio and insulin resistance was reported (Curtis et al., 2012). This supplement might not be considered to be 'chocolate' in line with EU law (European Union, 2004), and it was difficult in this study as with the studies combining cocoa flavanols with sterol esters (Allen et al., 2008; Polagruto et al., 2006; Solà et al., 2012) to assess the proportion of the effect due to the cocoa or the other potentially active ingredients. Curtis et al. (2012) also postulated that supplementing with cocoa flavanols and isoflavones reduced the estimated 10-year risk of cardiovascular disease, in fact the data indicated there was no net increase for the active compared to a 1.1% increase for the placebo.

1.4.2.7 - Clinical trials data: other biological markers

Chocolate and cocoa rich in flavanols have also been assessed for their effects on other markers of health. These have included the ability to protect the skin from UV damage, (Williams, Tamburic & Lally, 2009) and the emerging area of cognitive function in the elderly (Crews Jr., Harrison & Wright, 2008), who additionally reported reductions in cardiovascular risk. Further effects were a reduced risk of dementia (Desideri et al., 2012) and stroke (cerebral vascular accidents (CVA)) (Larsson, Virtamo & Wolk, 2012). Effects of cocoa flavanols upon changes in muscle function at a structural level

were reported (Taub et al., 2012) in a very small and uncontrolled study, along with the potential to enhance muscle recovery following exercise (Gilson et al., 2010).

There is also emerging data to suggest that cocoa polyphenols may have effects upon the gastrointestinal tract flora, with increases in *bifidobacteria* and *lactobacilli* (both $p<0.05$) along with decreases in potential pathogens (Tzounis et al., 2011). These effects were seen in conjunction with simultaneous decreases in C-reactive protein and triglycerides. This provides a further potential mode of action for cocoa flavanols as prebiotics, this might in turn influence enterocyte metabolism and result in systemic reductions in disease risk (Lomax & Calder, 2009).

1.4.2.8 - Systematic reviews and meta-analyses

Over thirty standard reviews, systematic reviews and meta-analyses have been published on the health effects of cocoa and chocolate up to the end of June 2012. However, only 11 of these reviews along with a costing study undertaken by Zomer et al. (2012) have followed a systematic methodology, and only these will be considered in this Chapter. Two of these have already been discussed in section 1.5.1, as part of the epidemiological data on the effects of cocoa on health, where they were, the only reviews to report hard endpoints, including death or myocardial infarction (Buitrago-Lopez et al., 2011; Khawaja, Gaziano & Djoussé, 2011).

The first systematic review on the effects of chocolate in the prevention of cardiovascular disease was published by Ding, Hutfless, Ding and Girotra (2006), and considered both short term feeding trials along with *in vitro* and epidemiological data. This review focused upon stearic acid, which is found in cocoa butter. The data on this saturated fatty acid suggested it was at least neutral in its effects upon cardiovascular risk, while the flavanols in chocolate were likely to be beneficial, with a crude estimate that 50g chocolate might reduce cardiovascular risk by 10.5% (95% confidence interval 7.0-13.5%). However, this estimate included the caveat that it was based on a number of

extrapolations. This was followed up by the meta-analysis of Taubert, Roesen and Schomig (2007) who considered cocoa and tea intake. The conclusion was that although cocoa may reduce blood pressure, tea intake had no effect. This is despite the significant reduction in blood pressure seen with cocoa ($p<0.01$ for both systolic and diastolic blood pressure), but not for tea. However, although the cocoa and tea studies were analysed separately, no formal subgroup analysis was undertaken.

A rigorous review by Hooper et al. (2008) considered the effects upon cardiovascular risk of a range of flavonoid-rich foods which include cocoa flavanols. This again found that chocolate improved endothelial function (both acutely and chronically) and reduced blood pressure. A range of similar beneficial effects were also described for soy protein isolates and both green and black teas.

Increased interest of the effects of cocoa and chocolate developed in 2010, demonstrated through the publication of three meta-analyses; two focused on blood pressure (Desch et al., 2010a; Ried et al., 2010a) and one on lipid profile (Jia et al., 2010). Ried et al. (2010a) suggested that normotensive individuals did not see a significant improvement in blood pressure, in response to cocoa and chocolate where Desch et al. (2010a) reported a more global improvement was seen across the 10 studies analysed. The meta-analysis assessing the effects of cocoa consumption on lipid profile suggested that although total cholesterol was reduced, improvement in LDL cholesterol was only seen in studies of a lower quality (lower Jadad score (Jadad et al., 1996) and there were no changes in HDL cholesterol (Jia et al., 2010). The meta-analysis of Jia et al. (2010) was also limited by the lack of studies investigating participants with increased cardiovascular risk including T2DM, and therefore an atherosclerotic profile.

This pattern of publication was repeated in 2011, with four further studies; two as previously discussed, focused on the epidemiological data (Buitrago-Lopez et al., 2011; Khawaja, Gaziano & Djoussé, 2011). The other two studies reported the effects on lipid

profile (Tokede, Gaziano & Djoussé, 2011) and multiple cardiovascular risk factors (Shrime et al., 2011). Tokede Gaziano and Djoussé (2011) reported the same benefits following cocoa and chocolate supplementation as Jia et al. (2010) but did not consider study quality across the 10 studies they selected. Shrime et al. (2011) suggested that the data from cocoa consumption studies gave short-term significant improvements in endothelial function, blood pressure, lipid profile and insulin resistance. However this paper had a number of methodological errors, for example the inclusion of data from trials that did not meet the reviewers own exclusion criteria.

The high interest in systematic reviews and meta-analyses continued into 2012 (data collection for the review concluded 30 June 2012), with Zomer et al. (2012) suggesting that chocolate supplementation might prove to be a cost effective intervention in reducing cardiovascular disease. Ellinger, Reusch, Stehle and Helfrich (2012) using a nonlinear regression model with a Bayesian approach demonstrated in 16 randomised controlled trials there was a significant reduction in blood pressure with 25mg dose of epicatechin. This methodology attempted to consider dose effects, which many of the other systematic reviews did not explore. However, it did not address the effect of the formulation of the supplement, whether consuming chocolate is preferential to cocoa. Hooper et al. (2012) followed up their previous work of 2008 (Hooper et al., 2008) focusing solely upon chocolate and cocoa. This confirmed previous findings showing an improvement in endothelial function being improved. Additionally, the authors claimed it to be the first published meta-analysis of improvements in insulin and insulin resistance despite the work of Shrime et al. (2011). Hooper et al. (2012) also suggested that larger, long-term studies were needed which were independent of the cocoa and confectionary industries, to investigate the cardiovascular benefits of cocoa flavanols thoroughly. The overall conclusion of both Shrime et al. (2011) and Hooper et al. (2012) is that the optimal effect of cocoa and chocolate appeared to be in interventions

containing 500mg of polyphenols. This is the same dose that has recently been accepted by EFSA as having a significant effect upon maintaining healthy endothelial function (EFSA, 2012a).

1.4.3 - Summary of Review

The history of this area of scientific exploration is less than twenty years old, from the initial *in vitro* work of Waterhouse, Shirley and Donovan (1996), to the first human studies being reported in 2000 (Wang et al., 2000, Rein et al., 2000a; 2000b). During this time, the beneficial effects of cocoa and chocolate have been demonstrated on a number of health markers. These include antioxidant status, which has been largely discredited as a false and simplistic mode of action to alternative mechanisms and oxidative stress/improved eicosanoid metabolism, lipid profile and resistance to oxidative damage, blood pressure and possibly insulin resistance. Most of these studies have been in healthy individuals, some with cardiovascular disease or risks. Only two studies have been undertaken in individuals with T2DM (although three others included some individuals as part of a high risk group). Of these two studies, one used cocoa as the intervention (Balzer et al., 2008) and did not assess effects upon diabetes, focusing on endothelial function, and the other used an interventional product with at least two potentially biologically active compounds (isoflavones in addition to cocoa flavanols) in a chocolate like formulation (Curtis et al., 2012). Thus there are no published data on the effect of chocolate upon measures of cardiovascular risk in T2DM (outside that presented in this thesis).

The data systematically reviewed, suggest that many of the studies lack rigour in their design, being at risk of confounding to either a lack of control arm, inadequate controls (not matched for appearance, energy content or macronutrients) or randomisation and the concealment of the blinding. In their personal review of the first decade of research into cocoa and health Cooper, Donovan, Waterhouse and Williamson (2008)

summarised and proposed an 11 point checklist for the planning of future trials; these included the design of the trials, the need to consider the matrix or formulation used; the use of chocolate in preference to cocoa (this will be considered in the exploratory review with meta-analysis presented in Chapter Three), transparency; both in terms of independency of the trial from industry and the need to register human trials publically, careful selection of biomarkers; especially those assessing antioxidant effects and finally, publication of null or negative results.

This introduction has considered the potential of cocoa flavanols, administered both in cocoa and chocolate as being beneficial to health. The claim has been accepted by the NDA panel of EFSA as a health claim relating to endothelial function (EFSA 2012a). The literature and currently registered studies leave highlighted six areas proposed in Chapter One, of benefit:

Blood pressure

Lipid profile

Insulin resistance and glycaemia

Endothelial function

Oxidative stress (rather than antioxidant capacity)

Inflammation

These six aspects of cardiovascular risk; three established and routinely used in clinical care, and three emerging risk factors will be examined through the exploratory review with meta-analysis and experimental studies which comprise the original data presented in this thesis.

1.5 Aims and Objectives of Experimental Work

The comments of Fraga (2005), published eight years ago, suggested clear beneficial effects of cocoa and chocolate in diabetes. However, despite this, no systematic clinical trials have been undertaken to investigate the effects of chocolate in T2DM.

1.5.1 Aim of Thesis:

Systematically investigate the effects of chocolate on the cardiovascular risk and glycaemic control of individuals with T2DM.

1.5.1.1 Objectives:

- Investigate the effect of the matrix (either chocolate or cocoa) on markers of cardiovascular risk and glycaemic control using an exploratory review with a meta-analysis methodology.
- Investigate the modulating effects of chocolate on cardiovascular risk over a two-month period.
- Investigate the acute effects of chocolate in individuals with T2DM during transient hyperglycaemia.
- Investigate the potential of milk chocolate to reduce cardiovascular risk in T2DM over a three-month period.

1.5.2 Hypothesis:

Primary hypothesis

Null Hypothesis: Chocolate rich in cocoa flavanols does not reduce cardiovascular risk in individuals with T2DM and leads to weight gain as part of a clinical trial.

Chapter Two: General Methods and Materials

Aim and Scope of Chapter:

- To summarise experimental approaches used within this thesis.
- To consider the role and design of clinical trials in producing evidence for the efficacy of interventions in healthcare.
- To consider the role of the placebo/control in clinical trials of food products.
- To consider the six types of marker that were defined in Chapter One for assessing the effect of chocolate in type 2 diabetes mellitus (T2DM).
- To consider the safety assessments and participant reported outcome measures.

To address these aims this chapter will be split into three sections:

2.1 - Introduction to study design.

2.2 - A review of clinical trial methodologies relating to food.

2.3 - General procedures and methodologies.

2.1 – Introduction to Study Design

An exploratory review (Section 1.4), with limited meta-analysis (Chapter 3), on the effect of chocolate on measures of diabetes control and cardiovascular risk was undertaken first to determine gaps in the knowledge base. This meta-analysis focussed on biological markers of health in one of six areas noted in Chapter One; Section 1.3.3:

1. Blood pressure
2. Lipid profile
3. Diabetes control and insulin resistance
4. Endothelial function
5. Oxidative Stress
6. Inflammation

In addition to these measures, weight change was determined as a marker for the additional energy load consumed that may adversely affect diabetes control.

Following this meta-analysis *de novo* clinical trial data was obtained from three randomised clinical trials in participants with T2DM summarised in Table 2.1.1, namely:

1. A pilot proof of concept study (Chapter Four – Study One).
2. An acute response study using transient hyperglycaemia to induce metabolic stress (Chapter Five – Study Two).
3. A three-arm double-blinded randomised controlled trial, which included a flavanol, enriched milk chocolate (Chapter Six – Study Three).

Table 2.1.1: Summary of study protocols, which constitute the experimental data presented within this thesis.

	Exploratory Review and Meta-analysis	**Pilot Proof of concept study**	**Acute response study**	**Three-arm study**
Chapter	Chapter Three	Chapter Four	Chapter Five	Chapter Six
Design	Exploratory Review and Meta-analysis	Randomised Controlled Trial Double blind Intervention Cross-over	Randomised Controlled Trial Double blind Intervention Cross-over	Randomised Controlled Trial Double blind Intervention Parallel
Regulatory framework	PRISMA Guidelines (Adapted for a single author due to the nature of this piece of work, search limited and estimation of variance of mean difference limited)	ICH NHS ethics/ NHS Research & Development Trials registration ISRCTN 25655161	ICH NHS ethics/ NHS Research & Development Trials registration ISRCTN 35988358	ICH NHS ethics/ NHS Research & Development Trials registration NCT01617603
Funding	None	Diabetes Research funds / chocolate provided as a gift	Barry Callebaut	Nestlé 07.52.NRC
Publication	One Abstract only to date (Accepted) Not suitable in current state for publication due to limitations in methodology	Mellor et al., (2010) + 3 abstracts	Mellor et al., (In press) + 2 abstracts	Three abstracts only to date
Participants	RCTs with well-defined interventions	T2DM (lifestyle or oral medications only)	T2DM (lifestyle or metformin only)	T2DM (lifestyle or metformin only)
Duration	All	8 weeks (2 months) + 4 weeks (1 month) washout	3 hours + 1 week washout	12 weeks following a 4 week run in period
Chocolate	Chocolate or Cocoa in any dose Control matched in terms of appearance/ taste and energy/ macronutrients	45g per day Formulated high polyphenol chocolate Dyed white chocolate	13.5g dose Acticoa (3% polyphenol) Acticoa (0.9% polyphenol)	20g per day (40g acute feeding dose) Milk chocolate (20mg epicatechin) Nestlé Noir (20mg epicatechin) Chocolate (1mg epicatechin)
Primary Outcomes	Effects of chocolate or cocoa on health	Improvement in lipid profile	Improved endothelial function	Reduced insulin resistance
Secondary Outcomes	Chocolate showing superior effect compared to cocoa	Weight change Glycaemic profile Blood pressure Insulin resistance	Oxidative stress, insulin and glycaemic response	Lipid profile, blood pressure, inflammation and oxidative stress

2.2- Clinical Trial Design

2.2.1- Hierarchy of Clinical Evidence

Traditionally when evaluating and generating data regarding evidence for health outcomes, a hierarchy of evidence has been developed (Ho, Peterson & Masoudi, 2008). This is often represented as a pyramid (figure 2.2.1.1), which is capped with a further tier of systematic reviews and meta-analyses of well-designed randomised controlled trials. The evidence for how this shapes the current evidence can be seen in Chapters One (introduction and literature review) and Three (exploratory review and meta-analysis). For the purposes of this chapter, it is being used to highlight the strength of the methods selected for the generation of the new data, which are presented in Chapters Three, Four, Five and Six.

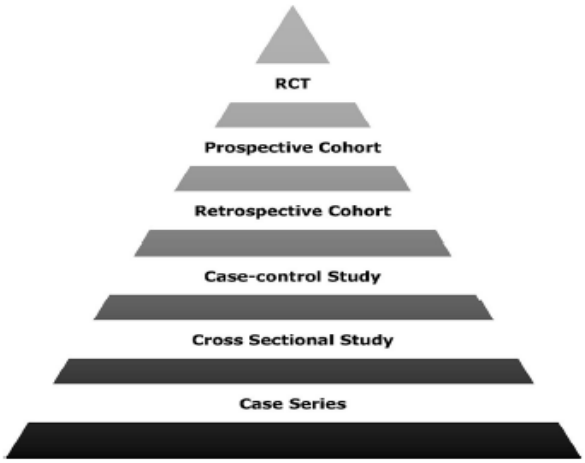


Figure 2.2.1.1: Pyramid or hierarchy of clinical evidence (adapted from Ho, Peterson and Masoudi 2008).

The selection of an exploratory review with meta-analysis for Chapter Three is to consider the weight of the data in the peer-reviewed literature, and how the experimental data presented in the preceding three chapters potentially influences it. This is partially to confirm previous meta-analyses findings of a benefit of cocoa and chocolate, although a number of these have included mixed interventions, including

other biologically active ingredients or exercise. The secondary outcome is perhaps more important; a sub-group analysis investigating whether chocolate offers greater efficacy compared to cocoa.

2.2.1.1 - Randomised Controlled Trials

Although observational studies, supported by the mechanistic studies provided by basic scientific work, there is no clinical trial data to date, available which supports a beneficial causative effect of chocolate in individuals with T2DM.

2.2.2- Clinical Trial Conduct

Conduct and reporting of clinical trials is well described in the case of those using pharmaceutical agents or investigational medicinal products. In Europe and the United States of America, these are tightly regulated by legislation. This is closely linked to the guidelines developed by the International Committee for Harmonisation (ICH), which set about the development of good practice, and ethics in medical research (ICH, 2005). However, when considering the work reported in this thesis, which investigated the health effects of foods or other nutritional products; no such regulations, guidelines or legislation exist.

The absence of regulation is in part circumvented by the recommendations of the International Committee of Medical Journal Editors (ICMJE) in 2005 who stipulated that all clinical trials prior to submission should have their protocol registered. This has become part of research culture in the United Kingdom, where National Health Service Research Ethics Committees (NRES) strongly consider it an ethical obligation, but not a statutory stipulation, that clinical trials should be registered (NRES, 2009). This also has its basis in pharmaceutical trials. In the USA, following the passing of the Food and Drug Administration (FDA) Amendment Act 2007 (FDA, 2007), all trial results needed to be registered.

The requirement is not simply aimed at pharmaceutical research targeting publication in highest quality medical journals as these recommendations have since been adopted by approximately a thousand titles. Additional what is considered to be a clinical trial has been expanded, since June 2007 the ICMJE adopted the WHO definition of clinical trial as:

"Any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes."

Where the meaning of a health-related intervention includes any intervention used to modify a biomedical or health-related outcome. This does not just include pharmaceutical agents, but extends to surgical procedures, devices, behavioural treatments, dietary interventions, and process-of-care changes. The health outcome being assessed includes any biomedical or health-related measures obtained in patients or participants, including pharmacokinetic measures and adverse events (ICMJE, 2009).

As trial registration is generally seen as best practice, this was completed for all the clinical trials reported in this thesis, as shown in table 2.1.1. This is especially important for trials investigating interventions linked to potential intellectual property (not only pharmaceutical agents, but also medicinal devices and foods amongst others) where there is potential for commercial exploitation and therefore a tendency not to report negative findings. So, even if a trial is not fully reported in the form of a peer-reviewed paper, the registry entry will remain in the public domain.

Although not a legal requirement, studies were conducted in accordance with ICH Good Clinical Practice Guidelines (1996) as would be expected in accordance with European law (European Union, 2001; 2005) and then their subsequent adoption into UK law (Statutory Instrument, 2004; 2006a; 2006b) for trials involving investigational medicinal products. The rationale for this was that the centre in which the work was undertaken was primarily engaged in pharmaceutical trials, and the nature of these

guidelines meant the conduct and data quality would be of the highest possible standard. As two of the studies were commercially funded but investigator led, it meant that the study protocol (although independently designed by myself with the supervisory team) together with the data, subject was to a high degree of scientific scrutiny throughout the data collect and analysis. This included full trial monitoring and blinded data scrutiny in the case of both of these trials funded by Barry Callebaut BV and Nestlé.

2.2.3 - Parallel Compared with Crossover Design

All studies followed a controlled experimental design, which assessed changes in anthropometric and biomedical markers along with the use of quantitative questionnaires. This method was chosen so to define the study population, also it is excepted that observational and cohort methodologies would not be sensitive enough to show the subtle changes that would be expected within the time frame of the thesis (six years). It is also considered that only randomised controlled trials have the ability to demonstrate a potential causative effect, with observational studies limited to suggesting associations (Ho, Peterson & Masoudi, 2008).

Cooper et al. (2008) suggested that future studies investigating the health effects of cocoa and chocolate should employ a crossover design as part of a randomised controlled study. Ideally this should also include a placebo or control, which, is blinded to both the participant and the investigator. The latter aspect will be considered later in this chapter (2.2.2.10).

The first two clinical trials (Chapters Four and Five) attempted to follow the recommendations of Cooper et al. (2008). These were chosen as the recommendations to follow, for a number of logistical reasons in addition to enhancing their scientific validity. The primary one being that it was a way of undertaking the study within the constraints of the initially limited available population. This changed through the course of the thesis following the initiation of a potential trial participant database developed in

conjunction with the local diabetes network. Also considering the effect size seen in the studies reviewed in Chapter One, and whether the effect may be of a lesser extent in individuals with T2DM, it was felt that using individuals as their own control would be likely to reduce the risk of inter-participant variation at baseline. This was felt to be most likely to have occurred in the study reported in Chapter Five, as this involved an oral glucose challenge, as glycaemia can vary between individuals. Therefore, it was felt that a crossover methodology was vital as it would be almost impossible to match individuals in terms of their glycaemic response prior to randomisation and that could lead to a large number of individuals being excluded, resulting in potentially lengthy and expensive screening procedures, due to inter-individual variation.

Despite the favouring of crossover-designed studies, they are not without their limitations and sources of confounding. Lathyris, Trikalinos and Ioannidis (2007) in a meta-analysis of crossover arm studies concluded both study designs tended to produce the same outcome, although the crossover design tended to show a more conservative estimate of effect. The parallel arm design tended to be limited when sample sizes were small. The crossover design is limited, in that although it may require fewer participants, they take longer to complete, generally a washout period is required between interventions and it assumes the participants are clinically and biologically stable throughout the study.

The issue with respect to the length of the washout period, is estimated from the half-life of the interventional product, which in the case of cocoa flavanols is short, with biological activity no longer evident after 22 hours (Spadafranca et al., 2010). However, biological markers being assessed may not revert to baseline for several weeks, e.g. glycosylated haemoglobin (HbA1c), which has a half-life of 28.7 days (Allgrove & Cockrill, 1988). Therefore for acute effect studies a minimum washout of 24-48 hours is required, and for chronic studies four weeks would be logical if including assessments

of glycaemic control. This would mean that the minimum length for a crossover study would be five months (20 weeks), which would allow two half-lives to see an effect upon HbA1c, and one half-life period for the washout. So when considering the inclusion criteria; if participants need to have been on stable medication for at least three months prior to enrolment, this would require participants to be clinically stable for a total of least eight months. With the nature of diabetes being a progressive disease, it may mean that some of the change seen might be the effect of the disease rather than lack of effect of the intervention or its potentially negative effect. An example of the progressive nature of diabetes, could be the increase in estimated cardiovascular risk after 1 year seen with the control intervention, as the intervention prevented this progression (Curtis et al., 2012).

The final consideration required in crossover-designed studies is to fully account for potential order effects. Theoretically, if the study is adequately randomised this should not be seen. Theoretically crossover studies can be confounded by carry over effects from one arm to the other. This can be controlled for statistically but with careful randomisation this should not be necessary.

In the case of food trials including the type presented in this thesis, which employed chocolate as the intervention product it could be considered that unlike drugs, which are often in a pill form and who's form result in little or no psychological or sociological attachment by the participant, food might evoke an emotive response. Therefore in clinical trials using foods a crossover methodology this could introduce a degree of confounding due to the emotional attachment of individuals to one of the interventional foods. It is possible that participants might have preconceived ideas from the media about the chocolate they expect to consume and that this might reflect how they respond to both the proposed 'active' and 'placebo' bars.

The potential participant response to having two different chocolates in a crossover study was one of the reasons why in the third study (Chapter Six) a parallel-randomised design was used. This avoids bias from preconditioning which is potentially seen in a crossover study design, although biases from previous life experiences could not be eliminated.

Due to the nature of the funding of the studies presented within this thesis, the chocolate formulations used were different in each study (summary characteristics of the chocolates are shown in Table 2.2.7). All three studies were doubly blinded at the point of randomisation, although there were subtle differences between the suggested 'active' chocolate and the placebo or comparator chocolate which may lead to the participants becoming unblinded. The investigators encouraged participants not to discuss their thoughts about the study chocolate until the end of the study in order to reduce biasing influences towards the investigator. The potential bias of participants 'unblinding' themselves was partially addressed using taste trials in the studies reported in Chapters Four and Five. For the parallel designed study in Chapter Six, Nestlé as a sponsor did not see this as an issue, so no taste trials were conducted. The rationale being that a milk chocolate was one of the 'active' interventions so would not be as easily detected as a traditional high polyphenol dark chocolate, which has a bitter taste. The risk was also moderated by the studies parallel design.

2.2.3.1 - Randomisation

All three studies were randomised prior to the enrolment of the first participants. The provider of the chocolate held the randomisation codes in all three studies. In the cases of Chapter Four (Study One) and Chapter Six (Study Three) this was Nestlé, Nestec York, UK and Nestlé Research Centre, Lausanne, Switzerland respectively and for Chapter Five (Study Two) Barry Callebaut BV, Lebbeke-Weize, Belgium. The

concealment of the randomisation was maintained until the initial analysis of the data was complete, at which point the code was broken.

2.2.4 - Regulatory Approval

All studies underwent full ethical review by the Hull and East Riding NHS Local Research Ethics Committee, and obtained approval prior to their commencement. Research governance approval was obtained through the Research and Development Department of Hull and East Yorkshire Hospitals NHS Trust.

All studies were conducted in accordance with the Declaration of Helsinki of the World Medical Association (WMA, 2000) and Good Clinical Practice (EMEA, 2002). The work in Chapter Four was supported by an unrestricted gift of the chocolate for the study by Nestec PTC, York, UK and was funded through the Diabetes Charitable fund. Funding and sponsorship from Barry Callebaut BV, Lebbeke-Weize, Belgium, supported the work in Chapter Five, although the author (along with the supervisory team) of the thesis was responsible for the trial design. The work in Chapter Six was supported, funded, sponsored and monitored by Nestlé Research Centre, Lausanne, Switzerland. The protocol and study design for the work presented in Chapter Six was developed by the author of the thesis in conjunction with their supervising team with support from the Nestlé Research Centre, Lausanne, Switzerland.

All three trials were registered on public data bases of randomised clinical trials; the work in Chapter Four being ISRCTN 25655161, Chapter Five; ISRCTN 35988358 (both available from http://www.controlled-trials.com/isrctn/) and Chapter Six; NCT01617603 (http://clinicaltrials.gov/ct2/home).

2.2.5 - Population

Participants in all three studies had T2DM and were taking (if applicable) stable medication for at least three months prior to enrolment with different patients invited to

participate in each studies. Further details and of the inclusion and exclusion criteria are described in Chapters Four, Five and Six. All participants were recruited from within the Hull and East Riding Diabetes Network in which they were patients.

Participants had all given consent to be contacted regarding research when they were referred to the networks diabetes programme. It is a recommendation that all individuals with diabetes receive structured education about how to self-manage their condition (NICE, 2003, 2008; Diabetes UK & Dept. of Health, 2005). This was selected as a key criterion, as all participants would have received the same basic information regarding their diabetes and the recommended dietary treatment of their diabetes, thus reducing lifestyle variability issues.

2.2.6 - Demographics

The population age range for all three studies was between 40 and 80 years. To reduce the potential for confounding from insulin deficiency, apart from the initial study (Chapter Four), which had a body mass index (BMI) range of 19-45 kgm^{-2}, the subsequent studies focused on overweight and obese individuals with a BMI range of 25-40kgm^{-2}. The limits on weight were largely set to avoid extremes of metabolism, including the sympathetic over activity and inflammation associated with morbid obesity (Pontiroli, Pizzocri, Paroni & Folli, 2006).

All studies included both male and postmenopausal female participants. The rationale for only including postmenopausal women was to reduce potential causes of variation in insulin resistance associated with cyclical changes in female sex hormones and currently relatively few premenopausal women have T2DM (Geer & Shen, 2009).

For the work in Chapters Four and Five there was no allocation of the proportion of males to females in the study. This was not considered to be a potentially significant source of confounding problem as both these studies involved a crossover design. With the larger three arm Study Three (Chapter Six), which had a parallel design, to reduce

any bias, equal age range and gender biases were assigned to each arm of the study, although the size of the randomisation blocks was not disclosed.

2.2.7 - Investigational Products - Chocolates

All chocolates, except the Nestlé Noir used in Chapter Six, were not commercially available. The chocolate in using in Chapter Four was formulated as an unrestricted gift for this study by Nestec, York; for the work presented in Chapter Five the chocolate comprised of research samples provided by Barry Callebaut BV, based on the Acticoa™ cocoa and has been used in other studies (Almoosawi et al., 2010; 2012) and for Chapter Six, the control iso-energy chocolate and high flavanol milk chocolate were specially formulated by Nestlé in Lausanne, Switzerland.

2.2.9 - Sampling Methods

Participants were invited to take part in the studies following their expression of interest in the research. This was undertaken by the posting of information to individuals, who were then contacted by telephone calls. Interested individuals had the nature of each study explained to them and were sent written information, and if they still were interested they were invited to attend to give full written consent to take part in the study and then underwent screening for suitability.

The sample possibly were not randomly obtained as initially the participants might have been clustered due to referrer bias as they were initially obtained from healthcare professionals referring their patients for diabetes education. This may have been compounded by self-selection bias, as some individuals may due to their nature be more likely to volunteer to participate in trials than others.

Table 2.2.7: The energy and polyphenolic content of the chocolates used in the experimental work presented in this thesis

Chapter (Study)	Source of Chocolate	Type	Polyphenol content per daily 'dose' Folin-Ciocalteau (GA)	Epicatechin per daily 'dose'	Estimated energy per day
Chapter Four (Study One) Note daily dose (3*15g)	Nestec/ Nestlé, York, UK	70% non-fat cocoa solid	783mg	55.4mg (note paper quoted catechins hence difference)	326kcal
		Dyed 'white' type chocolate	175.5mg	<0.2mg	325kcal
Chapter Five (Study Two) Single dose of 13.5g	Barry Callebaut BV, Belgium	Acticoa™	472.5mg	Not known	95kcal
		Matched chocolate	10.9mg		95kcal
Chapter Six (Study Three) Note daily dose 20g	Nestec/ Nestlé, Switzerland	Dark (Nestlé Noir)	Not known	19mg	109kcal
		Milk High polyphenol		19.1mg	95kcal
		Low polyphenol		1.1mg	115kcal

Note: the initial studies (Taubert et al., 2003; Grassi et al., 2005a) polyphenol content is quoted as approx. 500mg with no actual data to support. It is considered that 34-37% of the total polyphenol content is monomeric. This would suggest approximately 160mg of monomeric polyphenols (epicatechins and catechins) and in the Nestlé Noir/ Milk High polyphenol a total polyphenol of approximately 60 mg. Due to lack of the availability of the equipment and costs, studies were limited by having to rely on the published data provided by the manufacturers.

2.2.10 - Placebo and Blinding

The concept of the placebo in this type of study is a challenging one. Unlike pharmaceutical trials where the known 'active' under investigation is given as a single compound in a matrix of inert compounds, the placebo will be an identical formulation that is designed to look and taste exactly the same. With foods this is not as easy to achieve.

The other major challenge is in varying the 'active' product and the control only in terms of a single bioactive compound. In chocolate it is hypothesised that these are the polyphenols, with epicatechins being seen as the predominant 'active' components. This is based on the assumption that these can be measured circulating following their consumption, often in association with a beneficial effect and thus implying a mechanism of action (Rein et al., 2000a; Engler et al., 2004; Schroeter et al., 2006; Spadafranca et al., 2010). However it has been suggested that other components might have an effect on health, these range from theobromine (Kelly, 2005; van den Bogaard et al., 2010), fatty acid composition (Mursu et al., 2004), through to the effect on the endocannabinoid system (Di Marzo et al., 1998). Some of these effects might even be psychological effects, linked to the organoleptic qualities of chocolate starting with the taste and mouth feel. This is before considering any effects of restriction have been imposed on chocolate consumption in individuals with T2DM. To attempt to negate these effects, taste tests and study designs were adapted and described in the individual chapters relating to each of the studies.

2.3 - General Procedures and Methodologies

This section will focus on the common procedures across the three studies. In the first part it will describe the physical measurements associated with anthropometry and the second part will focus upon the biomarkers of cardiovascular risk and glycaemic control.

2.3.1 - Physical Measurements

A trained member of the research team undertook all physical measurements, weight, height, circumferences and blood pressure; 92% of participant visits were conducted the author of the thesis. All measurements carried out following WHO protocols (WHO, 2008a)

2.3.1.1 - Height

Height was measured using a SECA 206 (SECA, Birmingham, UK) roll tape according to accepted practice to the nearest millimetre.

2.3.1.2 - Weight

Weight was taken using SECA 877 (SECA, Birmingham, UK) which weighed in 100g increments up to 150kg and up to a maximum of 200kg. The scales were audited during the course of the work for this thesis achieving the necessary <1% error needed to be designated as fit for purpose. Weight was taken at all office visits for each of the studies presented in this thesis. Weight was recorded to the nearest 100g.

2.3.1.3 - Calculating Body Mass Index

In combination with the height, using the formula:

$$\frac{\text{Weight (kg)}}{\text{Height (m)} * \text{Height (m)}}$$

This was used to calculate Body Mass Index (BMI) or Quelelet's Index, BMI was then used to classify the participants, initially to assess their suitability for the studies and secondly to define participants level of obesity, on a scale from underweight through to obese (WHO, 2012).

2.3.1.4 - Waist circumference

Waist measurements were taken to provide additional information regarding levels of obesity of the participants taking part in the studies. The methodology followed was that recommended by the World Health Organisation (WHO, 2008b), defining waist as the midpoint between the uppermost border of the iliac crest (top of the hip bone) and the lower border of the costal margin (rib cage). A constant tension, non-elastic tape was placed around the abdomen at the level of this midway point and a reading taken when the tape is snug but does not compress the skin. This measurement was taken directly over the skin, with the participant asked to expire normally (measurement taken at the end of normal expiration) with the arms relaxed by their sides. Measurements were taken to the nearest millimetre and repeated until two measurements were within 5mm of each other prior to recording in the case report form.

2.3.1.5 - Hip circumference

The investigator stood to the side of the participant, and the tape was wrapped carefully around maximum circumference of the buttocks. The participant was asked to stand with their feet together and their weight evenly distributed over both feet and their arms relaxed by their sides. The tape was checked to insure that it was horizontal around the body and snug without constricting. The measurement was taken over a thin layer of clothing and participants were asked to wear similar or the same clothes at each visit. Measurements were taken to the nearest millimetre and repeated until two measurements were within 5mm of each other prior to recording in the case report form.

2.3.2 - Markers of Cardiovascular Risk – Established Clinical Markers

2.3.2.1 - Blood pressure

Blood pressure can be measured as a spot reading at clinical office visits or using an ambulatory blood pressure monitor. In Chapters Five and Six the protocol planned to use 24 hour ambulatory blood pressure monitoring. Initially this was planned for a subgroup of the participants represented in Chapter Five, but was subsequently abandoned due to bruising of participants. For Chapter Six it was not possible to undertake this type of monitoring as the visit schedule for the study and issues with participant attrition were identified after undertaking measurements on the first three participants.

As with the anthropometric measurements WHO protocol was followed for all three studies (WHO, 2008a). For all three studies, blood pressure was measured using a calibrated NPB-3900, Nellcor, (Puritan Bennett, Pleasanton, CA, USA). Participants were asked to rest in a seated position for a minimum of 15 minutes. An appropriately sized cuff for the individuals was selected and placed on the non-dominant arm. Three blood pressure measurements were taken, with the mean of the second and third calculated and recorded in the case report form. If the two measurements varied by more than 10mm Hg, the participant was asked to rest and the process repeated after a further 15 minutes.

2.3.2.2 - Lipid profile

Full lipid profile (total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides with low density lipoprotein (LDL) cholesterol calculated using Friedewald equation (Friedewald, Levy & Fredrickson, 1972) were measured in fasting participants at screening in all studies, and before and at the end of the chronic chocolate supplementation in Chapters Four and Six. In addition triglycerides were repeatedly measured during the meal tests of Chapters Five and Six.

Lipids were measured in plasma which was drawn via a cannula or butterfly and needle from the median-cubital or cephalic veins. Blood was drawn into a serum separator vacutainer blood collection tube. In the case of fasting samples this was labelled and sent to the Chemical Pathology Laboratories at Hull and East Yorkshire Hospitals NHS Trust for analysis. In the case of the samples drawn during the meal test these were centrifuged in a refrigerated centrifuge for 15 minutes at 3500RPM. Serum was then drawn off and placed in two labelled aliquots, which were frozen at -80^{o}C before being batch analysed. Batch analysis was also undertaken at the Chemical Pathology Laboratories at Hull and East Yorkshire Hospitals NHS Trust. Where there were cases of missing data from samples taken on the day of the visit, but stored frozen aliquots of serum were available, these were sent at the end of the studies as part of the batch analyses full lipid profiles were measured on fasting samples.

Assays were run using a Unicel DxC 800 analyzer (Beckman-Coulter, High Wycombe, UK) in a Clinical Pathology Accredited laboratory. This uses a spectrophotometric methodology to determine total cholesterol, HDL cholesterol and triglycerides with up to 92 further analytes.

Computer software in the pathology reporting system then calculated LDL cholesterol, with the caveat that a raised triglyceride level may prevent this being valid or reported. The rationale for not relying on the data for the LDL cholesterol when triglycerides are raised is due to the nature of the Friedewald equation (Friedewald, Levy & Fredrickson, 1972):

*LDL Cholesterol=Total Cholesterol–HDL Cholesterol - (Triglycerides/2.2)**

**All data in mmol/l*

2.3.2.3 - Glycaemia and insulin resistance

Glycosylated haemoglobin (HbA1C) (Selvin et al., 2010) and glucose (Kannel & McGee, 1979) were measured to assess glycaemic control, with both additionally having the potential as markers of cardiovascular risk (Chapter One).

2.3.2.3.1 - Glucose

Glucose was sampled both fasting in all three studies, and in Chapters Five and Six sequentially as part of the meal test protocols. Where possible the blood was drawn into a vacutainer containing sodium fluoride. If this was not possible then a capillary sample was taken and analysed next to the participant using a Hemoecue (Hemcue AB, Stockholm, Sweden). Samples were drawn into a vacutainer containing sodium fluoride, were then inverted to mix, and then chilled on ice. The fasting samples were sent to the Chemical Pathology Laboratories at Hull and East Yorkshire Hospitals NHS Trust, where they were analysed using a Unicel DxC 800 analyzer (Beckman-Coulter, High Wycombe, UK). For the meal test samples, the vacutainers were centrifuged in a refrigerated centrifuge for 15 minutes at 3500RPM. Serum was then drawn off and placed in two labelled aliquots, which were frozen at -80^{o}C before being batch analysed. Batch analyses were also undertaken at the Chemical Pathology Laboratories at Hull and East Yorkshire Hospitals NHS Trust.

2.3.2.3.2 - HbA1c

HbA1c was analysed at screening and the end of chronic feeding. This was drawn in a venous sample using an EDTA vacutainer tube or a capillary EDTA tube (BD microtainer) if venous access was a problem. These samples were then sent to the Chemical Pathology Laboratories at Hull and East Yorkshire Hospitals NHS Trust for analysis. HbA1c was measured on a HA-8140 analyser (Menarini Diagnostics, Florence, Italy) using HPLC methodology, which assesses potential confounding from

variant haemoglobins. Most of the HbA1c data was collected prior to the change from DCCT aligned results to IFCC units, which began in June 2009. Conversion from DCCT units to IFCC used the equation; IFCC-HbA1c =(DCCT-HbA1c-2.15)*10.929) (Diabetes UK, 2011).

2.3.2.3.3 - Insulin

Venous blood samples were drawn into vacutainer serum separator tubes at the same time samples were taken for analysis for glucose. Samples were centrifuged in a refrigerated centrifuge for 15 minutes at 3500RPM. Serum was then drawn off and placed in two labelled aliquots, which were frozen at -80^{o}C before being batch analysed. Batch analyses were undertaken at the Chemical Pathology Laboratories at Hull and East Yorkshire Hospitals NHS Trust. Serum insulin was assayed using a competitive chemiluminescent immunoassay performed using the Siemens Immulite 2000 analyzer (Euro/DPC, Llanberis, UK).

2.3.2.3.4 - Insulin resistance

Assessments of insulin function can either be dynamic assessing change over time in response to an oral or venous macronutrient load, or static, measured at baseline. The 'gold standard' methodology is to use the hyperinsulinaemia-euglycaemic insulin clamp methodology (Defronzo, Tobin & Andres, 1979), though this was not possible from a logistic perspective.

Instead, calculated measurements comparing fasting insulin were used, as dynamic methods were unsuitable owing to the addition of the study chocolate prior to the 75g glucose load in Chapter Five. The measurements selected were the Homeostatic Model Assessment (HOMA) (Rudenski, Matthews, Levy & Turner, 1991; Matthews et al., 1985) and Quantitative Insulin Sensitivity Check Index (QUICKI) (Katz et al., 2000).

2.3.2.4 - Endothelial function

Endothelial function can be measured either functionally or using biomarkers in an attempt to associate the two in Chapter Five both were measured, whereas in Chapter Six only a functional measurements were undertaken.

2.3.2.4.1 – Reactive Hyperaemia Peripheral Artery Tonography (RH-PAT) (EndoPAT)

Many studies investigating the effects of cocoa or chocolate upon cardiovascular risk have assessed endothelial function. Only five studies, starting with Fisher, Hughes, Gerhard-Herman, and Hollenberg (2003) used a peripheral tonometry (RH-PAT) methodology; whereas the majority of studies have used flow-mediated dilation (FMD). The 'gold standard' methodology of angiography with acetylcholine infusion as recognised by the Food and Drink Administration (FDA) for assessing endothelial function of coronary artery endothelial function is not practical in a routine research clinic environment. Flow mediated dilation is in part limited by potential operator error, in that it relies on the investigator measuring the effect. Both FMD and RH-PAT are based on measuring the dilation of blood vessels following an occlusion. (RH-PAT) is based on the site of the assessment being the fingers; meaning that sites for blood sampling were more accessible. Assessments were made on both the occluded and non-occluded arms measurements and were automated meaning the data could be readily quality assured. For this reason, the EndoPAT 2000 (Itamar Medical, Caesarea, Israel) (RH-PAT) was selected. Nohria et al. (2006) demonstrated that changes in measurements made using this methodology are nitric oxide dependent.

Manufacturers recommendations for protocol were adapted for Chapters Five and Six. Participants were invited into a climate-controlled room, which had its temperature set at 22^{o}C. The room was free from environmental distractions, with low level lighting and was kept quiet. After asking participants to relax for 15 minutes blood pressure was measured according to the protocol outlined in section 2.3.2.1, following a further five

minutes rest the EndoPAT 2000 probes were placed on the index finger of each hand and on the left arm a suitably sized blood pressure cuff was place around the upper arm. The probes were attached to the EndoPAT 2000, and the computer to which the EndoPAT was attached was programmed to start the test. Following five minutes of recording to assess baseline readings, the blood pressure cuff was inflated to 50mmHg above the recorded systolic blood pressure or 200mmHg which ever was the greater. The occlusion was maintained for five minutes, with the degree of occlusion being checked by amplifying the size of the signal. When the occlusion was released, recording continued for a further five minutes, the difference between size of the flow before and after the occlusion was use to calculate RH-PAT. This was then moderated using a comparison corrected for by flow measured from the control arm.

The EndoPAT 2000 assessment was taken prior to cannulation; it was considered that following 60 minutes, the trauma associated would have subsided. The cannula was inserted in the right arm so would have only effected the control arm. Throughout the study visits, which included EndoPAT 2000 assessment, the participants were asked to remain in the climate-controlled room, resting in an environment involving minimal stimulation.

2.3.2.4.2 - Adhesion molecules

Adhesion molecules were only assessed in Chapter Five. These were measured in serum, which were drawn into serum separator vacutainer tubes, prior to the consumption of chocolate and at the end of the test. The samples were centrifuged in a refrigerated centrifuge for 15 minutes at 3500RPM. Serum was then drawn off and placed in two labelled aliquots, which were frozen at -80^{o}C before being batch analysed. Batch analyses were undertaken in the Biomedical Research Laboratories, Postgraduate Medical Institute, University of Hull. All assays were carried out by Enzyme-linked immunosorbent assay (ELISA) using kits provided by Bender MedSystems (Vienna,

Austria). Calibration curves were estimated using Microsoft Excel using polynomial equations, where necessary having to achieve an R^2 of at least 0.995. Microsoft Excel was then used to convert data into ng/ml.

2.3.2.5 - Oxidative stress

2.3.2.5.1 - Malondialdehyde and TBARS

Despite the limitations of these markers (Mensink et al., 2003), they were assessed for Chapters Four and Six, in order to allow comparison of the data with published data. These were measured in fasting plasma, which were drawn into EDTA vacutainer tubes. The samples were processed as described in Section 2.3.2.4.2. The level of TBARS, which is deemed to be equivalent the amount of malondialdehyde (MDA) was assayed for using an ELISA kit provided by R&D Systems (Minneapolis, MN, USA). Calibration curves were estimated as described in Section 2.3.2.4.2, data was presented in ng/ml.

2.3.2.5.2 - Isoprostanes (15-F2t-isoprostane)

For Chapters Five and Six, urinary 15-F2t-isoprostane α (formerly known as 8-iso-PGF2α and 8-epi-prostaglandin F2) were measured. All participants in these two studies undertook 24-hour urine collections prior to each visit, starting at 08:00 the day before and finishing at 08:00 when arriving at the clinical trials facility. This was the point when they emptied their bladder prior to their commencing the study visit. From this point the second 24-hour urine collection began. Total volume for each collection was recorded and aliquots taken and frozen at -80^{o}C until batch analysis could be undertaken on the completion of the study. For Chapter Five, the urine collection was undertaken the day before the study visit, then the day of the study. In the case of Chapter Six, the urine collection was undertaken for the 24 hours prior to visits 1 and 4.

Urinary 15-F2t-isoprostane-α was measured by ELISA kit (Oxford Biochemical Research, Oxford. USA). Data was presented as both absolute and corrected for

creatinine. Calibration curves were estimated using Sigmaplot V11 (Systat Software, Chicago, IL, USA). Data was then converted using Microsoft Excel to mg per 24 hour (absolute value) and mg/mol (creatinine ratio)

2.3.2.6 - Inflammatory markers

For all studies C-Reactive Protein (CRP) was measured, for Chapter Six additionally interleukins (IL1RA and IL6) and Tumour Necrosis Factor (TNF-α) were also measured.

2.3.2.6.1 - C-reactive protein

C-reactive protein was measured using a high sensitivity assay so more accurately it should be described as high sensitivity-C-Reactive Protein (hs-CRP). This was measured from serum that had been collected in serum separator vacutainer tubes, these were analysed using a Unicel DxC 800 analyzer (Beckman-Coulter, High Wycombe, UK) at the Chemical Pathology Laboratories at Hull and East Yorkshire Hospitals NHS Trust. This assay was carried out for fasting samples as part of routine pathology samples, and for meal tests, samples were centrifuged, pipetted into aliquots and stored prior to batch analyses as previously described in Section 2.2.3.3.2.

2.3.2.6.2 - Interleukins (IL1RA and IL6) and TNF-α

These were measured using heparinised plasma collected in vacutainer tubes and processed as described in Section 2.3.2.4.2. All assays were undertaken using ELISA kits, for IL1RA (R&D Systems), IL6 and TNF-α (both BenderMedSystems). Calibration curves were estimated using Microsoft Excel using polynomial equations where necessary with the curve having to achieve an R^2 of at least 0.995. Microsoft Excel was then used to convert data into pg/ml.

2.3.2.7 - Participant reported outcomes

Chapters Four and Six included questionnaires which were incorporated to obtain additional information about changes in mood, function, sleep pattern and appetite. As

the instruments were not the same for both studies, these will be described in detail in the relevant chapters.

2.3.2.8 - Assessment of background diet

In all three studies background diet were monitored and participants were instructed to consume chocolate evenly through the day if part of chronic feeding. During the study participants were asked to refrain from altering their intake of polyphenol rich foods (details presented in Appendix II). Just before and during the acute phase investigations, participants were asked to abstain from these foods all together.

Dietary intake for Chapter Four was assessed by recall undertaken by myself, the author of this thesis and a registered dietitian, but was not formally analysed. For Chapters Five and Six, these were formally recorded and analysed using Microdiet (Downlee System Ltd, Chapel-en-le-Frith, Derbyshire, UK) to assess change over course of the study. The methodologies of recording dietary intake were all over a maximum of 48 hours. For Chapter Five participants also recorded all their physical activity levels, food and drink consumption for 24 hours before and after each study visit. In Chapter Six 24-hour dietary recalls undertaken by the study dietitian (myself). A conscious decision was made not to follow a more formal diet diary methodology over four or more days as this it was considered, might lead to a change in food intake.

2.3.2.9 - General data handling and statistical methodology

2.3.2.9.1 - Data handling

All three studies considered in this thesis were supported by the use of a case report form. For Chapters Four and Five, these were internally designed and the data monitoring was the responsibility of the Research and Development Department of Hull and East Yorkshire Hospitals NHS Trust. For Chapter Six, data were collected in a case report form provided by the sponsor, with case notes and data monitored by the sponsor to insure compliance with Good Clinical Practice (ICH, 1996). All data (anthropometry, biochemistry and

questionnaire data) were transcribed into Microsoft Excel for transfer to sponsors for analysis.

All data were managed in accordance with the Data Protection Act 1998 and institutional requirements. The study visits were also recorded within the participant's medical notes, which also included a copy of the informed consent. Finally the participant's (with their permission) General Practitioners were informed regarding their participation in the studies.

2.3.2.9.2 - General statistical methodology

The data analyses reported within Chapters Three (exploratory review with meta-analysis), Four and Five (Studies One and Two) were undertaken by myself with advice from Mr Alan Rigby, (Statistician, University of Hull). Due to contractual restrictions associated with Chapter Six (Study Three) statistical analyses were in part, undertaken by the sponsors' statistician.

All three interventional studies were powered based on previously published studies using cocoa (Balzer et al., 2008) and were at least *post hoc* tested using power calculation software (N-Query, Statistical Solutions, Sargus, MA, USA or G*Power (Faul, Erdfelder, Lang and Buchner, 2007). Data were processed primarily using Microsoft Excel, and in the case of Chapters Four and Five analysed using PASW Statistics 18.0 (IBM, New York, NY, USA), Microsoft Excel and GraphPad Quick Calcs (GraphPad, La Jolla, CA, USA). For Chapter Six, data was transferred from the case report forms and Microsoft Excel onto ClinTrial 4.2 (Domain Pharma, Lexington, MA, USA). The data were then analysed within the sponsor organisation before producing a report in LaTeX (LaTeX Project). The nature of the tests for normality and methods of statistical analysis used in each study will be described as part of the relevant chapters.

2.3.3 - Summary

This Chapter details the methodologies used for the study design underpinning the structure of this thesis, the assays used and overall description of data handling and analysis methods.

Chapter Three: An Exploratory Review with Meta-Analysis - the Effects of Cocoa Flavanols on Markers of Cardiovascular Risk and Glycaemic Control: Which is Better Chocolate or Cocoa?

This chapter considers the data from the published randomized controlled trials published to then end of June 2012 and prior to the subsequent chapters (Four, Five and Six) that describe the experimental work from the series of themed clinical trials.

This review focuses on the potential benefits of cocoa and chocolate in the management of diabetes and the potential reduction in associated complications. It aims to investigate if there are any differences as a result of the matrix of formulation of the product in which the cocoa flavanols are consumed. It will assess whether it is preferable to consume these flavanols in the form of a cocoa drink or in chocolate.

Aim and Scope of Chapter:

- To undertake a meta-analysis of the published randomized controlled trials reporting effect of flavanols on markers of cardiovascular disease risk and glycaemia.
- To attempt to assess the effect of the formulation of the product (either cocoa or chocolate) on efficacy using subgroup analysis.

Table 3.0 sets out the PICOS (Participants, Interventions, Comparisons, Outcomes and Study Design) approach to this chapter and how the data will be used to meet the above aims

Table 3.0: Study questions and PICOS (Participants, Interventions, Comparisons, Outcomes and Study design) for meta-analysis and review.

Study Question(s)	**Do chocolate and cocoa formulations rich in polyphenols reduce markers of cardiovascular risk and glycaemic control?** **Is chocolate rich in polyphenols more effective in reducing cardiovascular risk and glycaemic control markers than cocoa formulations rich in polyphenols?**
Participants	**Healthy and individuals with risk factors of cardiovascular disease including type 2 diabetes** **Excluded studies which recruited pregnant women and children.**
Interventions	**Cocoa or chocolate formulations rich in polyphenols, flavanols or epicatechins where the polyphenol, flavanols or epicatechin content has been defined**
Comparisons	**A comparator cocoa or chocolate formulation which is low in polyphenols, flavanols or epicatechins where the polyphenol, flavanols or epicatechin content has been defined**
Outcomes	**Change in risk markers of cardiovascular risk or diabetes control, only numerically presented data will be used due to the risk of published figures may not have accurately presented scales:** **1. Blood pressure (systolic and diastolic blood pressure)** **2. Lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides)** **3. Glycaemia (fasting glucose and HbA1c)** **4. Endothelial function** **5. Oxidative Stress (isoprostanes)** **6. Inflammation (subject to comparable data being available)** **7. Weight**
Study design	**Randomised controlled trials published in the peer reviewed literature investigating the effects of chocolate and/or cocoa on cardiovascular risk up until 30th June 2012**

To address these aims, the meta-analysis will be presented in four parts:

3.1 - Introduction.

3.2. - Methodology.

3.3 - Results.

3.4 - Discussion.

3.1 – Introduction

There is strong epidemiological evidence for the potential benefits of foods rich in flavanols and related compounds as has been previously reviewed in Chapter One. However, it needs to be acknowledged that the absorption and metabolism of these compounds is somewhat complex (Crozier, 2009). In foods these compounds exist as monomeric catechins (mainly epicatechins) and oligomeric flavanols, typically varying from dimers to decamers. The ratio of these depends on a range of factors; from the country of origin of the cocoa, through to the method of processing the cocoa beans (mainly fermentation and roasting) (Hurst et al., 2011). Typically, 34-37% of the flavanols in cocoa are monomeric and as such have greater potential in terms of bioavailability (Langer et al., 2011; Hii et al., 2010; Wollgast & Anklam, 2000).

The stability of these compounds in post-harvest food is also an important consideration. In chocolate and cocoa the flavanols (especially flavano-3-ol) content has been reported as being stable for at least two years (Hurst, Payne, Miller & Stuart, 2009). Such that chocolate has been described as an ideal matrix for the preservation and potentially the delivery of flavanols (McShea et al., 2008).

There are a number of variables that need to be considered when comparing the potential for solubilised cocoa or chocolate as a delivery to act as vehicles for flavanols. Beyond the food chemistry, there are nutritional considerations linked to the energy, fat (especially saturated fatty acids) and sugar content which may be metabolically significant. However these points are potentially of no consequence if the end product is unacceptable to the consumer. The critical first point is to consider the bioavailability and biological activity of the cocoa flavanols and how this may relate to the food product formulation.

3.1.1 - Influences upon Absorption

Animal and human studies indicate there are a range of conditions through which the absorption of monomeric flavanols can occur (Hackman et al., 2007; Schramm et al., 2003). Following a systematic search, there appears to be only one small study that has attempted to compare chocolate and cocoa matched for carbohydrate, epicatechins and other potentially biologically active compounds (e.g. caffeine and theobromine) (Baba et al., 2000). This was a crossover study of five healthy males, which, unfortunately was not designed with the necessary power to determine a difference in absorption between the cocoa and chocolate. It claimed that the only difference between the cocoa and the chocolate was the contribution of cocoa butter to the fat and energy content of the chocolate.

Baba et al. (2000) suggested that there was no significant difference in uptake from the gut between cocoa and chocolate, although the clearance of epicatechin metabolites in the chocolate group was 29.8±5.3% of the ingested epicatechins, compared with 25.3±8.1% (p=0.329) for the cocoa. However, the levels of non-conjugated epicatechins appeared to be higher with cocoa at the one and two hour points following ingestion (0.10±0.03 compared to 0.22±0.06 μmol/l and 0.15±0.04 compared to 0.22±0.02 μmol/l respectively). This single study presents a mixed picture of the effects of cocoa and chocolate on the bioavailability of epicatechin. If urinary recovery is used as a measure of bioavailability, a study with a sample size of 21 would have an 80% power to detect a significant difference between chocolate and cocoa at the 0.05 level (G*Power 3 (Faul et al., 2007)). This suggests there is potential for a difference in the absorption of epicatechins from chocolate and cocoa that warrants further study.

There are a number of mechanisms to explain why flavanols administered in the form of chocolate may have enhanced absorption compared with flavanols administered in a

cocoa drink. These include a combination of physical and chemical properties of the cocoa or chocolate, along with the nutrient and non-nutrient effects on gastrointestinal physiology.

A common feature of dietary polyphenols including flavanols, is their need to be hydrolysed, as in the plant, they mostly exist in a glycoside form. This can occur as either an acid or enzymic hydrolysis. The length of time and amount of hydrochloric acid secreted in the stomach will influence the acid hydrolysis, and the second will be influenced by the levels of enzymes such as lactase phloridzin hydrolase and cytosolic β-glucosidase in the intestine (Crozier, 2009). Initially, based on data from *in vitro* studies, it was considered that increased time in the stomach might hydrolyse a greater proportion of procyanidins and thus increase the pool of epicatechin and other monomeric flavanols available for absorption (Spencer et al., 2000). These data were somewhat refuted by (Rios et al., 2002) who suggested that in humans fed via a nasogastric tube there were no differences seen with respect to degradation of procyanadins after a gastric transit of ~50-60minutes.

The potential for acid hydrolysis in the stomach of both the glycosides and the procyanadins to increase bioavailability of the polyphenols from chocolate or cocoa, could be of considerable importance. It has been widely described that stomach emptying is quicker for liquids than solids, which could infer that the increased viscosity of chocolate compared to cocoa drinks might slow gut transit time. The difference in the fat content might also lead to an increase in the time the food is in the stomach. Gut peptides, including cholecystokinin produced by the duodenal mucosa under the influence of fat are known to supress gastric emptying (Liddle, Morita, Conrad & Williams, 1986). This highlights two potential mechanisms on how chocolate might act to slow gastric emptying when compared to cocoa. However to date, no published work has tested this hypothesis. To this end, more work is required to explore

this idea, and the nature of the metabolites identified by Baba et al. (2000) after the three-hour point for chocolate, which were not seen, with cocoa.

The product formulation or matrix also has a number of physical effects that could affect bioavailability. From the studies reported, the quality of the cocoa powder used was not always clear. This included its ability to be dispersed in the medium (typically water) (Fogliano et al., 2011). Cocoa is not very soluble even in hot water and this might be a barrier to bioavailability, with the hydrophobic cocoa particles being less readily digested, preventing the procyanadins and monomeric flavanols from being hydrolysed to more bioavailable forms (Fogliano et al., 2011). In chocolate, these physical characteristics are very different; the cocoa tends to be evenly distributed within the product, the effect of which is to increase surface area available for hydrolysis and digestion, resulting in increased bioavailability. It is logical that the high fat content will provide a hydrophobic matrix, but the particle size is likely to be much smaller. It is not clear which of these two factors is more important in influencing bioavailability.

The effect of food ingredients other than cocoa has concentrated on the effects of adding milk or altering the sugar (usually sucrose) component of the end product. The sucrose or other carbohydrate content may be of interest in terms of its biological activity, in addition to its potential effects on bioavailability. In human studies, where cocoa was administered with or without sugar (sucrose), it appears that sugar might enhance flavanols absorption (Schramm et al., 2003). The same group continued this work to investigate the effects of other carbohydrate-rich foods with cocoa, which produced a similar enhancing effect on flavanols absorption.

Where the sugar used in chocolate was changed from sucrose to maltitol (Rodriguez-Mateos et al., 2012) with the aim of reducing the glycaemic burden and energy density, it appears to reduce the absorption of the flavanols. This was especially evident at the

one and two hour time points, which are of importance when considering potential postprandial effects which are investigated in Chapters Five and Six. In terms of the nutritional management of diabetes, this is of further interest, as historically maltitol along with other sugar alcohols have been used in the manufacture of confectionary for people with diabetes. This is despite the sugar alcohols having an equal energy content and in quantities greater than 25 grams per day, increase the risk of osmotic diarrhoea and not being recommended in the evidence based nutritional guidelines of Diabetes UK (Dyson et al., 2011).

From a bioavailability perspective there is a case to suggest chocolate might be preferable to cocoa alone. The rational for this could be explained by differences in composition between the two products. A property that could account for this effect is that the flavanols are co-administrated along with carbohydrate (including sucrose). The rationale for using sugar alcohols (polyols) in the study by Rodriguez-Mateos et al. (2011), was that high intakes of sugar and its accompanying energy (which is associated with chocolate) are undesirable from a public health perspective, let alone when trying to manage T2DM. What is unclear is whether flavanols are biologically active when provided with any type of carbohydrate preferentially (either as starch, sucrose or other sugars e.g. lactose).

3.1.2 - Influences upon the Biological Action of Carbohydrate on Flavanols Absorption

There is evidence to suggest that although carbohydrate may facilitate flavanol absorption, this might not necessarily translate into an enhancement of biological efficacy. To date only one study (Njike et al., 2011) has tempted to address this issue however, it used a complex crossover design, resulting in the need for *post hoc* analysis, and might have been prone to error. This was reported by Faridi et al. (2008) (acute effects) and Njike et al. (2011) (six weeks feeding data). Both papers were based on the

published study protocol NCT00538083. In the first phase single doses of chocolate (placebo or dark (flavanols rich) chocolate) were administered; in the second phase, there were three arms where a single dose of placebo, sugared cocoa or sugar-free cocoa, were given. All 44 participants who completed the trial received both chocolates and all three cocoa formulations. The authors reported significant improvements in endothelial function and blood pressure with the dark chocolate compared to the cocoa-free chocolate bar, however, the focus of their conclusions was that the sugar-free cocoa improved endothelial function to a greater extent than the sugared cocoa. From this it was suggested that sugar content might negatively impact on the beneficial effects seen with cocoa flavanols (Faridi et al., 2008). However they did not explore or consider differences between the chocolate and cocoa phases.

A *post hoc* t-test based on the data of Faridi et al. (2008) (GraphPad, La Jolla, CA, USA) suggested that for endothelial dysfunction there was significantly greater improvement in flow mediated dilation for the dark chocolate compared to the sugared cocoa ($p=0.0076$, standard error of difference (SED) = 0.82) with no significant difference between the dark chocolate and the sugar-free cocoa ($p=0.1325$, SED =0.913). The principle difference between the sugared cocoa and the chocolate was that the dark chocolate contained approximately two-thirds less carbohydrate (in the form of sucrose) than the cocoa (39g compared to 104g). Although these participants were overweight they were otherwise 'healthy', and the authors did not report any assessment of metabolic syndrome or insulin resistance. The difference in carbohydrate content, only resulted in a 30% reduction in the energy content (327kcal compared with 460 kcal); a reflection of the significantly greater fat content of the chocolate (27g compared with 2g) for the cocoa.

3.1.3 - Potential Mechanisms

Type 2 diabetes and other insulin resistant states are associated with hyperinsulinaemia and hyperglycaemia (which characterises the former). Both pathological states have been associated with a reduction in nitric oxide bioavailability. As described in Chapter One, an increase in the bioavailable pool of nitric oxide is considered to be the primary mechanism of cocoa flavanols, with the systemic effects seen via reduced endothelial dysfunction and blood pressure. The unifying theory of Brownlee (2005) supports this mechanism, since the uncoupling of the mitochondria resulting in superoxide generation along with activation of NADPH oxidases results in an overall increase in oxidative stress (Dworakowski et al., 2008; Munzel et al., 2008). The net effect of which is a reduction in the amount of bioavailable nitric oxide, leading to endothelial dysfunction and hypertension. Ironically, ingesting carbohydrate with cocoa flavanols might increase their bioavailability but reduce their biological effects.

3.1.4 - Linking Plasma Flavanols to Biological Effects

In vitro and animal work have demonstrated mechanistic pathways to link the dosage of cocoa flavanols to their effects. The same methodology has not always been as thoroughly applied in humans. A number of studies have assessed levels of flavanols and epicatechin metabolites as part of clinical trials; generally this has been to measure compliance or check the presence of these metabolites following ingestion to link to the physiological effect. Engler et al. (2004) using a randomized double blind crossover study design with low and high flavonoid dose content chocolate, reported a synchronous rise in epicatechins and improvement in endothelial function following consumption of flavonoid rich chocolate. Few dose-finding studies have been undertaken to obtain an optimal concentration of cocoa flavanols, especially with respect to the form of presentation and accompanying nutrients or food components.

Despite this, as previously discussed in Chapter One, ESFA subject to EU commission has granted authorisation for chocolate manufacturer - Barry Callebaut BV, to use a proprietary claim for chocolate and cocoa containing 200mg of polyphenols (EFSA, 2012a).

The observations of Faridi et al. (2008) together with the bioavailability data present an interesting picture. To increase palatability, many of the studies using cocoa use a significant amount of sucrose to enhance compliance. This may have the effect of increasing bioavailability (Schramm et al., 2003), but might reduce biological activity (Faridi et al., 2008). This suggests that chocolate may be a better option since less carbohydrate may be needed to make it palatable when compared to cocoa formulations: The result being that chocolate has a formulation with enough carbohydrate to enhance absorption, but not enough to influence nitric oxide availability negatively and as such impede its beneficial effects. It is highly plausible that chocolate may have physical, biological and organoleptic properties to enhance cocoa flavanol intake, bioavailability and biological activity, although Ried et al. (2009) suggested 50g of chocolate was not acceptable to their study population, sales data of chocolate might refute this argument. The co-ingestion with other macronutrients might enhance bioavailability, whilst the relatively modest (compared to cocoa preparations) sugar content might enhance biological action.

3.2 - Methodology

The exploratory review in Chapter One, along with the material presented in this chapter have been conducted and presented in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) and Cochrane methodology guidelines (Moher, Liberati, Tetzlaff & Altman, 2009; Higgins & Green, 2011). The notable deviation is that this work is being produced primarily for this thesis, so instead of being done by a group of experienced reviewers, I have taken responsibility for the conduct of the review. As such it was therefore not possible to publish a protocol for the review, as has been done by others (Ried et al., 2010b).

3.2.1 – Study Selection

All clinical trials included had human participants and only included adults who were either healthy or had a chronic non-communicable disease (e.g. obesity, diabetes, cardiovascular disease). The key selection criterion was that they were randomised controlled trials of either parallel or crossover design. Initial searches were less restrictive and included trials that did not have a control arm or non-matched placebo. These were screened and accepted or rejected based on the afore mentioned selection criterion.

Interventions were chocolate or cocoa as the primary intervention, including studies using cocoa extracts and refined flavanols or epicatechin supplements. Studies were only selected if the investigators had attempted to match the high polyphenol/flavanol arm with a similar comparator or placebo. Each study had to meet at least two of the following four criteria; appearance or taste, weight or volume, energy content and carbohydrate load. The studies also define the amount of epicatechin, flavanols, procyanadins or polyphenols in both arms and no other potential biologically active compounds could be added to the cocoa or chocolate, other than those which occur naturally. Studies or study arms, which included physical activity (as this may lead to

change in the level of oxidative stress) isoflavones or sterol esters as part of the chocolate formulation, were therefore excluded. These were developed as key criteria to define the validity control of the material presented in this chapter.

3.2.2 – Biomarkers and Clinical Measurements

The study focused upon the primary outcomes of the selected trials then six categories as defined in Chapter One, were used to categorize the data from these outcomes, being:

1. Glycaemia or insulin resistance (e.g. HOMA, HbA1c, glucose or insulin)
2. Blood pressure (either ambulatory or measured in the clinic room)
3. Lipid profile (Total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides)
4. Endothelial function (Flow mediated dilation, reactive hyperaemia and soluble markers e.g. adhesion molecules)
5. Oxidative stress (e.g. urinary or plasma isoprostanes)
6. Inflammatory markers (e.g. C-Reactive Protein)

Additionally, weight changes were included from studies that reported baseline and end of study weights.

Markers were generally chosen on the basis of their clinical relevance to diabetes and its management or prevention, or linked to the recommendations of the PASSCLAIM group with respect to accepted surrogate markers which might be appropriate for a health claim associated with a food (Riccardi et al., 2004; Mensink et al., 2003; Mann et al., 2004). It was not possible to identify hard endpoints for macrovascular or microvascular complications of diabetes, or in fact the onset of diabetes in high-risk groups. This was a reflection of the short nature and small size of interventional studies using chocolate and cocoa.

3.2.3 – Search Strategy

A structured search strategy was carried out to the end of June 2012. This included Medline (http://www.ncbi.nlm.nih.gov/pubmed) and EMBASE (http://www.embase.com/), the Cochrane Library (CENTRAL) (http://www.thecochranelibrary.com/view/0/index.html), and the WHO clinical trial database (International Clinical Trials Registry Platform) (http://apps.who.int/trialsearch/). Bibliographies of included studies and previously published reviews, systematic reviews and meta-analyses were checked. Searches were limited to English. For a full description of the search strategies and search terms and search strategy along with the PRISMA checklist, see Appendix I.

Titles and abstracts and potential full-text articles were assessed for inclusion. Due to time constraints, it was not possible to contact the authors of studies for further data or clarification. The author of this thesis extracted the data used in the analysis from the numerical data presented in the papers. The quality of the graphical presentations was deemed insufficient to allow adequate estimation of the size of any effect and the measure of spread around the mean, it was considered the reliability of the reproduction in print of the scales could additional introduce error.

3.2.4 – Inclusion Criteria

Initially the aim was to stratify studies by disease of the participants, however with the exception of the work presented in Chapters Four, Five and Six, only one other study on individuals with T2DM as defining characteristics that met the inclusion criteria was available. With only a single study feeding cocoa to individuals with T2DM (Balzer et al., 2008), it was not possible to rely on this approach, so it was necessary to include all clinical trials. There was no exclusion criterion set for study duration; studies that were both acute (a few hours) and continuous or prolonged feeding (2-26 weeks) were included. The only exception was for studies of a minimum of 14 days where blood pressure and weight, were excluded.

Changes in continuous variables were initially aligned to match the units, and converted using standard equations if needed (e.g. for lipids from mg/dl to mmol/l), with the measure of spread standardised to the standard deviation. Changes from baseline to the end of study were recorded, as were the standard deviations using the assumption that variances were equal. Where more than one outcome point was reported, the data was extracted for baseline and the final endpoint of the study (or change). For parallel studies with more than one group, the control groups were used for all interventions. Where studies reported several doses, only the highest was selected for comparison with the control. In groups where the study population were stratified, e.g. lean and overweight, these data were split. This approach was applied for any study reporting acute and prolonged feeding data. Crossover studies were treated as parallel studies, with the total number of participants completing being recorded in both the intervention and control arms.

3.2.5 – Trial Quality and Assessment of Risk Bias

Trial quality was partially addressed by the strict inclusion criteria which formed the validity control. Review Manager 5.2 (Nordic Cochrane Centre, Copenhagen, Denmark) was used to assess the risk of bias with respect to selection (random sequence generation and allocation concealment), performance, detection, attrition and reporting bias. Additionally an assessment of other bias was made with respect for the similarity with respect to appearance and taste of the intervention and control. In addition, the influence of commercial interest was assessed, to see if the studies described their source of funding and/or interventional products, and whether this was linked to a cocoa or chocolate manufacturer, or a company with commercial or intellectual property associated with them.

3.2.6 – Statistical Methods

Variability between studies was estimated by I^2, which is the proportion of total observed variability due to genuine variation, as apposed to random error within studies. This was considered significant when $I^2 > 50\%$ (Higgins et al., 2003). Where this was observed, a

random effects model was selected to assess the mean differences between groups, and the data were interpreted with caution.

The extracted data was converted where necessary to SI units to prior to undertaking the analysis. Where data was presented with a 95% confidence interval or standard error of mean, these were converted to standard deviation using standard equations. A calculation of the mean change was then estimated, along with its variation of change (standard deviation, using variance sum law 1) from studies where the baseline and post intervention data was presented using standard formula. This included Almoosawi et al. (2012) who reported change to a post intervention baseline (which was not used) and for the purposes of this analysis an estimate based upon the pre-intervention baseline was used in order to be comparable with the other studies. The data selected for analysis constituted of the baseline compared to the end of chocolate supplementation. Where more than one dose of polyphenol rich chocolate was used, the lowest (control) was compared with the highest polyphenol content chocolate. Where possibly comparisons were made between products most similar with respect to nutrient profile.

The differences between intervention and control were estimated with Review Manger 5.2 software (Nordic Cochrane Centre, Copenhagen, Denmark), using mean differences in random-effects meta-analysis. For all outcomes where there were greater than eight published cohorts the data were explored by a sub-group analysis to discover if there were differences in effect between chocolate and cocoa. Where studies gave both cocoa and chocolate as the intervention, this was considered as chocolate. Funnel plots were run for all analyses to access for publication or small study biases. Results were reported as the mean difference between intervention and control with 95% confidence interval, p value and assessment of heterogeneity.

3.3 - Results

A total of 1351 studies were found following a Medline, Embase and Cochrane Library (CENTRAL) search for clinical trials linked to chocolate or cocoa up until the end of June 2012 (see PRISMA flowchart, figure 3.3.1). Following the removal of duplicates, there were 243 relevant titles and abstracts, which were screened. All abstracts were screened for relevance to the outcomes and criteria described in the methodology and a total of 69 full papers of clinical trials were selected for inclusion. These excluded the work of the author (only where I was the lead author), as this work is presented in the subsequent chapters of this thesis. A total of 31 studies met all of the inclusion criteria, and reported relevant outcomes with adequate description of the intervention and control.

The 69 studies (not all reported relevant outcome data) reviewed represented a total of 2772 participant exposures to cocoa or chocolate and this represented 17750 participant years of cocoa or chocolate consumption. Of the 31 included studies, which reported relevant outcomes, represented 1285 participant exposures and a total of 2752 participant years (table 3.3.1; for summary data). This might imply that the trials meeting the inclusion criteria represented approximately half of the total participant exposures, but some tended to have a shorter duration. Ten of the studies declared no link to industry, with thirteen studies being linked to Mars/Masterfoods, five to Nestlé and three to Hershey. The remaining studies were linked to non-multinationals or trade bodies. Twelve of the studies utilised a parallel design and the remainder used a crossover design. It was also noted that there were a number of authors contributing to a number of papers, with seven authors contributing more than one paper as either first or second author.

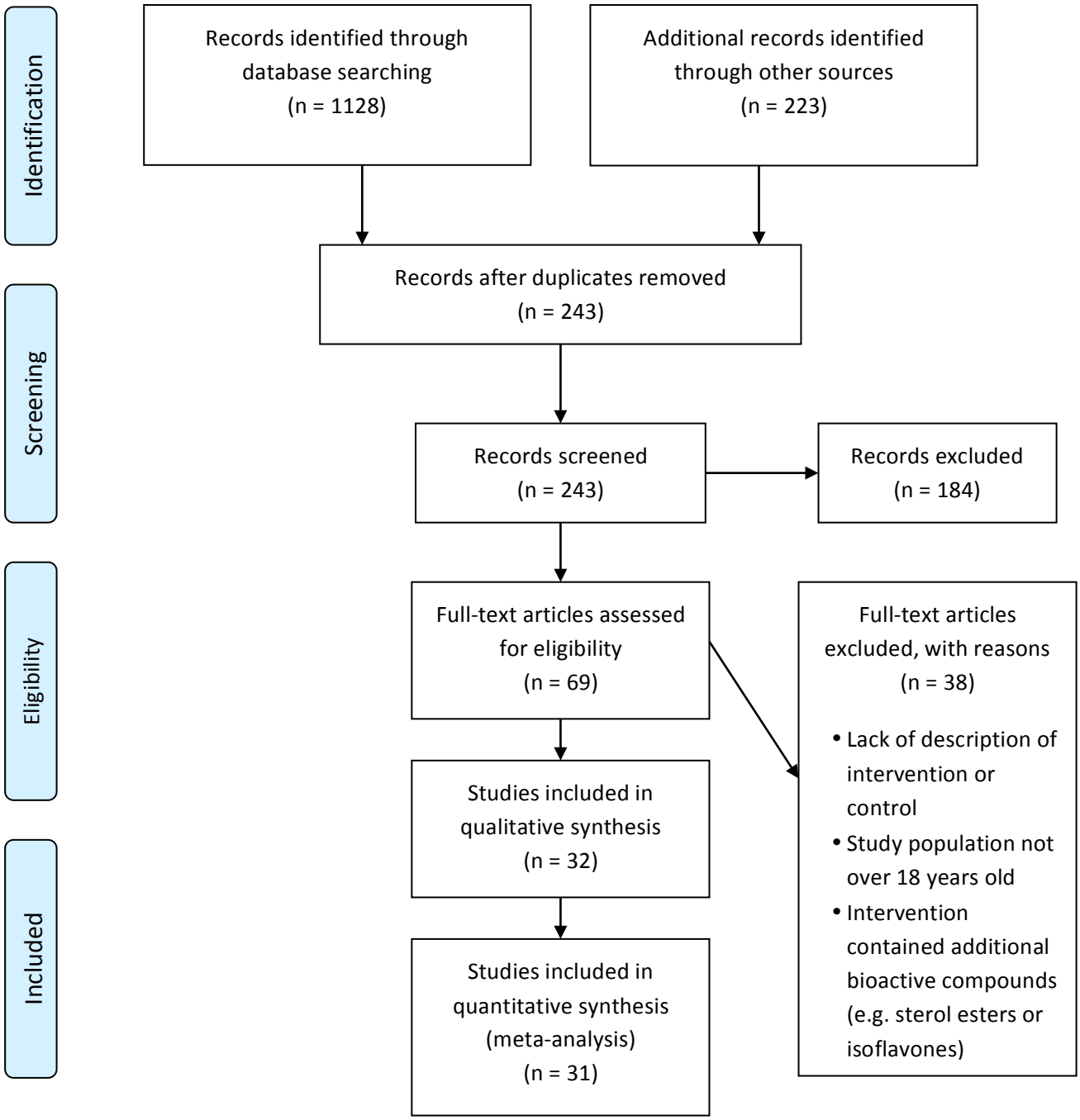


Figure 3.3.1: The PRISMA flowchart for the selection of the studies for the meta-analysis included in this chapter. (Moher et al., 2009)

Sixteen of the studies described their participants as being healthy, although in the case of one study, the population was elderly and three others had overweight or obese participants. Two studies focused on cardiovascular disease, a further two studies investigated the effects of cocoa or chocolate upon heart failure and a single study investigated heart transplant patients. In terms of risk factors, one study considered the effect of cocoa upon individuals with T2DM; a further study investigated the effects of chocolate in participants with impaired glucose tolerance and hypertension, eight studies focused upon participants with hypertension alone and two studies recruited participants with dyslipidaemias. Of the remaining studies, one recruited smokers and the other participants with chronic fatigue syndrome.

The interventions included two studies that used tablets containing cocoa polyphenols, one that utilised both chocolate and cocoa and one that included bread with supplementary cocoa. Of the twelve studies using cocoa as the intervention, eight clearly defined this as being mixed with water, one with cream, and one described a dairy-based cocoa drink. Eighteen studies described their intervention as chocolate; of these, one utilised milk chocolate, with the remaining studies described their chocolate as dark or high in polyphenol (and therefore flavanols).

The polyphenol content of the control intervention was described as zero in 19 studies; however, in all cases the source of the analysis was not defined. Two of the studies reported a control intervention with greater than 100mg of polyphenols/flavanols. These studies therefore had a greater dose of polyphenols in the control arm than in the intervention arm (one study in the case of Sathyapalan et al. (2010) and four studies in the case of Monahan et al. (2011)). Studies which included sham eating, or a placebo which was not well matched to the flavanols rich cocoa or chocolate, e.g. milk or sugar solution were excluded from any analysis.

The primary outcome of the studies varied, as not all the studies were registered: Only eleven were found to have pre-publication study registration, and not all matched their

proposed primary outcomes to those reported in the final peer reviewed publication. Fourteen studies had endothelial function as the primary outcome, five had a reduction in blood pressure and two had an improvement in lipid profile. Five studies had a focus upon composite measures; in one study this was cardiovascular risk, another focused upon insulin resistance and three considered metabolic syndrome. Of the remaining nine studies, five focused upon oxidative stress, two on the effects of cocoa polyphenols upon platelet aggregation, one on probiotic effects of cocoa and one on reduction in symptoms of fatigue and function.

Table 3.3.1a: Summary data of the studies which met the inclusion criteria for the meta-analysis including number of participants, design, source of funding and characteristics of the interventions and comparators. Key - X – Crossover design, P-Parallel design, NS – Not Stated. Note for enhanced presentation, citations have been limited to the lead author only, full citations are available in Appendix 1.

Lead Author	Year	Design	Duration (d)	n placebo	n active	Placebo Polyphenols mg/ day	Placebo Epicatechin mg/ day	Active Polyphenols mg/ day	Active Epicatechin mg/ day	Intervention	Comparator	Comment	Funding
Allgrove	2011	X	14	20	20	0.0	0.0	NS	38.7	Dark Chocolate	Cocoa liquor free Chocolate	Energy matched no reference to colour or taste	Nestle
Almoosawi (overweight)	2012	X	28	21	21	0.0	0.0	500.0	19.0	Dark Chocolate	Colour, taste and nutrient matched Chocolate	Well matched	NS
Almoosawi (obese)	2012	X	28	21	21	0.0	0.0	500.0	19.0	Dark Chocolate			NS
Baba	2007	P	28	31	37	NS	0.0	NS	129.0	Cocoa in water	Cocoa matched flavour	Matched	Meiji Seika Kaisha Ltd
Balzer	2008	X	30	10	10	NS	16.8	NS	203.0	Cocoa in water	Nutrient matched Cocoa	Unclear	Mars
Davison, K.	2008	P	84	11	12	18.0	NS	451.0	NS	Dairy cocoa in water	Nutrient matched Cocoa	No reference to taste/ colour	Mars
Davison, K.	2010	P	42	14	13	33.0	0.0	1052.0	208.0	Cocoa drink	Nutrient matched Cocoa	No reference to taste/ colour	Mars
Davison, G.	2012	X	0.2	14	14	0.0	0.0	NS	96.8	Dark Chocolate	Nutrient matched Chocolate	No reference to taste/ colour	Nestle
Engler	2004	P	14	10	11	0.0	0.0	213.0	46.0	Dark Chocolate	Low flavanols dark Chocolate	No reference to taste/ colour	NS
Faridi (Chocolate)	2008	x	0.1	44	44	0.0	0.0	821.0	21.5	Dark Chocolate	Placebo Chocolate	Unclear	Hersheys
Faridi (Cocoa)	2008	x	0.1	44	44	8.8	0.0	805.2	48.4	Cocoa drink	Placebo Cocoa	Unclear	Hersheys
Farouque (Long-term)	2006	P	42	20	20	19.6	4.7	444.0	107.0	Chocolate and cocoa in milk	Energy matched Chocolate and Cocoa	Unclear detail with respect to the drink	Mars
Farouque (acute)	2006	P	0.1	20	20	19.6	4.7	444.0	107.0				Mars
Flammer (acute)	2011	P	0.1	10	10	0.0	0.0	624.0	36.0	Chocolate	Nutrient matched Chocolate	Unclear	Nestle
Flammer (Long-term)	2011	P	28	10	10	0.0	0.0	624.0	36.0				Nestle
Flammer	2007	P	0.1	11	11	0.0	0.0	624.0	36.0	Dark Chocolate	Nutrient matched Chocolate	Unclear	Nestle
Fraga	2005	X	14	28	28	0.0	0.0	168.0	39.0	Milk Chocolate	White Chocolate	Difference in taste and appearance	Mars

Table 3.3.1a: Continued

Lead Author	Year	Design	Duration (d)	n placebo	n active	Placebo Polyphenols mg/ day	Placebo Epicatechin mg/ day	Active Polyphenols mg/ day	Active Epicatechin mg/ day	Intervention	Comparator	Comment	Funding
Grassi	2005 b	X	15	10	10	0.0	0.0	500.0	66.0	Dark Chocolate	White Chocolate	Difference in taste and appearance	NS
Grassi	2005 a	X	15	15	15	0.0	0.0	500.0	66.0	Dark Chocolate	White Chocolate	Difference in taste and appearance	NS
Grassi	2008	X	15	19	19	0.0	0.0	1080.0	110.9	Dark Chocolate	White Chocolate	Difference in taste and appearance	NS
Heiss	2005	X	0.1	11	11	11.5	1.0	181.0	21.0	Cocoa drink	Matched Cocoa	Unclear	Mars
Monahan	2011	X	0.1	23	23	330.0	0.0	1470.0	96.0	Cocoa drink	No Cocoa drink	Unclear	Hersheys
Muniyappa	2008	X	14	20	20	8.0	1.0	338.0	87.0	Cocoa drink	Placebo drink	Similar packaging, taste and colour	Mars?
Murphy	2003	P	28	15.	13	6.0	NS	234.0	NS	Cocoa polyphenol tablets	Placebo tablet	Not specified, but likely to be well matched	Mars
Mursu	2004	P	21	15.	15	0.3	0.0	556.8	227.0	Dark Chocolate	White Chocolate	Difference in taste and appearance	NS
Njike	2011	X	42	44	44	8.8	0.0	805.2	48.4	Cocoa drink	Placebo Cocoa	Unclear	Hersheys
Sathyapalan	2010	X	54	10	10	175.5	2.3	783.0	55.4	Dark Chocolate	Simulated energy matched Chocolate	Taste test referred described	NS
Shiina	2009	P	14	19	20	0.0	NS	550.0	NS	Dark Chocolate	White Chocolate	Difference in taste and appearance	Meiji Seika Kaisha Ltd
Taubert	2003	X	14	13.0	13	0.0	NS	500.0	NS	Dark Chocolate	White Chocolate	Difference in taste and appearance	NS
Taubert	2007	P	126	22.0	22	0.0	0.0	30.0	5.1	Dark Chocolate	White Chocolate	Difference in taste and appearance	NS
Tzounis	2011	X	28	20.0	20	NS	3.0	NS	89.0	Dairy Cocoa drink	Matched dairy Cocoa drink	Similar taste and coded packaging	Mars
Wang-Polagruto	2006	P	42	8	9	43.0	NS	446.0	NS	Cocoa drink	Cocoa drink (matched)	Coded packaging, unclear regarding taste	Mars
Westphal	2011	X	0.2	18	18	14.7	0.7	918.0	146.0	Cocoa drink	Matched Cocoa drink	Unclear	NS
Wiswedel	2004	X	0.3	10	10	14.0	NS	187.0	NS	Cocoa drink	Matched Cocoa drink	Unclear	Mars

Table 3.3.1b: Summary data of the studies which met the inclusion criteria for the meta-analysis including the population group, primary and secondary outcomes. Note for enhanced presentation, citations have been limited to the lead author only, full citations are available in Appendix 1.

Lead Author	Year	Population	Primary Outcome	Secondary Outcomes
Allgrove	2011	Healthy males	Oxidative stress	Heart Rate, Oxygen Uptake, Glucose, Free-fatty acids, Inflammatory markers, Insulin and counter regulatory hormones
Almoosawi (overweight)	2012	Healthy women, overweight	Metabolic syndrome	HOMA, Glucose, Lipids, Blood pressure, Weight
Almoosawi (obese)	2012	Healthy women, overweight	Metabolic syndrome	HOMA, Glucose, Lipids, Blood pressure, Weight
Baba	2007	Normo- and hypercholesterolaemic men and women	Lipids	Oxidised LDL cholesterol, Apolipoprotein
Balzer	2008	Diabetes men and women	Endothelial function	Lipids, Glucose, HbA1c
Davison, K.	2008	Healthy overweight and obese men and women	Metabolic syndrome	Blood pressure, Lipids, HOMA, Glucose
Davison, K.	2010	Men and women with hypertension	Blood pressure	Cortisol, NEFA, Glucose, Inflammatory factors
Davison, G.	2012	Healthy males	Oxidative stress	
Engler	2004	Healthy adults	Endothelial function	Blood pressure, Lipids, LDL cholesterol oxidation, Oxidative Stress
Faridi (Chocolate)	2008	Men and women with hypertension	Endothelial function	Blood pressure
Faridi (Cocoa)	2008	Men and women with hypertension	Endothelial function	Blood pressure
Farouque (Long-term)	2006	CAD patients	Endothelial function	Lipids, Glucose, CRP, LDL cholesterol oxidation
Farouque (acute)	2006	CAD patients	Endothelial function	Lipids, Glucose, CRP, LDL cholesterol oxidation
Flammer (acute)	2011	CHF	Endothelial function	Lipids, Glucose, Inflammatory factors, Oxidative Stress
Flammer (Long-term)	2011	CHF	Endothelial function	Lipids, Glucose, Inflammatory factors, Oxidative Stress
Flammer	2007	Heart transplant	Platelet aggregation	Glucose, Blood pressure, Inflammatory factors, Oxidative Stress
Fraga	2005	Healthy males	Oxidative stress	Lipids, Blood pressure, Lactate and oxygen consumption

Table 3.3.1b: Continued

Lead Author	Year	Population	Primary Outcome	Secondary Outcomes
Grassi	2005b	Men and women with hypertension	Blood pressure	Lipids, Weight
Grassi	2005a	Healthy adults	Blood pressure	Lipids, Weight
Grassi	2008	IGT hypertensives	Insulin resistance	Blood pressure, Endothelial function
Heiss	2005	Smokers	Endothelial function	
Monahan	2011	Healthy older adults	Endothelial function	Insulin, Glucose, Blood pressure
Muniyappa	2008	Hypertensives	Endothelial function	Weight, Blood pressure, Lipids, Glucose, Insulin, Inflammatory markers
Murphy	2003	Healthy adults	Platelet aggregation	Oxidative stress, Adhesion molecules
Mursu	2004	Healthy adults	Lipids	Liver function, Oxidative stress
Njike	2011	Men and women with hypertension	Endothelial function	Glucose, Lipids, Blood pressure
Sathyapalan	2010	Chronic fatigue syndrome	Reduced fatigue	Weight
Shiina	2009	Healthy adults	Cardiovascular risk	Lipids, HbA1c, Oxidative stress
Taubert	2003	hypertensives	Blood pressure	
Taubert	2007	hypertensives	Blood pressure	Weight, Lipids, Oxidative stress, Glucose
Tzounis	2011	Healthy adults	Probiotic effects	Blood pressure, Lipids, Glucose, CRP
Wang-Polagruto	2006	Hypercholesterolaemic women	Endothelial function	Adhesion molecules
Westphal	2011	Healthy adults	Endothelial function	Lipid, free fatty acids, Blood pressure
Wiswedel	2004	Healthy adults	Oxidative stress	Antioxidants and Oxidative Stress

In terms of duration, ten studies reported data in an acute or post-prandial state and 20 investigated supplementation for fourteen days or more with the longest duration of feeding 126 days (mean 22 days; median 15 days). For the purposes of the meta-analysis all data were included, except in the case of blood pressure and weight where a minimum of 14 days was used as the criterion for selection.

3.3.1 - Study Validity

The risk of selection, performance, detection, attrition, reporting bias and the bias introduced from the adequacy of the matching of the control to the intervention were categorised into low, unclear and high risk of bias. The matching of the intervention to the control product appeared to insufficiently describe or a high risk of bias in about 70% of the studies. High risk of bias was more common in studies using chocolate, however there were a number of examples of taste trials and careful matching of the products used in both arms of studies using chocolate. Figure 3.3.1.1 shows the summary data, which suggested that there is a high risk of bias particularly with respect to blinding and matching of the intervention to the control, however about 90% appeared to have low levels of reporting bias, largely a reflection of reporting baseline and post intervention data. Figure 3.3.1.2, presents the judgments about bias for the studies individually.

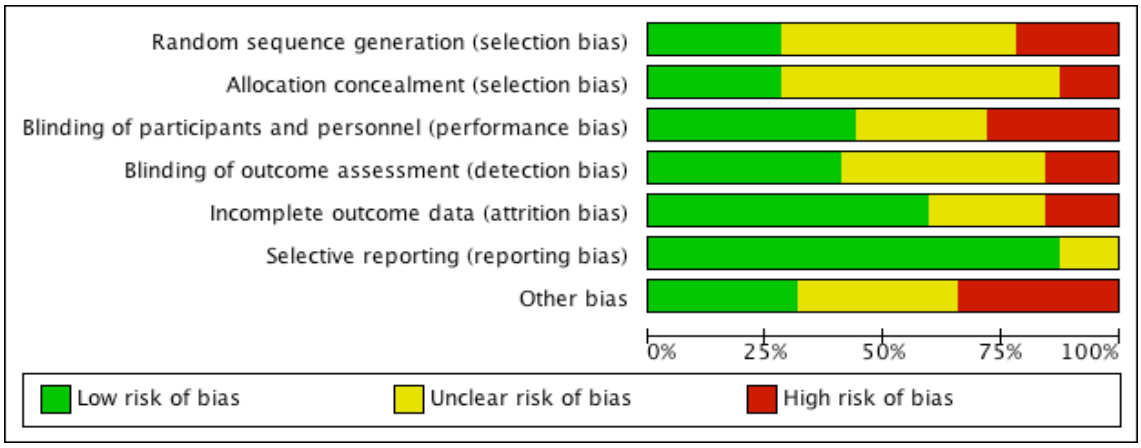


Figure 3.3.1.1. Risk of bias graph, judgements about each risk of bias item presented as percentages across all included studies.

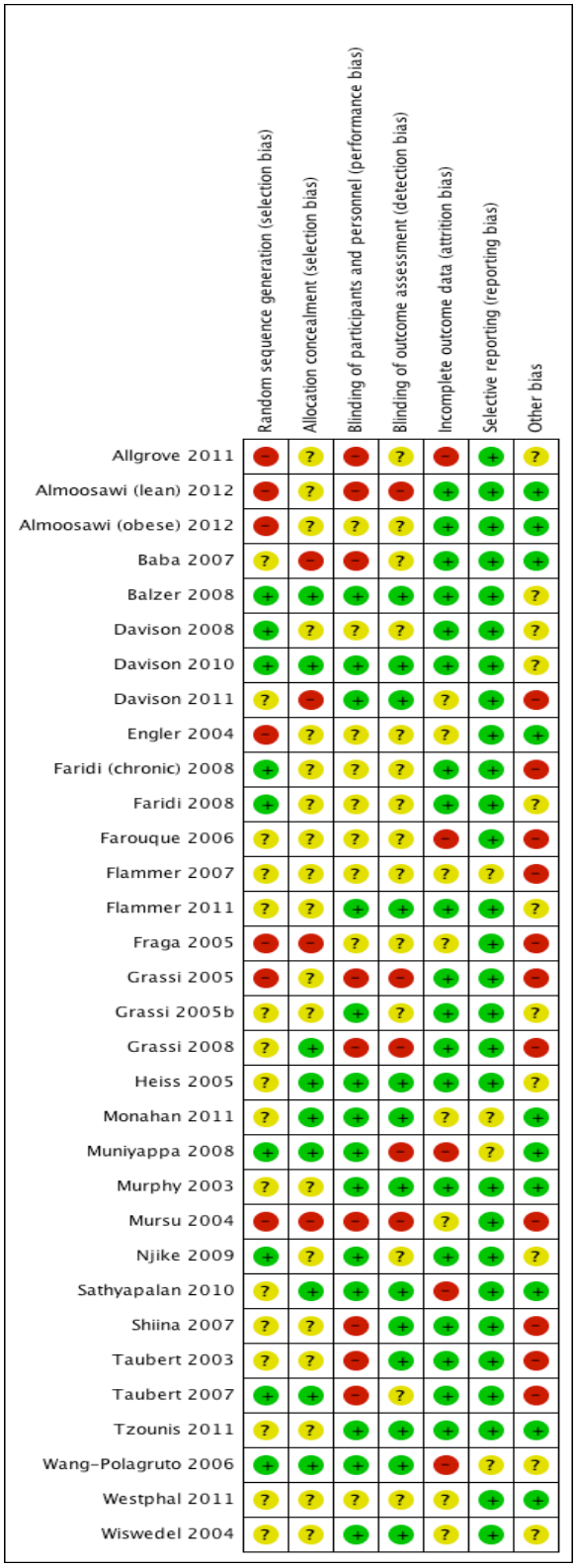


Figure 3.3.1.2: Risk of bias summary judgements about each risk of bias item for each included study.

The studies varied greatly with respect to their design, investigational product and conduct. Participant blinding was not clear in all studies; it was reported as being adequate by the researchers in 15, and by the participants in 12 of the studies. This difference was primarily due to the use of white chocolate as a control where there was a risk that the participant might be unblinded by the nature of their prior knowledge and experience. As previously described, all but ten of the 33 studies were associated with industry or trade bodies, and that 14 of the publications shared seven authors which also might represent the possibility of additional bias. Where data was extracted as baseline and end of study (and standard deviation/ standard error or 95% confidence interval) rather than mean change, the analysis resulted in a larger 95% confidence interval for the mean difference. This increased the degree of heterogeneity in a number of the forest plots.

The nature of the formulations varied greatly in terms of amount of product, mean weight (g)± standard deviation across 33 studies was 65.9±40.1g for the control and 67.7±38.7g active with a range from 5.6 to 180 g. This led to a difference in energy and carbohydrate content; from negligible carbohydrate and energy in the cocoa polyphenol capsules, to 2352Kj (560kcal) where there was in excess of 100g of carbohydrate. When cocoa supplementation was compared with the chocolate, there was a significant difference in the energy ($p=0.027$) but not the amount of carbohydrate ($p=0.213$) content. The mean (± standard deviation) energy and carbohydrate content of the cocoa interventions (placebos and interventions combined) were 872±653Kj (208±155kcal) energy and 34.2±35.8g carbohydrate compared to 1341±808kj (319±192kcal) energy and 24.3±19.5g carbohydrate for chocolate interventions. This related to a mean ± standard deviation percentage of energy provided by carbohydrates in the cocoa intervention studies of 68.2±13.8% and for the chocolate intervention studies a significantly lower proportion, at 38.8±13.1% ($p<0.0001$).

3.3.2 - Effect of Cocoa and Chocolate on Blood Pressure

3.3.2.1 - Systolic blood pressure

Data from sixteen trial data sets were included in the meta-analysis (figure 3.3.2.1). This represented 16 published trials and 576 participants. The meta-analysis suggested a significant reduction in systolic blood pressure with high polyphenol chocolate or cocoa (-3.46mmHg; 95% Confidence Intervals -4.80, -2.11, I^2 =84%; p<0.0001) after a minimum of 14 days supplementation.

3.3.2.1.1 - Sub-analysis of chocolate supplementation

Data from nine data sets (extracted from eight published trials) representing 313 participants suggested a significant improvement in systolic blood pressure with high polyphenol chocolate (-4.72mmHg; 95% Confidence Intervals -6.27, -3.17, I^2 =87%; p<0.0001) after at least 14 days supplementation. This was in contrast to the data for cocoa, which did not appear to influence systolic blood pressure (-0.59mmHg; 95% Confidence Intervals -3.21, 2.03, I^2 =68%; p=0.66)

Analysis of the sub-groups within the meta-analysis suggested a significant difference between studies utilising chocolate and cocoa. The meta-analysis suggested that chocolate was more effective in improving systolic blood pressure than cocoa in well-controlled clinical trials lasting at least 14 days (p=0.008; Chi^2 =7.07, I^2 =85.9).

3.3.2.1.2- Potential small study and publication bias for systolic blood pressure

The funnel plot in figure 3.3.2.1 and the measure of heterogeneity, suggest that the data are very heterogeneous, and there are a number of studies suggestive of small study bias by the nature of their standard error of mean differences. Although Taubert et al. (2003) was included in the review, as there was not enough numerical data, it was not included in the analysis for blood pressure.

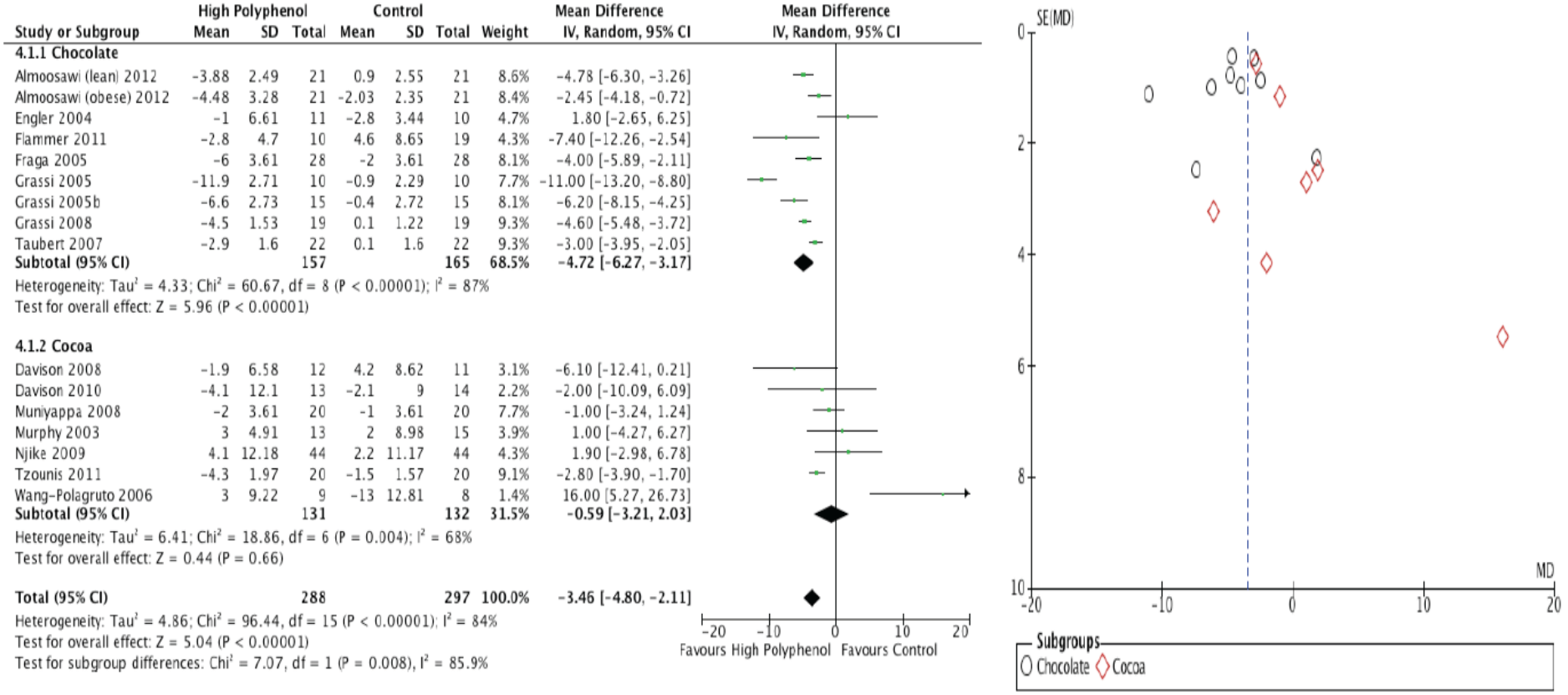


Figure 3.3.2.1: Meta-analysis results with forest plots for systolic blood pressure from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa. Please note for Almoosawi (lean) refers to BMI<25kgm^{-2} and Almoosawi (obese) refers to the subset with a BMI≥25kgm^{-2} (overweight and obese).

3.3.2.2 - Diastolic blood pressure

Data from sixteen trial data sets were included in the meta-analysis (figure 3.3.2.2). This represented 16 published trials and 576 participants. The meta-analysis suggested a significant improvement in diastolic blood pressure with high polyphenol chocolate or cocoa (-2.11mmHg; 95% Confidence Intervals -3.30, -0.91, I^2 =87%; p<0.0001) after a minimum of 14 days supplementation.

3.3.2.2.1 - Sub-analysis of chocolate supplementation

Data from nine data sets (extracted from eight published trials) representing 313 participants suggested a significant improvement in diastolic blood pressure with high polyphenol chocolate (-3.05 mmHg; 95% Confidence Intervals -4.46, -1.63, I^2 =90%; p<0.0001) after at least 14 days supplementation. This was in contrast with the data for cocoa, which did not appear to significantly influence diastolic blood pressure (-0.53 mmHg; 95% Confidence Intervals -2.17, 1.10, I^2 =54%; p=0.52).

Analysis of the sub-groups within the meta-analysis suggested that chocolate was more effective in improving diastolic blood pressure than cocoa in well-controlled clinical trials lasting at least 14 days (p=0.006; Chi^2 =7.68, I^2 =87.0%).

3.3.2.2.2- Potential small study and publication bias for diastolic blood pressure

The funnel plot in figure 3.3.2.2 and the measure of heterogeneity, suggest that the data are very heterogeneous and there are a number of studies suggestive of small study bias by the nature of their standard error of mean differences. This is particularly notable for the studies using cocoa based interventions where three studies showed very little effect but a larger standard error.

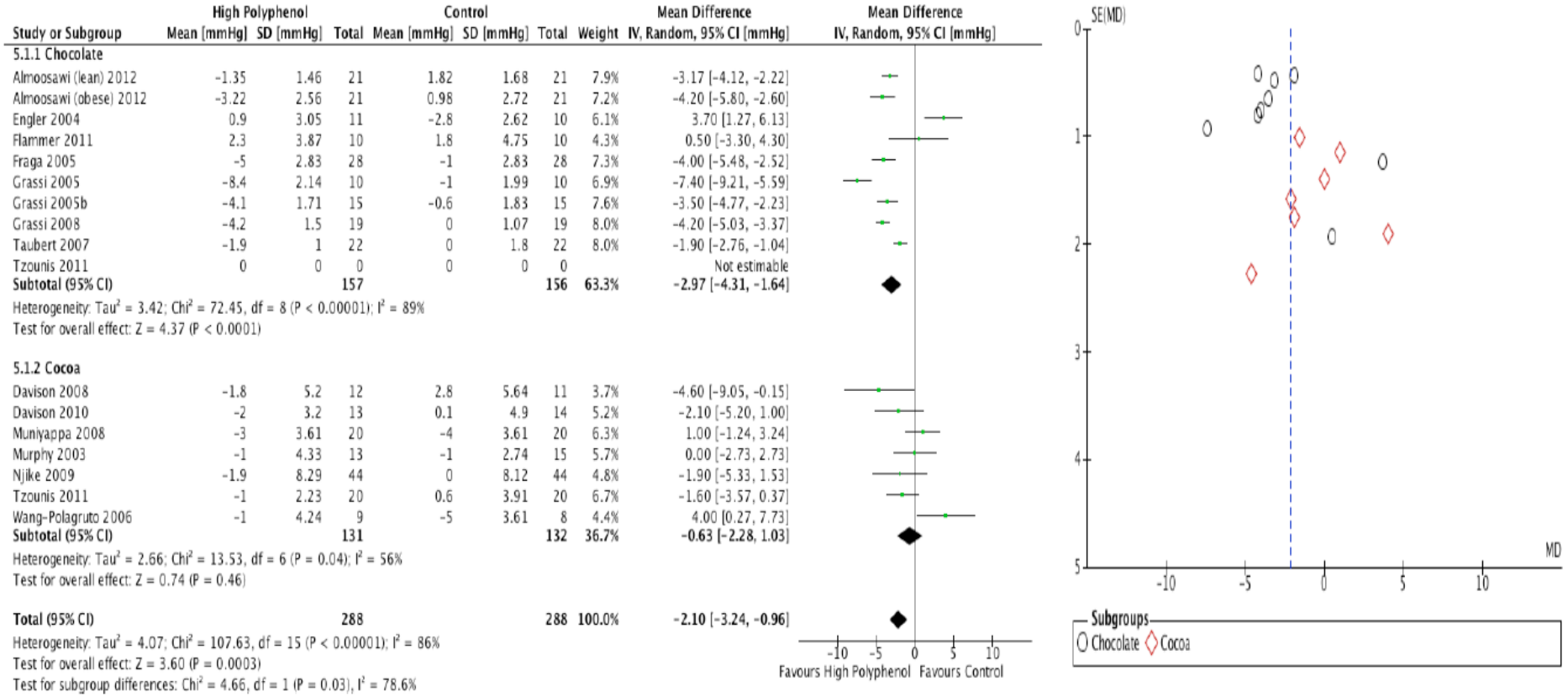


Figure 3.3.2.2: Meta-analysis results with forest plots for diastolic blood pressure from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa. Please note for Almoosawi (lean) refers to BMI<25kgm^{-2} and Almoosawi (obese) refers to the subset with a BMI≥25kgm^{-2} (overweight and obese).

3.3.3- Effect of Cocoa and Chocolate on Fasting Lipid Parameters

3.3.3.1 - Total cholesterol

Data from fourteen trial data sets were included in the meta-analysis (figure 3.3.3.1). This represented 561 participants. The meta-analysis suggested a small but statistically significant reduction in total cholesterol with high polyphenol chocolate or cocoa (-0.14 mmol/l; 95% Confidence Intervals -0.23, -0.05, I^2 =89%; p=0.003).

3.3.3.1.1 - Sub-analysis of chocolate supplementation

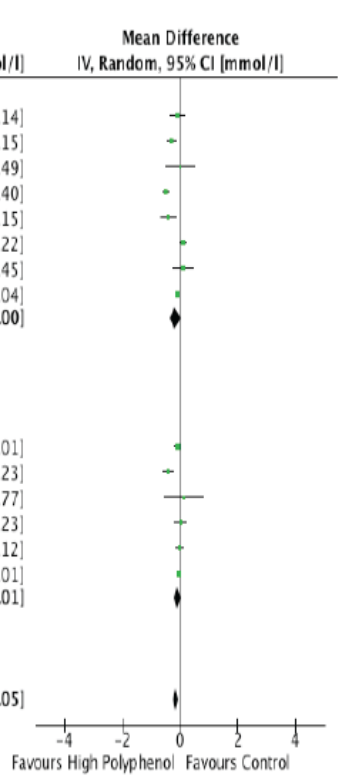
Data from eight published trials representing 261 participants included in the meta-analysis suggested no significant improvement in total cholesterol with high polyphenol chocolate (-0.17mmol/l; 95% Confidence Intervals -0.34, 0.00, I^2 =92%; p=0.06). The meta-analysis of studies utilising cocoa as the vector, also suggested no significant improvement in total cholesterol (-0.09 mmol/l; 95% Confidence Intervals -0.19, 0.01, I^2 =72%; p=0.07).

Analysis of the sub-groups within the meta-analysis suggested there were no significant difference between studies utilising chocolate and cocoa (p=0.43; Chi^2 =0.62, I^2 =0.0%).

3.3.3.1.2 - Potential small study and publication bias for total cholesterol

The funnel plot in figure 3.3.3.1 and the measure of heterogeneity, suggest that the data are very heterogeneous and there are a number of studies suggestive of small study bias by the nature of their standard error of mean differences. The funnel plot highlights two studies, which included chocolate with very small standard error or mean difference, otherwise the plot appears as would be expected.

Study or Subgroup	High Polyphenol Mean [mmol/l]	SD [mmol/l]	Total	Control Mean [mmol/l]	SD [mmol/l]	Total	Weight	Mean Difference IV, Random, 95% CI [mmol/l]
6.1.1 Chocolate								
Engler 2004	0.1	0.28	11	0.2	0.28	10	6.1%	-0.10 [-0.34, 0.14]
Farouque 2006	0.06	0.27	20	0.36	0.21	20	8.2%	-0.30 [-0.45, -0.15]
Flammer 2011	0.1	0.49	10	0.1	0.63	10	2.6%	0.00 [-0.49, 0.49]
Fraga 2005	-0.47	0.19	28	0.03	0.18	28	9.4%	-0.50 [-0.60, -0.40]
Grassi 2005	-0.4	0.29	10	0	0.27	10	6.0%	-0.40 [-0.65, -0.15]
Grassi 2005b	0.1	0.19	15	0	0.15	15	8.8%	0.10 [-0.02, 0.22]
Mursu 2004	0.12	0.47	15	0.02	0.51	15	4.2%	0.10 [-0.25, 0.45]
Taubert 2007	-0.06	0.08	22	0.03	0.08	22	10.2%	-0.09 [-0.14, -0.04]
Subtotal (95% CI)			131			130	55.5%	**-0.17 [-0.34, 0.00]**
Heterogeneity: $Tau^2 = 0.05$; $Chi^2 = 82.55$, df = 7 ($P < 0.00001$); $I^2 = 92\%$								
Test for overall effect: Z = 1.91 (P = 0.06)								
6.1.2 Cocoa								
Baba 2007	-0.13	0.18	37	-0.05	0.2	31	9.5%	-0.08 [-0.17, 0.01]
Balzer 2008	-0.16	0.26	21	0.25	0.31	20	7.6%	-0.41 [-0.59, -0.23]
Davison 2008	-0.16	0.76	12	-0.28	0.83	11	1.7%	0.12 [-0.53, 0.77]
Muniyappa 2008	-0.31	0.33	20	-0.34	0.33	20	6.9%	0.03 [-0.17, 0.23]
Njike 2009	0.13	0.21	44	0.15	0.41	44	8.5%	-0.02 [-0.16, 0.12]
Tzounis 2011	-0.43	0.06	20	-0.39	0.05	20	10.3%	-0.04 [-0.07, -0.01]
Subtotal (95% CI)			154			146	44.5%	**-0.09 [-0.19, 0.01]**
Heterogeneity: $Tau^2 = 0.01$; $Chi^2 = 17.90$, df = 5 ($P = 0.003$); $I^2 = 72\%$								
Test for overall effect: Z = 1.79 (P = 0.07)								
Total (95% CI)			285			276	100.0%	**-0.14 [-0.23, -0.05]**
Heterogeneity: $Tau^2 = 0.02$; $Chi^2 = 116.59$, df = 13 ($P < 0.00001$); $I^2 = 89\%$								
Test for overall effect: Z = 2.92 (P = 0.003)								
Test for subgroup differences: $Chi^2 = 0.61$, df = 1 (P = 0.43), $I^2 = 0\%$								



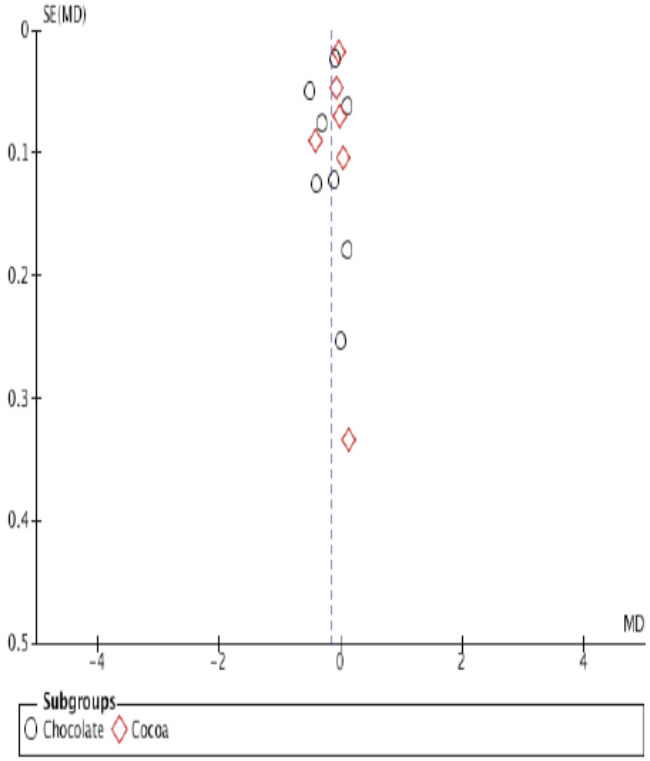


Figure 3.3.3.1: Meta-analysis results with forest plots for total cholesterol from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa.

3.3.3.2- HDL cholesterol

Data from fourteen trial data sets were included in the meta-analysis (figure 3.3.3.2). This represented 561 participants. The meta-analysis suggested the improvement in HDL cholesterol with high polyphenol chocolate or cocoa was not significant (0.04 mmol/l; 95% Confidence Intervals -0.00, 0.08, I^2 =92%; p=0.09).

3.3.3.2.1 - Sub-analysis of chocolate supplementation

Data from eight published trials representing 261 participants included in the meta-analysis suggested no significant improvement in HDL cholesterol with high polyphenol chocolate (0.05mmol/l; 95% Confidence Intervals -0.02, 0.12, I^2 =91%; p=0.17). The meta-analysis of studies utilising cocoa as the vector, also suggested no effect on HDL cholesterol (0.03 mmol/l; 95% Confidence Intervals -0.03, 0.09, I^2 =92%; p=0.29). Analysis of the sub-groups within the meta-analysis suggested there were no significant differences between studies utilising chocolate and cocoa (p=0.66; Chi^2 =0.19, I^2 =0.0%).

3.3.3.2.2 - Potential small study and publication bias for HDL cholesterol

The funnel plot in figure 3.3.3.2 and the measure of heterogeneity, suggest that the data are very heterogeneous and there are a number of studies suggestive of small study bias by the nature of their standard error of mean differences.

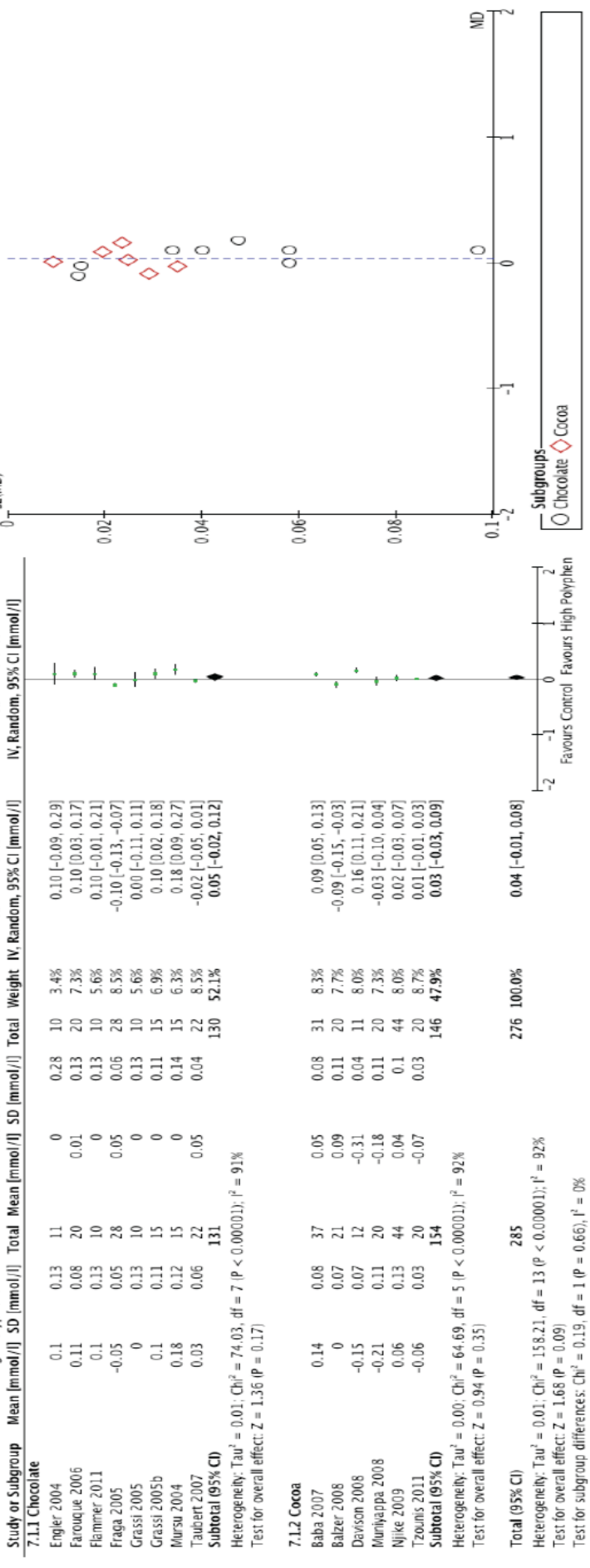


Figure 3.3.2: Meta-analysis results with forest plots for HDL cholesterol from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa.

3.3.3.3 - LDL cholesterol

Data from fourteen trial data sets were included in the meta-analysis (figure 3.3.3.3). This represented 561 participants. The meta-analysis suggested a small significant reduction in LDL cholesterol with high polyphenol chocolate or cocoa (-0.17 mmol/l; 95% Confidence Intervals -0.31, -0.02, I^2 =98%; p=0.03).

3.3.3.3.1 - Sub-analysis of chocolate supplementation

Data from eight published trials representing 261 participants included in the meta-analysis suggested no significant improvements in LDL cholesterol with high polyphenol chocolate (0.01mmol/l; 95% Confidence Intervals -0.18, -0.20, I^2 =95%; p=0.91). The meta-analysis of studies utilising cocoa as the vector, suggested a significant reduction in LDL cholesterol (-0.39 mmol/l; 99% Confidence Intervals –0.67, -0.12, I^2 =99%; p=0.006). The degree of heterogeneity could be a reflection of the different study populations and durations of study and that LDL cholesterol is calculated and would be influenced by variation in triglyceride levels.

Analysis of the sub-groups within the meta-analysis suggested that cocoa rich in polyphenols were more effective at lowering LDL cholesterol than chocolate rich in polyphenols (p=0.02; Chi^2 =5.50, I^2 =91.8%).

3.3.3.3.2 - Potential small study and publication bias for LDL cholesterol

The funnel plot in figure 3.3.3.3 and the measure of heterogeneity, suggest that the data are very heterogeneous and there are a number of studies suggestive of small study bias by the nature of their standard error of mean difference.

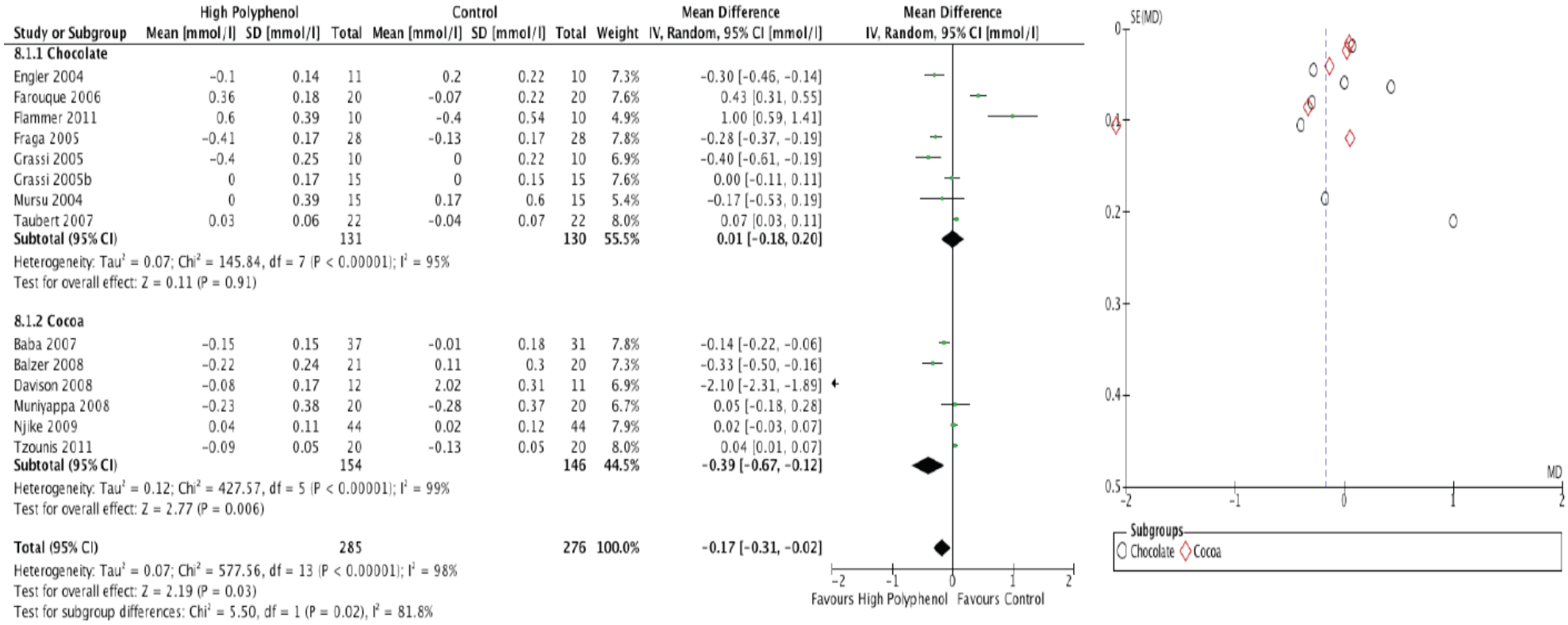


Figure 3.3.3.3: Meta-analysis results with forest plots for LDL cholesterol from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa.

3.3.3.4 - Triglycerides

Data from fourteen trial data sets were included in the meta-analysis (figure 3.3.3.4). This represented 561 participants. The meta-analysis suggested no significant improvement in triglycerides with high polyphenol chocolate or cocoa (-0.07 mmol/l; 95% Confidence Intervals -0.14, -.000, I^2 =89%; p=0.06).

3.3.3.4.1 - Sub-analysis of chocolate supplementation

Data from eight published trials representing 261 participants included in the meta-analysis suggested no significant improvements in triglycerides with high polyphenol chocolate (-0.05mmol/l; 95% Confidence Intervals -0.15, 0.06, I^2 =86%; p=0.39). The meta-analysis of studies utilising cocoa did suggest a significant reduction in serum triglycerides (-0.11 mmol/l; 95% Confidence Intervals -0.19, -0.02, I^2 =80%; p=0.01)

However, analysis of the sub-groups within the meta-analysis suggested there were no significant differences between studies utilising chocolate and cocoa (p=0.41; Chi^2 =0.69, I^2 =0.0%).

3.3.3.4.2 - Potential small study and publication bias for triglycerides

The funnel plot in figure 3.3.3.4 and the measure of heterogeneity, suggest that the data are very heterogeneous and there are a number of studies suggestive of small study bias by the nature of their standard error of mean difference.

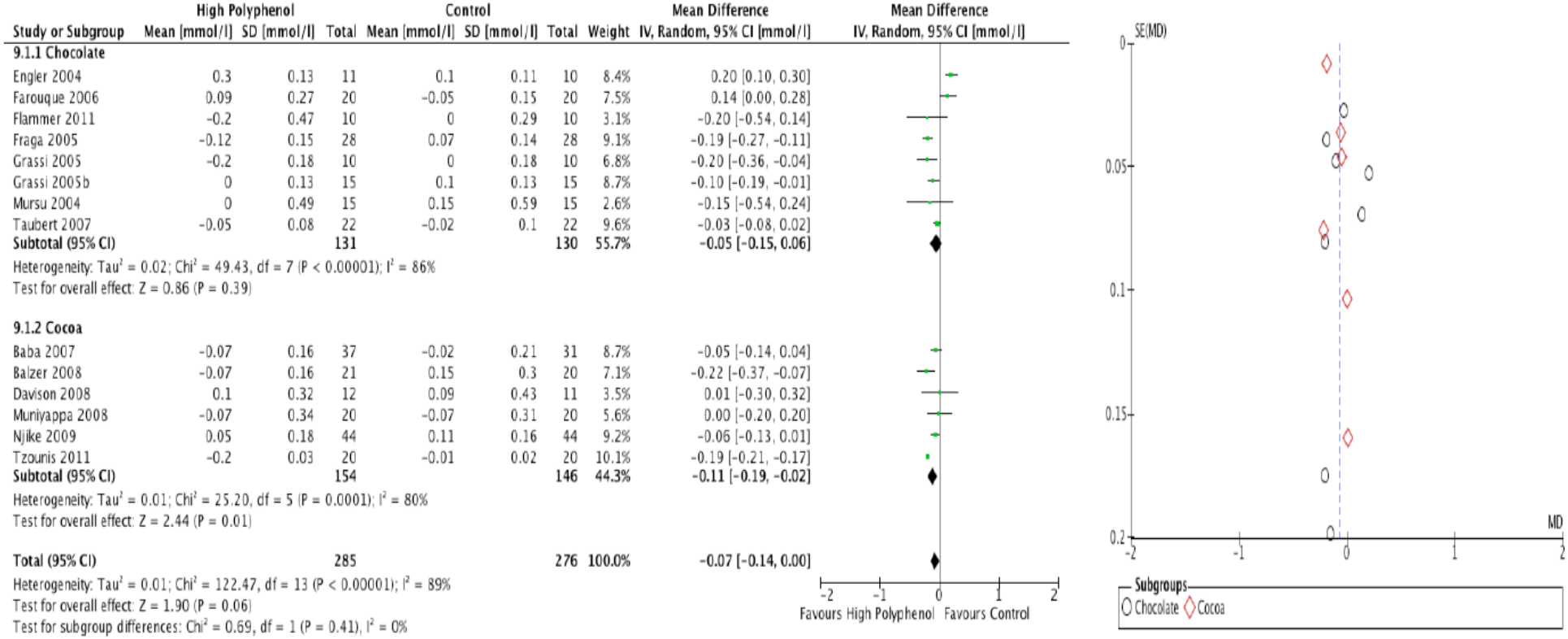


Figure 3.3.3.4: Meta-analysis results with forest plots for triglycerides from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa.

3.3.4 - Effect of Cocoa and Chocolate on Insulin Resistance and Fasting Glucose

3.3.4.1 - HOMA

A number of trials reported data linked to insulin metabolism, however in the case of two studies this was not presented numerically nor was not in a format that was readily accessible. This resulted in only three data sets (from two publications) being included. The meta-analysis (figure 3.3.4.1) represents 107 participants' data. This was not split into subgroups for cocoa or chocolate. No significant improvement in HOMA was seen (-0.55 mmol/l; 95% Confidence Intervals -1.15, 0.04, I^2 =93%; p=0.7). The funnel plot, suggested that the data might not be representative, due to its asymmetry.

3.3.4.2 - Fasting glucose

Data from ten trial data sets (extracted from nine publications) were included in the meta-analysis (figure 3.3.4.2). This represented 420 participants. The meta-analysis suggested no significant effect on fasting glucose with high polyphenol chocolate or cocoa (-0.06 mmol/l; 95% Confidence Intervals -0.22, 0.09, I^2 =92%; p=0.41).

3.3.4.2.1 - Sub-analysis of chocolate supplementation

Data from four published trials representing 188 participants included in the meta-analysis suggested no significant improvement in fasting glucose with high polyphenol chocolate (-0.28mmol/l; 95% Confidence Intervals -0.50, -0.07, I^2 =79%; p=0.01). The meta-analysis of studies utilising cocoa as the vector, also suggested, slightly negative effect on fasting glucose (0.11 mmol/l; 95% Confidence Intervals 0.03, 0.20, I^2 =33%; p=0.008). Analysis of the sub-groups within the meta-analysis suggested there was a significant differences between studies utilising chocolate and cocoa, (p=0.0008; Chi^2 =11.20, I^2 =91.1%). The funnel plot suggests there was little in the way of small study bias. The data of Allgrove et al. (2011) although had glucose as an outcome was not included as not enough data was available. Variation in variance of mean difference appears to be linked to a couple of studies with very small inherent standard deviation (Taubert et al., 2007; Davison et al., 2008).

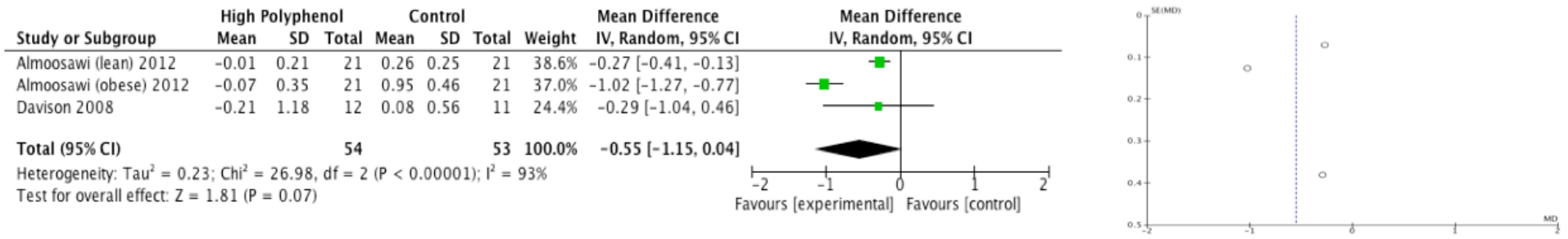


Figure 3.3.4.1: Meta-analysis results with forest plots for HOMA from clinical trials using interventions of chocolate or cocoa. Please note for Almoosawi (lean) refers to BMI<25kgm^{-2} and Almoosawi (obese) refers to the subset with a BMI≥25kgm^{-2} (overweight and obese).

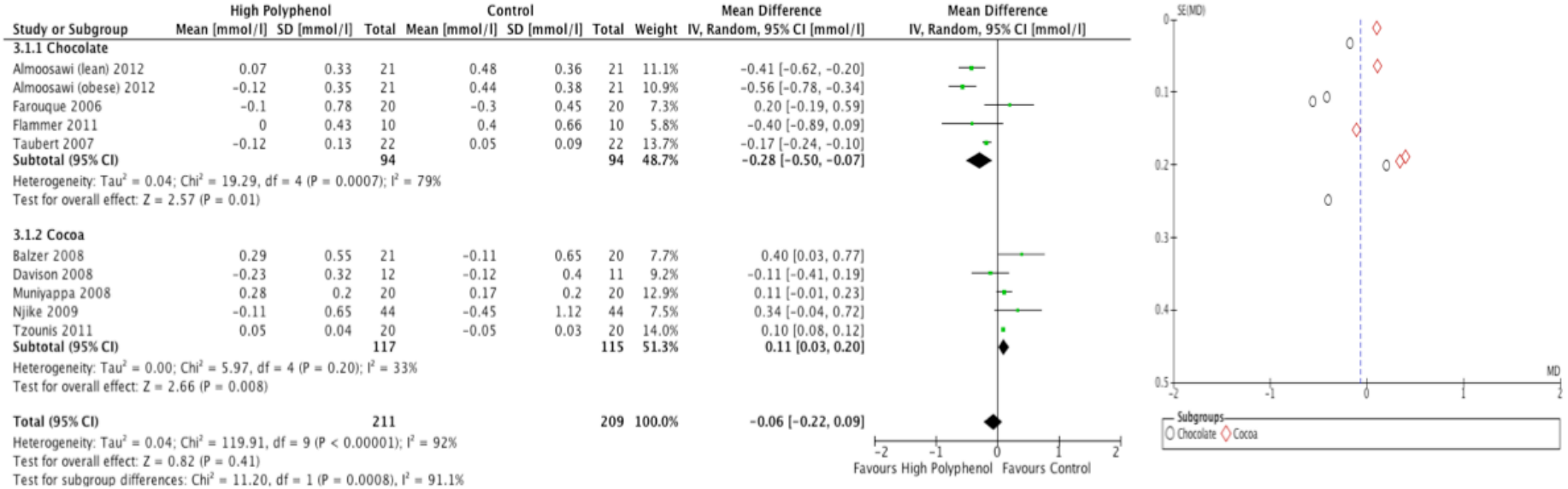


Figure 3.3.4.2: Meta-analysis results with forest plots for fasting glucose from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa. Please note for Almoosawi (lean) refers to BMI<25kgm^{-2} and Almoosawi (obese) refers to the subset with a BMI≥25kgm^{-2} (overweight and obese).

3.3.5 - Effect of Cocoa and Chocolate on Endothelial Function

Data from twelve trial data sets (extracted from ten published studies) were included in the meta-analysis (figure 3.3.5.1). Both acute and post-supplementation changes were included. This represented 554 participants. The meta-analysis suggested a significant improvement in endothelial function measured by change in flow-mediated dilation with high polyphenol chocolate or cocoa (1.62%; 95% Confidence Intervals 0.88, 2.36, I^2 =92%; p<0.0001).

3.3.5.1 - Sub-analysis of chocolate supplementation

Data from four published trials (extracted from five data sets) representing 211 participants included in the meta-analysis suggested a significant improvement in endothelial function with high polyphenol chocolate (1.74%; 95% Confidence Intervals 0.51, 2.96, I^2 =72%; p=0.005). The meta-analysis of studies utilising cocoa as the vector, also suggested a significant improvement on endothelial function (1.56%; 95% Confidence Intervals 0.62, 2.50, I^2 =94%; p<0.0001). Analysis of the sub-groups within the meta-analysis suggested there were no significant differences between studies utilising chocolate and cocoa (p=0.99; Chi^2 =0.00, I^2 =0.0%).

3.3.5.2 - Potential small study and publication bias for endothelial function

The funnel plot in fig 3.3.5.1 and the measure of heterogeneity, suggest that the data are very heterogeneous and there are a number of studies suggestive of small study bias by the nature of their standard error of mean differences. Although the paper of Westphal and Luley (2011) was identified for inclusion in the meta-analysis, the time frame and the inclusion of a meal lead to the decision to exclude its data from the final analysis. There is a degree of variability in the standard deviation of change of the studies presented here, this could reflect differing populations and methodologies, which could have led to greater variation.

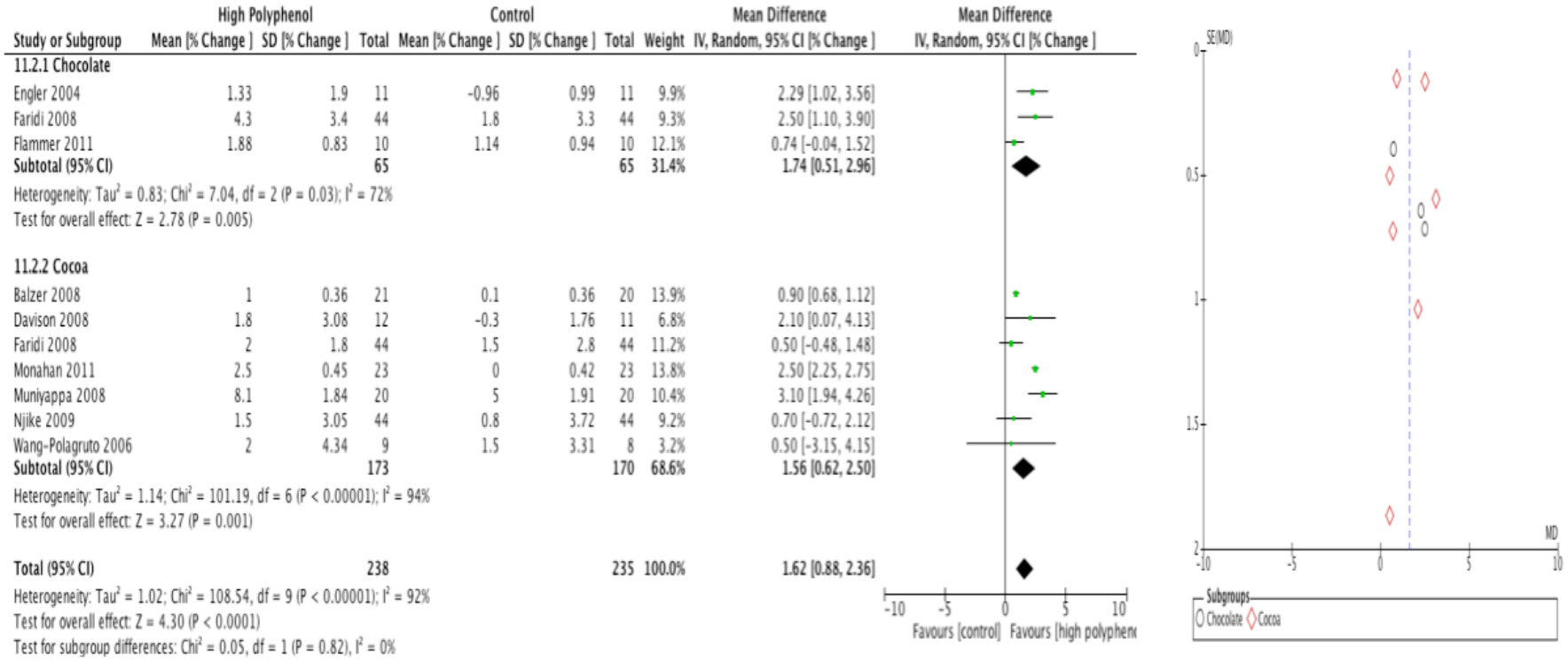


Figure 3.3.5.1: Meta-analysis results with forest plots for endothelial function (both acutely two hours post feeding and fasting following longer term daily cocoa or chocolate consumption), from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa.

3.3.6 - Effect of Cocoa and Chocolate on Oxidative Stress

Data from eight published studies were included in the meta-analysis (figure 3.3.6.1). Both acute and post-supplementation changes were included. This represented 213 participants. The meta-analysis suggested no significant effects upon oxidative stress as assessed by changes in urinary or serum isoprostane markers with high polyphenol chocolate or cocoa supplementation (0.95pg/ml 95% Confidence Intervals -12.98, 14.88, I^2 =88%; p=0.89).

3.3.6.1 - Sub-analysis of chocolate supplementation

Data from five published trials representing 137 participants included in the meta-analysis suggested non-significant improvement in oxidative stress with high polyphenol chocolate (-26.08pg/ml; 95% Confidence Intervals -55.74, 3.58, I^2 =94%; p=0.08). The meta-analysis of studies utilising cocoa as the vector, however suggested a non-significant increase in oxidative stress (51.70pg/ml; 95% Confidence Intervals -23.05, 126.45, I^2 =95%; p=0.18). It is important to note that this was based on only three studies and data from 76 participants. Analysis of the sub-groups within the meta-analysis suggested the difference between studies utilising chocolate and cocoa did not reach significance (p=0.04; Chi^2 =4.38, I^2 =77.1%) possibly a reflection of the heterogeneity of the data resulting from differing methodologies used in the studies.

3.3.6.2 - Potential small study and publication bias for oxidative stress

The funnel plot in figure 3.3.6.1 suggested there was an even distribution. There is a degree of variability in the standard deviation of change of the studies presented here, this could reflect differing populations and methodologies, which could have led to greater variation. Although oxidative stress was assessed in Allgrove et al. (2011), it was only presented as a figure with no numerical data, and was excluded

3.3.7 - Effect of Cocoa and Chocolate on Inflammatory Markers

Only four studies that met the inclusion criteria, reported data on inflammatory markers, these in turn reported three different markers. Therefore it was not possible to include these in the meta-analysis.

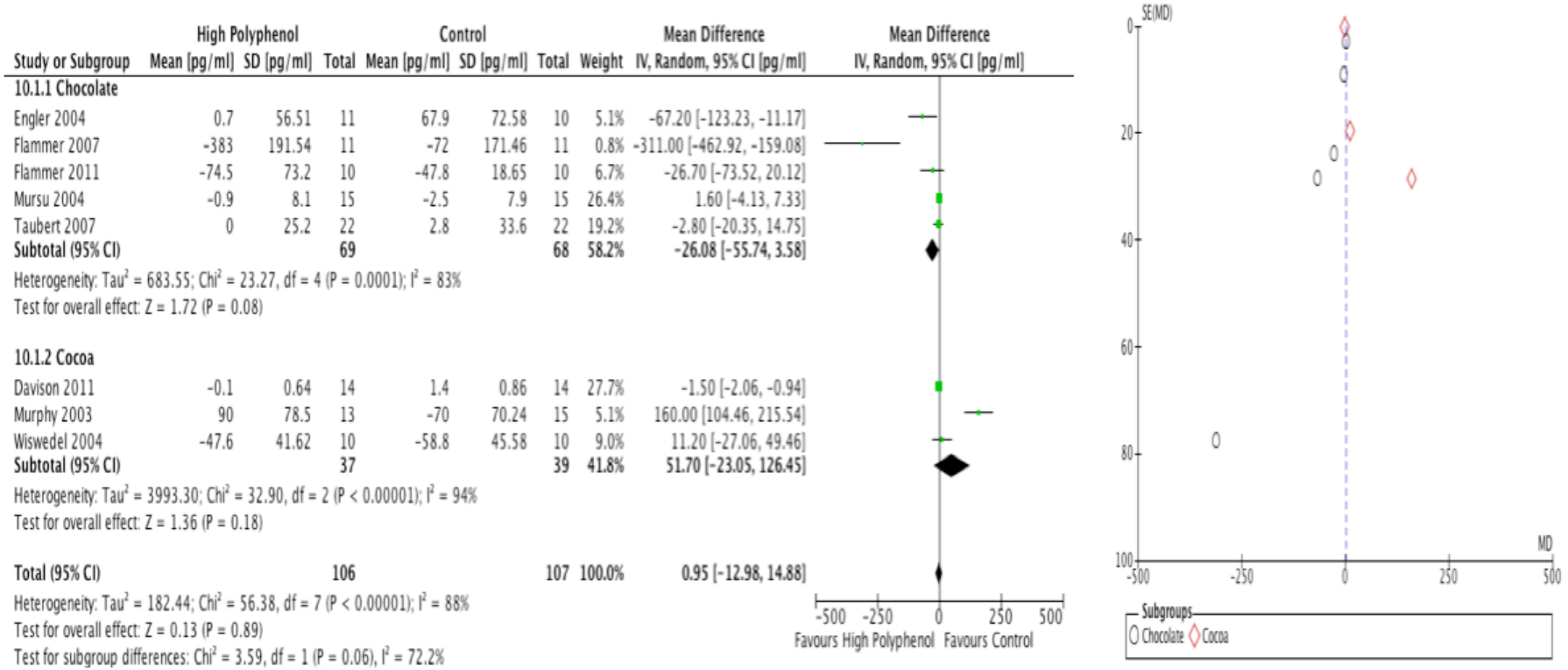


Figure 3.3.6.1: Meta-analysis results with forest plots for oxidative stress (measured using urinary or serum isoprostane markers) from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa.

3.3.7 - Effect of Cocoa and Chocolate on Weight Change

Data from eight data sets (extracted from seven published studies) were included in the meta-analysis (figure 3.3.7.1). To meet the inclusion for the meta-analysis the intervention needed to be at least 14 days long. This represented 328 participants. The meta-analysis suggested no significant effect upon weight following supplementation with high polyphenol chocolate or cocoa (-1.15kg 95% Confidence Intervals -2.38, 0.08, I^2 =86%; p=0.07). There was the suggestion of a trend to weight loss, however some studies reported weight gain in the control arms. This was notable in the cocoa supplementation trials more than in the chocolate studies.

3.3.7.1 - Sub-analysis of chocolate supplementation

Data from three published trials representing 148 participants included in the meta-analysis suggested no significant effect on weight with high polyphenol chocolate feeding as part of controlled trials (-0.09kg; 95% Confidence Intervals -0.62, 0.45, I^2 =0.0%; p=0.74). The meta-analysis of studies utilising cocoa as the vector, appeared to have no significant effect upon weight (-2.07kg; 95% Confidence Intervals -5.57, 1.43, I^2 =93%; p=0.74). Analysis of the sub-groups within the meta-analysis suggested there were no significant differences between studies utilising chocolate and cocoa (p=0.27; Chi^2 =1.19, I^2 =16.2%).

3.3.7.2 – Potential small study and publication bias for weight change

In the funnel plot in figure 3.3.7.1 suggested there was a larger than expected change in weight, but this was largely due to the study of Davison et al. (2008), which might have led to a small study bias effect. This also accounts for the wide 95% confidence interval for the cocoa subgroup. There is a degree of variability in the standard deviation of change of the studies presented here, this could reflect difference in the degree of dietary restrictions within studies and natural variance in body weight of the participants.

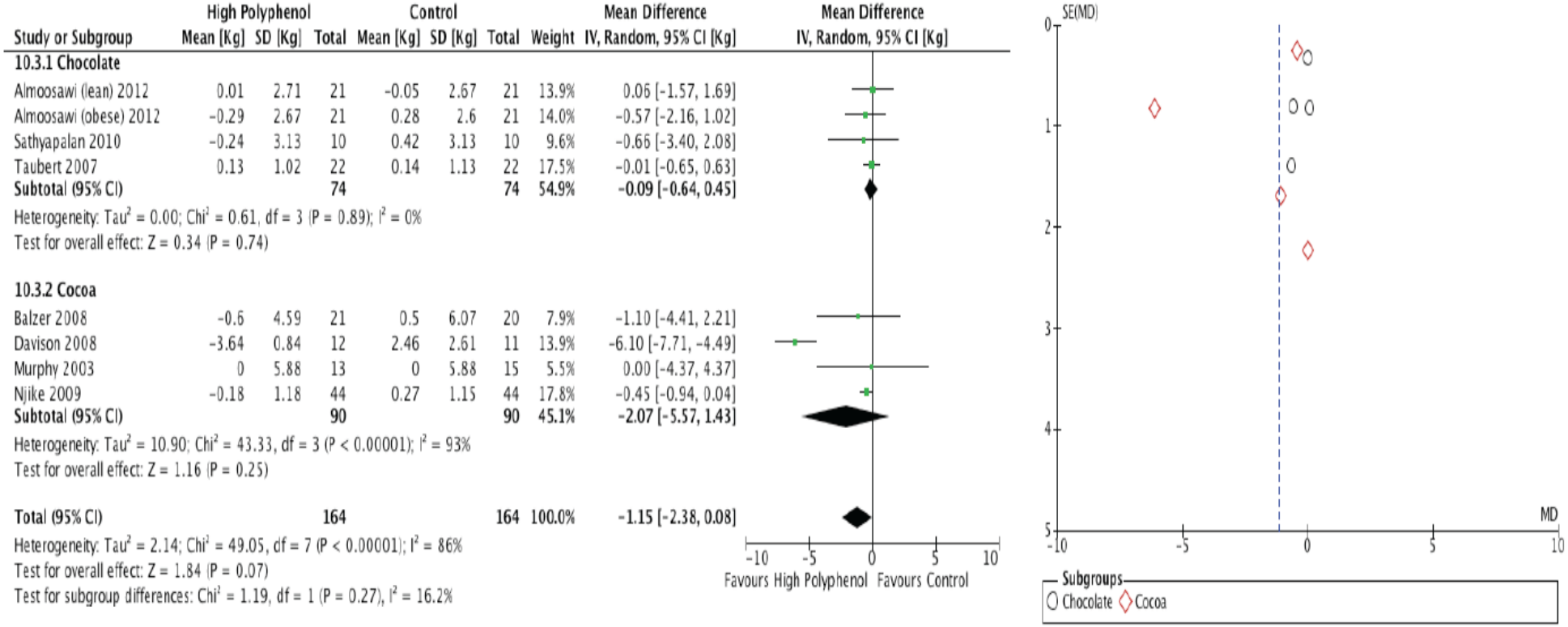


Figure 3.3.7.1: Meta-analysis results with forest plots for weight from clinical trials using interventions of chocolate or cocoa. Meta-analysis includes sub-group analysis comparing interventions utilizing chocolate with those using cocoa. Please note for Almoosawi (lean) refers to BMI<25kgm^{-2} and Almoosawi (obese) refers to the subset with a BMI≥25kgm^{-2} (overweight and obese).

3.4 Discussion

This exploratory review and meta-analysis identified 31 randomised controlled trials (representing the data from 1285 participants) where cocoa or chocolate supplementation has been used in an attempt to demonstrate a health benefit. All had a well-designed protocol and clear explanation of the intervention and control. Until the end of June 2012, eleven systematic reviews had been published which supported the potential beneficial effects of cocoa and chocolate polyphenols on a ranged of health outcomes. These range from the meta-analysis of blood pressure data from randomised controlled trials, through to cardiovascular disease reduction, from systematic reviews of epidemiological studies (reviewed in Chapter One). This review was limited by the lack of the use of indexing of the terms chocolate and flavanols, along with the lack of being limited to randomised controlled trials. In part the potential bias this may have induced would have been limited by the hand search and use of clinical trial registers to attempt to insure all relevant literature was collated. This together with the limitations of the method of analysis which relied on estimates of variance linked to assumptions about the distribution of the data and did not account for the data being paired and any correlations between the baseline and end of study data sets, leading to increased heterogeneity and larger confidence intervals for a number of studies. These factors need to be considered when interpreting this exploratory review, and more robust methodology incorporate, should this study be undertaken for consideration for publication.

This is an initial exploratory review and meta-analysis to consider the potential difference in effect of chocolate as a vector for delivering the cocoa polyphenols (epicatechins and procyanadins) compared to cocoa. The rationale for a difference in effect is, based on the practicalities of feeding cocoa, its chemo-physical properties and a number of biological and psychological factors linked to its consumption. The data supports the case that chocolate has greater efficacy than cocoa for two of the parameters associated with cardiovascular risk. However, for LDL cholesterol, cocoa appeared to have significantly greater efficacy. Perhaps

this is a reflection of the saturated fat content of chocolate, perhaps acting via increasing triglyceride levels as this marker in the cases of these studies has been estimated using the Friedewald equation (Friedewald, Levy, & Fredrickson, 1972). For none of the six parameters (blood pressure, lipid profile (excluding LDL cholesterol), insulin resistance and glycaemia, endothelial function, oxidative stress and inflammation) did any data support the greater efficacy of cocoa, whereas for chocolate it demonstrated clear statistical superiority for its effects on lowering blood pressure (both systolic and diastolic) and fasting glucose (with the data for cocoa showing a statistically significant increase).

The results for the subgroup analysis for oxidative stress did not reach significance ($p=0.06$), this could be linked to the heterogeneity of the data presented in the literature, or a limitation of the analysis which led to variability in the variance of the mean difference. This warrants further study both in thorough systematic review and more robust meta-analysis and clinical trials. As a potential mechanism for the effect of increased insulin and carbohydrate to increased oxidative stress has been well described in the hypothesis of (Brownlee, 2001; 2005). Alternatively, Mursu et al. (2004) suggested that cocoa butter might have anti-inflammatory or antioxidant capacity based on the reduction of LDL cholesterol oxidation from low polyphenol chocolate. Although it is clear that more work needs to be undertaken, which falls outside of the scope of this thesis.

The studies were mostly of a crossover design ($n=19$), compared to 12 parallel design studies. However, the study design did not appear to have a significant effect on the outcomes suggesting that both study designs are valid in conducting of clinical trials of this nature. As discussed in Chapter Two, there are advantages and disadvantages in both approaches. It is likely the effect of treating data as independent in estimating the variance of the mean difference has added to the variability seen in the forest plots and the high level of heterogeneity. This should be considered if this work were to be repeated as part of a systematic review with meta-analysis.

Although only one study was found which investigated the effects of cocoa polyphenols alone, it was in a study population entirely made up from participants with T2DM. Curtis et al. (2012) investigated the effects of cocoa polyphenols in combination with soy isoflavones in postmenopausal women with T2DM. However, this study was discounted, as it was not possible to extract the effect of the cocoa polyphenols from the effects of the soy isoflavones. The work of Balzer et al. (2008), which, until my studies reported later in this thesis, was the only one to have considered the effects of cocoa flavanols only in diabetes. The additional benefit of this meta-analysis was to try to identify potential effects of both the intervention and control arms. It was clear from the mean change data, that there was no indication of harm from either arm in the studies. This was a potential limitation of this type of work, as the forest plot reports the mean and 95% confidence interval of the difference between the intervention and control arms and it is possible that if the control arm produces a negative effect, a modest negative effect of the intervention will show as a positive mean difference effect.

This exploratory review and meta-analysis was limited by the nature of the studies included, a critical limitation being that interventions varied widely, with studies using from 5 to over 100g of chocolate per day. The nature and presentation of the polyphenols also varied widely; some studies used commercially available products, whereas others used specially formulated products. This has often been a criticism made by EFSA (2012b) of the data used to support health claims, in that the dose of the active component is not defined and the studies presented have a wide degree of heterogeneity. This was an artefact of the way the research is being conducted with a number of small groups working with manufacturers testing different formulations in small cohorts. To investigate the potential benefits of cocoa polyphenols on health more fully, ultimately larger studies will need to be undertaken, using the minimum of a standard dose of epicatechin and polyphenols, if it is not possible to use a standard chocolate formulation. Such investigations are unlikely to be carried out due to funding restrictions and commercial interests. The challenge of limited funding is reflected in

this thesis where lack of funding meant that different chocolate formulations had to be used in each of the three clinical trials.

It should be noted that the potential additional benefits of chocolate rich in polyphenols might be exaggerated due to the confounding introduced by the poor matching of control to intervention. With eight studies using white chocolate as the control to either dark or milk chocolate as the intervention. This introduces a high risk of bias, and the details are not available to assess the effect this might have on participant perception of benefit/ risk. It may depend on how the study was presented and the participant information provided on how this may influence the study outcome. For example a study presented as 'does dark chocolate lower blood pressure' may induce a response in favour of the dark chocolate above what might be expected. Therefore the potential additional benefits of chocolate rich in polyphenols compared to cocoa need to be further explored as part of well designed studies with carefully selected control chocolates.

Further limitations of the exploratory review and meta-analysis could be due to the widely different baseline characteristics of the participants, which varied from healthy volunteers to those with diabetes, cardiovascular disease and even heart failure and postoperative heart transplants patients. This might have influenced the data on hypertension in particular.

The final and perhaps key limitation of this exploratory review is that was is based entirely upon the numerically published data, due to time and resource limitations of this piece of work (one author and as a part component of a PhD thesis), it was not possible to contact study authors for clarification of data. Should this work to be repeated, a clearer search strategy would be employed using at least two researchers (one experienced in conducting systematic reviews), to insure that it was correctly index to reduce the risk of missing articles. The search strategy would also be adjusted so that it was more clearly limited to randomised controlled trials (which would need to be indexed to controlled trials and clinical trials) to insure a more precise search.

The use of more than one researcher would assist in identifying potential errors, e.g. where data is presented as mean± standard error of mean rather than standard deviation. This would potentially allow for the contacting of authors to reduce the reliance on calculating estimates of the variance of change and potentially use papers, which were excluded from this study due to the lack of numerical data being presented. Alternatively, instead of variance law 1 for the estimate of the variance of the change, variance law 2 could be used with the necessary sensitivity analyses where assumed correlation coefficients are deployed. This should reduce the variability in the estimate of variance for the mean differences, as it will account for the correlation and paired nature of the data, which this review did not.

3.4.1 - Summary

This exploratory review and meta-analysis suggests (subject to further work and analysis) that:

1. Chocolate consumption may provide greater efficacy when compared to cocoa with respect to markers of cardiovascular risk and diabetes control. This is clear for the greater beneficial effects seen with chocolate with respect to cocoa, for blood pressure, fasting glucose and oxidative stress, although cocoa may be improve LDL cholesterol. This may be in part be reflecting the effect of poor matching of control chocolates to the intervention product.
2. Chocolate preparations tend to have a low percentage of energy as carbohydrate, despite a higher mean energy content. This might make chocolate more suitable for people with T2DM, as the excess carbohydrate may adversely influence the metabolic disturbances and glycaemia associated with this condition.
3. There is no evidence from either the controls or interventions of negative effects on the parameters associated with cardiovascular risk or diabetes control.

This exploratory review and meta-analysis justifies the selection of chocolate as a vector for cocoa polyphenol delivery to investigate their potential health effects in individuals with T2DM as it may have mechanistic advantages supported by some of the findings of the sub-group analyses. However, it needs to be appreciated the high risk of bias which resulted from inadequate blinding in the studies and weaknesses in this review means that these findings need to be treated with caution.

Chapter Four: Proof of Concept Study: Investigating the Effects of High Cocoa Polyphenol Rich Chocolate on Cardiovascular Risk in Individuals with Type 2 Diabetes Mellitus

Aims and scope of chapter:

- To undertake a proof of concept study to investigate the safety of prolonged feeding of chocolate to individuals with type 2 diabetes mellitus (T2DM).
- To access the potential health benefits of consuming high polyphenol chocolate for individuals with T2DM.

Table 4.0, defines the study question and PICOS approach to achieving the above aims of this chapter.

Table 4.0: Study questions and PICOS for the proof for study one.

Study Question(s)	**Can chocolate rich in polyphenols reduce cardiovascular risk in individuals with type 2 diabetes mellitus?** **Can chocolate rich in polyphenols improve lipid profile; primarily increase HDL cholesterol in individuals with type 2 diabetes mellitus?**
Participants	**Twelve individuals with type 2 diabetes mellitus controlled by lifestyle alone, or in combination with oral hypoglycaemic medication.**
Interventions	**Chocolate rich in polyphenols (783mg total polyphenols/ 55mg epicatechins)**
Comparisons	**Chocolate low in polyphenols (176mg total polyphenols/ <2mg epicatechins)**
Outcomes	**Primary outcomes:** **1. HDL cholesterol** **2. Cholesterol:HDL cholesterol ratio** **Secondary outcomes:** **1. Change in weight** **2. Glycaemia (glucose or HbA1c)** **3. Blood pressure, insulin resistance and inflammation.**
Study design	**A double blind randomised controlled trial, with two - eight week intervention periods separated by a four-week washout period.**

To address these aims the clinical study will be presented in three parts:

4.1 - Background and study-specific methodology.

4.2 - Results, safety, efficacy and participant-reported outcomes.

4.3 - Discussion and conclusion.

4.1 – Background and Study Specific Methodology

Type 2 diabetes mellitus is associated with excess cardiovascular risk at least some of which can be moderated by lifestyle factors, including the diet. One of these risk factors, dyslipidaemia, is known to be a especially prevalent in individuals with T2DM (Haffner et al., 1998) and high intakes of refined carbohydrates such as sucrose are associated with worse glycaemic control, increased obesity and hypertriglyceridaemia (Laville & Nazare, 2009). Concern over the effect of sugar on glycaemia has been reduced by work on the glycaemic index (GI) (Brand-Miller, Hayne, Petocz, & Colagiuri, 2003). The concept of the GI has led to the realization that foods such as chocolate, although high in carbohydrate, and in particular, sugar/sucrose, may have a lesser effect on blood glucose than foods that are low in sugar, including potatoes and bread (Foster-Powell, Holt, & Brand-Miller, 2002).

It has been hypothesized that flavonoid compounds found in foods, including epicatechins found in high-cocoa-solid chocolates, decrease in mortality rate from coronary heart disease, cancer and stroke (Buijsse, Feskens, Kok, & Kromhout, 2006). Short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy participants (Grassi, et al., 2005a). Dark chocolate consumption has been reported to increase HDL cholesterol concentration and chocolate fatty acids may inhibit lipid peroxidation in healthy participants (Mursu et al., 2004).

The hypothesis was therefore that the daily consumption of chocolate (45 g daily) containing polyphenol-rich, high-cocoa solids improves cardiovascular risk factors when eaten chronically, whilst being safe for individuals with T2DM. Additionally, safety was assessed by monitoring weight changes and glycaemia along with incidence of adverse events.

4.1.1 - Research Design and Methods

The principle methodology was to assess the effect of consuming of 45g chocolate with a high cocoa solid content (and therefore rich in flavanols) daily versus a placebo chocolate for a total of five months. The protocol involved two months (eight weeks) of one chocolate, one-month (four weeks) washout prior to a further two months (eight weeks) consuming the alterative chocolate. This was judged to be a safe regime for individuals with T2DM and would hopefully be weight neutral and improve lipid profile and thus reduce the cardiovascular risk factors associated in this condition.

Cardiovascular risk factor reduction was assessed by:

Primary outcome;

- Increased HDL cholesterol, along with assessments of overall lipid profile improvement (HDL: Total cholesterol ratio). HDL cholesterol is known to be supressed in T2DM, and is not significantly improved even with medication (Krauss, 2004).

Secondary outcomes;

- Reduced insulin resistance, this is not proposed as a primary outcome, owing to the poly-pharmacy of the participant group (the inclusion criteria included all oral hypoglycaemia agents, which is in contrast to the studies reported in Chapters Five and Six).
- Reduced blood pressure, this was not deemed to be appropriate as a primary outcome, since many individuals with T2DM are often on antihypertensive therapies, with primary care incentivized to the use of ACE inhibitors (Information Centre, 2011).

Safety was assessed by:

- Weight remained neutral, since a common side effect of many therapeutic strategies in the management of diabetes (e.g. sulphonylureas and insulin) is weight gain (UKPDS Group, 1998a; Holman et al., 2008)
- Lack of an effect upon HbA1c, self monitored glycaemia and the continuous monitoring of interstitial glucose (using the Glucoday (A.Menarini, Wokingham, Berkshire, UK).

Participant Reported Outcomes were assessed by:

- Hospital Anxiety Depression Scale (HADS) (Bjelland, Dahl, Haug, & Neckelmann, 2002; Zigmond & Snaith, 1983)
- SF-36 (short form) (Ware, 2000)
- Pittsburgh Sleep Quality Index (PSQI) (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989)

The first two questionnaires were modified from a previous study investigating the effect of chocolate in chronic fatigue syndrome (Sathyapalan, Beckett, Rigby, Mellor, & Atkin, 2010). In designing the study, feedback was obtained feedback from a patient user group who questioned whether cocoa taken as a pre-bed time drink might improve sleep quality leading to the addition of the questionnaires.

4.1.2 – Participants

A total of 19 individuals with T2DM were screened for inclusion in the study. The conduct of the study can be seen in the CONSORT flowchart (figure 4.1.2.1). The diagnosis of T2DM was based on the WHO/IDF guidelines (2006). An exclusion criterion of Hba1c >9.0% (DCCT aligned) or >75mmol/mol was used. In addition, rescue criteria were employed, so that an increase in HbA1c of 1% or 11mmol/mol during the study or a weight gain of greater than 2kg between study visits would have led to the participant being withdrawn from the study for their own safety.

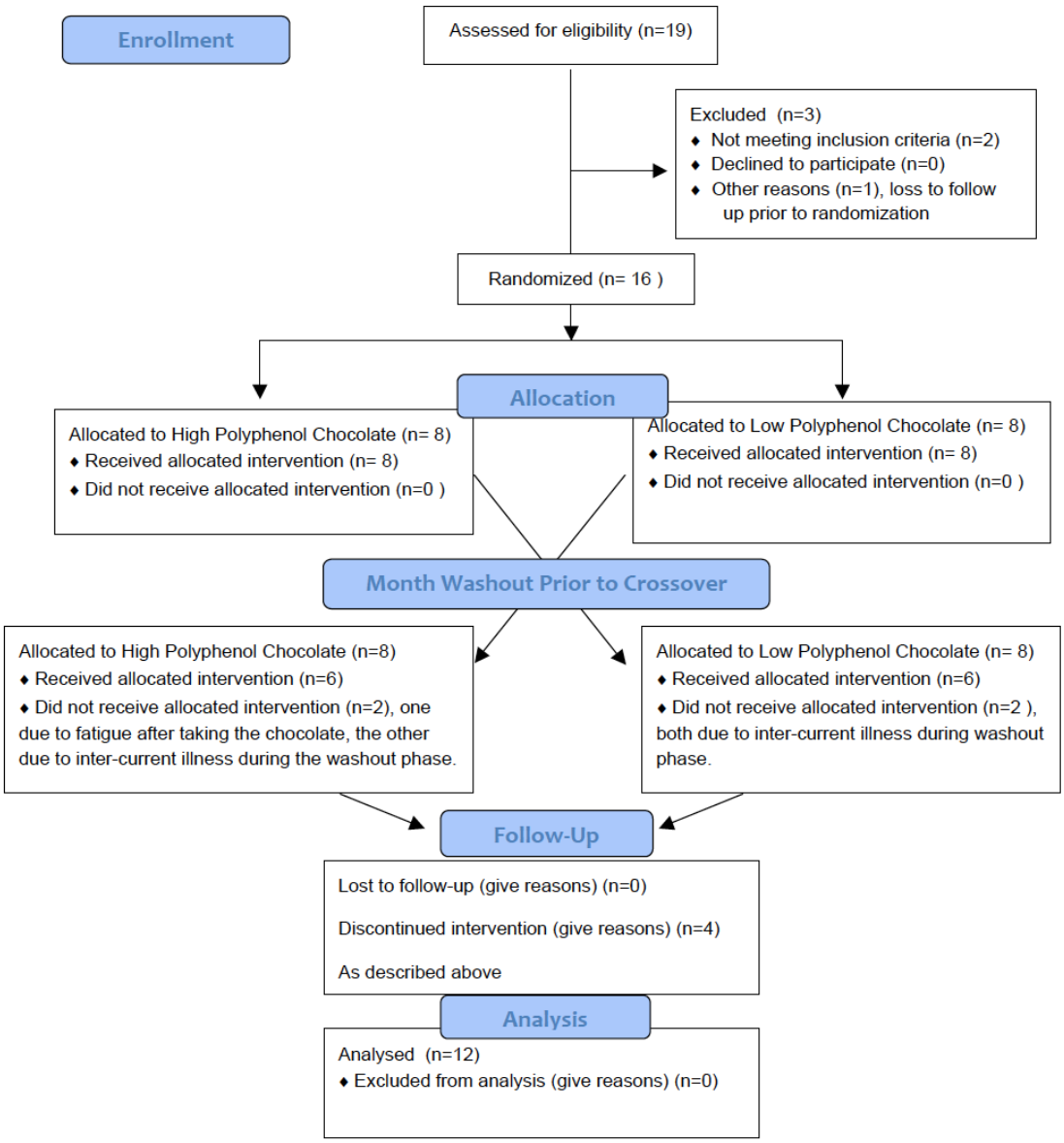


Figure 4.1.2.1: CONSORT flow diagram of the conduct of the trial, including the outcome for all participants.

Of the total of 19 participants recruited to the study; three were excluded; two because of concurrent medications that had been altered in the last three months, and one subject was excluded because they were lost to follow up following the initial study visit. Twelve participants completed the study, with four participants not completing. One withdrew because of reported loss of energy within a week of starting the low polyphenol chocolate arm of the study. The other three who did not complete the study were withdrawn due to inter-current illness associated with their pre-existing conditions

(bowel disease and two respiratory illness), all of which occurred during the washout phase between chocolate interventions.

4.1.3 - Study Design

A randomized, placebo-controlled crossover study methodology was employed. Following screening, five participants were assigned the 'placebo' chocolate and seven the 'active' flavanols rich chocolate for eight weeks, and advised to follow their same dietary patterns, with the exception of excluding all other chocolates and cocoa products. This was followed by a four-week washout period where no chocolate was dispensed and the participants were instructed not to consume any chocolate or cocoa products. Participants then returned for reassessment and were dispensed the alternate chocolate to the one their received in the first eight weeks.

A copy of the randomization code was kept secure by the hospital pharmacy and was available to the investigator only in case of an emergency. All participants gave written informed consent and the Hull and East Riding NHS Research Ethics Committee and Hull and East Yorkshire Hospitals NHS Research and Development Department approved the protocol. The chocolate was provided as an unrestricted gift from Nestlé PTC, York and the study was paid for out of charitable funds. The trial was registered (ISRCTN 25655161) and conducted in accordance with the Declaration of Helsinki (WMA, 2000).

The active product was flavanol (polyphenol) rich chocolate containing 85% cocoa solids (derived from a high cocoa liquor content) compared with a comparator control chocolate containing cocoa butter alone with no non-fat cocoa solids (cocoa liquor) (simulated iso-calorific chocolate). The control chocolate was low in polyphenols, but was dyed to the same colour as the high cocoa liquor/polyphenol rich chocolate (with a recipe specially prepared by Nestlé PTC York. UK). Details of the chocolate formulations can be found in Section 2.2.7 of Chapter Two, and tables 4.1.3.1 and

4.1.3.2. Individual 15g foil wrapped bars were provided and participants were asked to consume one bar three times daily. Participants were advised not to consume extra chocolate during the study period; apart from this, they were asked not to change their diet. To assess the ability of potential participants to be able to identify which chocolate is the 'active' and which is the control, a taste trial was conducted was described by Sathyapalan et al. (2010).

Nestlé PTC in York performed the analyses of the chocolates. The control chocolate, a simulated iso-calorific chocolate that was dyed to match the colour of the flavanols rich chocolate, contained 2293Kj/100g (547 Kcal/100 g)(1033Kj (246Kcal) for the 45g chocolate daily dose of three 15g bars) whereas the dark, flavanol rich chocolate with cocoa powder contained 2289Kj/100g (545 Kcal/100g) (1029Kj (245Kcal) for the daily dose). Calculations were done using the Genesis for Windows (Esha Research, Salem, OR, USA). The glycaemic index of dark chocolate was estimated at 41 whereas for simulated iso-calorific chocolate it was 34 – 44 based upon the data of Foster-Powell et al. (2002).

The composition of high cocoa liquor/polyphenol rich chocolate and the simulated iso-calorific chocolate are given in table 4.1.3.1, and the polyphenol content of each, shown in table 4.1.3.2. The percentage of non-fat cocoa solids (cocoa liquor) in polyphenol rich chocolate was 33.17%. Microbiological analyses were carried out on a cross section of samples and showed that salmonella or *enterobacter laceae* were absent.

Table 4.1.3.1: Polyphenol analysis* high cocoa liquor/ polyphenol rich chocolate and simulated iso-calorific chocolate (cocoa liquor free / low polyphenol)

Polyphenols	High Cocoa Liquor / Polyphenol Rich chocolate	Dose for 45g (3 bars) Daily Dose per participant	Simulated iso-calorific chocolate Cocoa Liquor Free / Low Polyphenol)	Dose for 45g (3 bars) Daily Dose per participant
Folin	17.4 mg ECE/g	783mg ECE	3.9 mg ECE/g	176mg ECE
Catechin	0.37 g/kg	16.6 mg	<0.05 g/kg	<2mg
Dimer B2	0.80 g/kg	36.0mg	<0.05 g/kg	<2mg
Epicatechin	1.23 g/kg	55.3mg	<0.05 g/kg	<2mg
Trimer C	0.58 g/kg	26.1mg	<0.05 g/kg	<2mg
Tetramer D	0.33 g/kg	14.9mg	<0.05 g/kg	<2mg
Dimer B5	0.32 g/kg	14.8mg	<0.05 g/kg	<2mg

*** All analysis undertaken and provided by Nestlé PTC, York, UK**

All participants attended a structured, group patient education programme (XPERT) and were on stable medication for their diabetes, hypertension, lipids and gout, for three months prior to entry into the study. Participants were encouraged to incorporate the chocolate into their normal habitual diet as advised during the XPERT programme, which all participants undertook at least one month before being enrolled onto the study (Deakin, Cade, Williams, & Greenwood, 2006). At each visit, dietary compliance was monitored by 24-hour dietary recall undertaken by myself (data not presented), as a registered dietitian. Participants were instructed to consume the chocolate at each of the following points in the day, either mid-morning, mid afternoon and in the evening or as a supper snack. The returned empty packets, marked with the date, and time each bar was consumed, together with the any uneaten chocolate, were used to monitor compliance with the study and the intake of chocolate.

Table 4.1.3.2: Nutritional composition of chocolate bars, shown per 100g, per bar and per thrice-daily dose.

Energy and Nutritional Profile						
Nutrient	**Per 100g**		**Per bar**		**Par 3 bars (daily dose)**	
	High polyphenol	**Low polyphenol**	**High polyphenol**	**Low polyphenol**	**High polyphenol**	**Low polyphenol**
Energy (kcal/ Kj)	547/2297	540/2268	82 / 344	81/340	326/ 1032	323/1020
Fat (g) **Of which saturates (g)**	45.4 25.3	31.3 16.0	6.8 3.8	4.7 2.4	20.4 11.4	14.1 7.2
Carbohydrate (g) **Of which sugars (g)**	28.9 23.8	60.0 49.3	4.3 3.6	9.0 7.4	12.9 10.8	38.7 32.6
Protein (g)	7.3	8.0	1.1	1.2	3.3	3.6
Composition by Macronutrient	%	%				
Protein	5.43	5.72				
Carbohydrate	19.51	41.78				
Fat	75.05	52.44				

4.1.4 Study Measurements

Following an overnight fast, weight and blood pressure were measured and blood samples drawn at baseline and at the end of the intervention phase (eight weeks later), following the four weeks washout and at the end of the second phase (following a further eight weeks). All blood samples were drawn and processed as described in Chapter Two. Routine biochemistry, liver function tests and a full blood count were performed using the hospital pathology labs to screen for any changes as part of safety monitoring.

All participants were requested to test their own capillary glucose using a glucose meter supplied by the research team (Glucomen; Menarini Diagnostics, Wokingham, UK). Participants were asked to take a 7-point glucose profiles (pre-breakfast, 2-hours post breakfast, pre-midday meal, 2-hours post midday meal, pre-evening meal, 2-hours post evening meal and pre-bed) on two occasions, in two separate weeks during the period

they were consuming the chocolate. Each participant recorded a minimum of at least 28 readings. In addition, three participants volunteered to have interstitial glucose measurements made during each chocolate intervention arm and at the mid-point of the wash out period. This was undertaken using the Glucoday Continuous Glucose Monitoring System (CGMS) (A Menarini Diagnostics, Wokingham, UK).

4.1.5 - Statistical analysis and sample size calculation

For a two-sided 5% significance level, a sample of five participants per group was needed, assuming a 10% dropout rate for a pilot cross over trial. This gave the study 80% power to detect a 20% difference in HDL between treatments (assuming a common standard deviation of 0.25). This is similar to published studies investigating the effects of chocolate in healthy individuals. The results were considered significant if the two-tailed *P* value was <0.05. The sample size was derived using nQuery version 4 (Statistical Solutions Ltd. Cork, Ireland). Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA) and Microsoft Excel 2007 (Microsoft, Redmond, Washington, USA).

All data from were tested for normality, using Kolmogmorov-Smirnov, as advised by the University statistician (Mr Alan Rigby). Mean percentage changes obtained at the end of the 'active' chocolate phase were compared with the results at the end of the placebo phase, using the paired Student's *t*-test for normally distributed data and Wilcoxon's signed-rank test for data which violated normality. The period and the carryover effect that may have occurred from the crossover design were tested using the appropriate Student's *t* test.

4.2 – Results

4.2.1 – Safety Data and Biomedical Results

Table 4.2.1: Baseline characteristics of participants

Characteristic	
Age (Mean years ± standard deviation)	58.33±6.79
Gender ratio (Male: Female)	7:5
Duration of Diabetes (Mean years ± standard deviation)	4.17±1.95
Weight (Kg ± standard deviation)	89.4±21.6kg
BMI (Kgm^{-2}± standard deviation)	31.77± 9.90
Oral Diabetes Medication (Metformin Only: Other: None)	7:2:3
Lipid Lowering Medication (Yes : None)	9:3
Blood Pressure Medication (Yes : None)	7:5

Initial safety was assessed by reviewing the baseline to end of study data. This represented a total of four months (16 weeks) of consuming 45g chocolate per day, split into two periods of eight weeks of chocolate consumption separated by a four-week washout period. This was used in addition to assessing the effect of the control, as it was felt that this would indicate if prolonged consumption of any chocolate might have negative effects. This methodology is commonly used in pharmaceutical trials to assess safety in terms of exposure to a compound or product. Weight was unchanged at 89.4±21.8kg (mean± standard deviation) at the end of the study compared to 89.4±21.6kg (p=1.00) at baseline. The dietary recall data suggested that the chocolate tended to replace other snack foods and there was some reduction in portion size at the subsequent meal. Compliance with chocolate consumption was a median of 92% based on returned and dated chocolate wrappers (91% for the high polyphenol chocolate (range 89-98%) and 94% for the low polyphenol chocolate (range 89-98%).

There were no effects on glycaemia in terms of Hba1c 6.43±0.81% on study entry compared to 6.50±0.76% at the end of the study (p=0.84); Total cholesterol (4.84±1.49mmol/l versus 4.98±1.34mmol/l; p=0.84); HDL cholesterol (1.18±0.29mmol/l versus 1.26±0.27mmol/l; p=0.49); LDL cholesterol

(3.01±1.25mmol/l versus 3.20±1.21mmol/l; p=0.76) and triglycerides (1.18±0.85mmol/l versus 1.15±0.59; p=0.31). No effect was seen for systolic blood pressure (p=0.97) or diastolic blood pressure (p=0.46).

The nutrient and flavonol composition of the chocolates, given in tables 4.1.3.1 and 4.1.3.2, shows that the chocolates were matched for energy content; the main difference being in flavonol content. Since palatability of the chocolate was a key issue, the primary aim of formulation for the control chocolate was be similar to the flavanols rich bar. This necessitated a compromise and it was not possible to match the macronutrient content of the bars.

Further safety data were collected with all 12 participants recording self-monitored blood glucose (SMBG) readings, undertaken while they were consuming the chocolate at six points (pre and post-prandial) on two occasions through the two-month period. The data in figure 4.2.2.1 show that the glycaemic excursion was greater following breakfast for the low polyphenol chocolate compared to the high polyphenol chocolate. To investigate this further, continuous glucose monitoring (CGMS) shown in figure 4.2.2.2 was undertaken using the Glucoday on the three participants who gave their further consent. The results appear to match the observations from the SMBG.

Table 4.2.2.1: Mean values for biochemical of cardiovascular risk. P values were obtained by t-test. All biochemical tests were tested for normality using the Kolmogorov-Smirnov test which none of the data violated.

	Pre-low polyphenol Chocolate (placebo)	Post-low polyphenol Chocolate	P - value	Pre-high polyphenol Chocolate (active)	Post-high polyphenol Chocolate	P -value
Systolic blood pressure (mmHg)	132±*5*	134±*6*	0.67	132±*5*	134±*5*	0.55
Diastolic blood pressure (mmHg)	80±*3*	82±*3*	0.10	82±*3*	84±*2*	0.51
HbA1c (%)	6.4±*0.8*	6.4±*0.7*	0.63	6.4±*0.8*	6.5±*0.8*	0.07
Glucose (mmol/l)	6.8±*1.5*	7.2±*1.3*	0.17	7.0±*1.4*	6.9±*1.5*	0.71
Insulin (uIU/l)	10.8±8.3	14.8±9.7	0.07	13.91±9.0	14.13±10.6	0.92
HOMA	3.4±3.3	4.6±3.2	0.08	4.5±3.5	4.5±3.9	0.95
Hs-CRP (mg/l)	2.6±*2.5*	2.4±*2.2*	0.72	3.0±*2.1*	2.0±*1.5*	0.22
Serum Cholesterol (mmol/l)	4.9±1.4	5.0±1.3	0.55	5.0±1.5	5.0±1.3	0.94
Serum Triglycerides (mmol/l)	141±0.89	1.30±0.61	0.52	1.33±0.5	1.20±0.65	0.32
Serum LDL Cholesterol (mmol/l)	3.02±1.21	3.15±1.13	0.18	3.20±1.42	3.18±1.24	0.83
Serum HDL Cholesterol (mmol/l)	1.18±0.26	1.20±0.30	0.78	1.17±0.27	1.24±0.26	0.04
Cholesterol:HDL	4.3±1.6	4.4±1.5	0.83	4.4±1.4	4.1±1.3	0.03

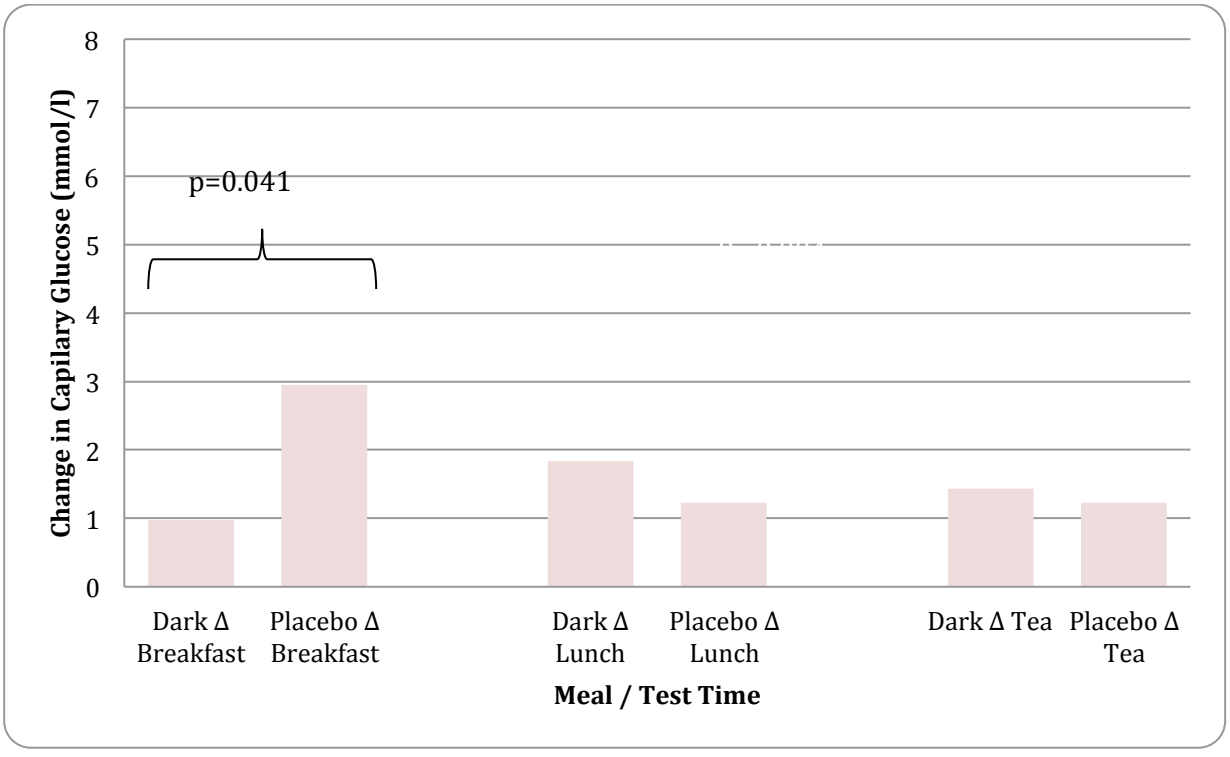


Figure 4.2.2.1: Self monitored capillary glucose monitoring (at least 12 recordings for each time point)

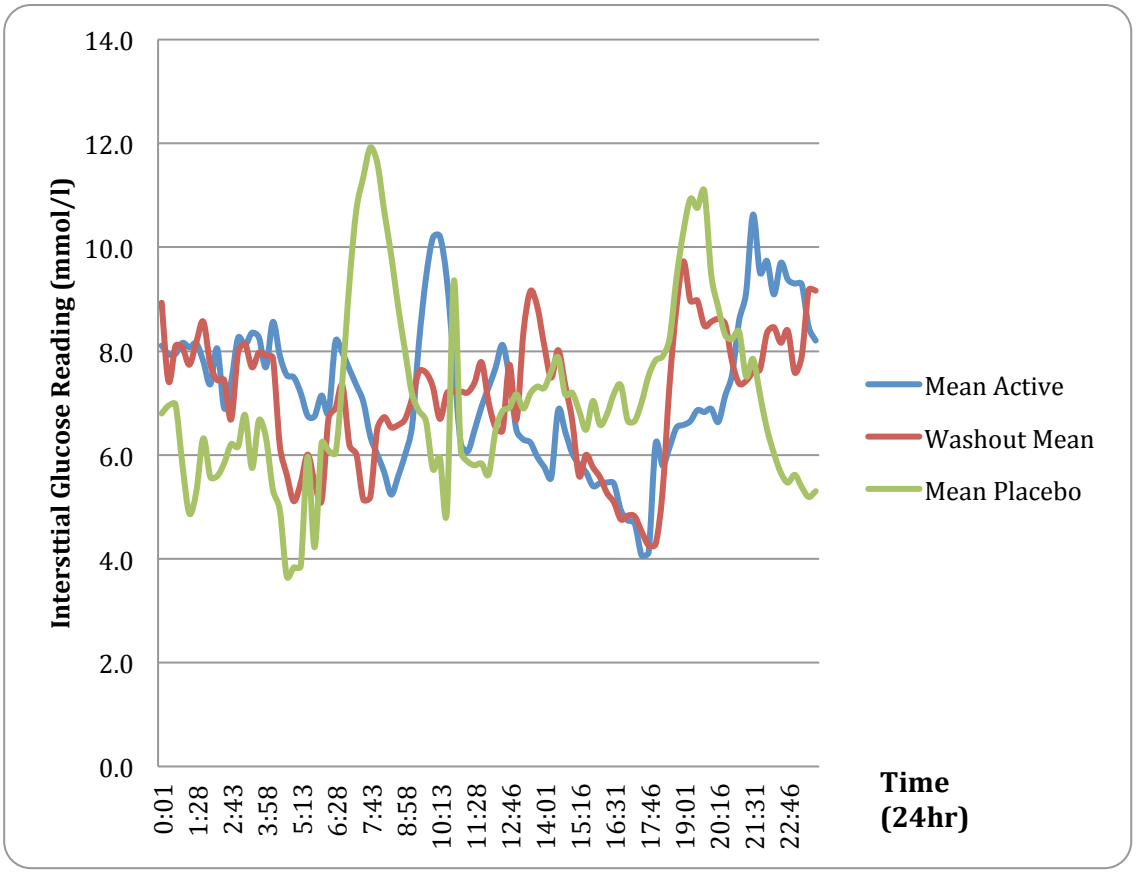


Figure 4.2.2.2: CGMS for 'active' chocolate, washout (twice for each of the participants) and placebo. Two participants undertook CGMS

The data in table 4.2.2.1 and figure 4.2.2.3 demonstrate a significant improvement in the lipid profile with high polyphenol chocolate in terms of an increase in HDL cholesterol and cholesterol: HDL ratio. This matched a non-significant reduction in triglycerides.

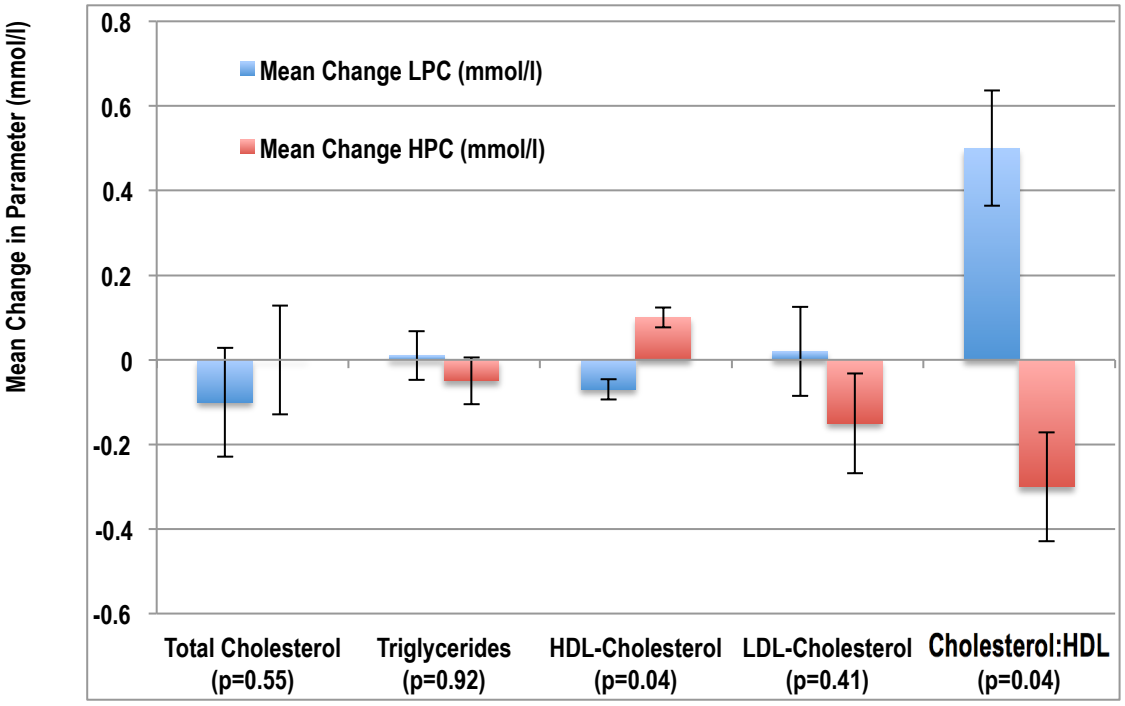


Figure 4.2.2.3: Difference in lipid profile parameters between high polyphenol chocolate compared to low polyphenol chocolate. All samples measured fasting at baseline and following 8-weeks of consuming 45g chocolate per day (3 times 15g bars) data presented as mean ± standard error of mean.

Along with the observed absolute changes in lipid parameters (table 4.2.1.1) the positive effect of high flavanol chocolate can be seen on HDL cholesterol levels and as an improved HDL:total cholesterol ratio. There did not appear to be an adverse effect on total cholesterol despite the 16g of saturated fatty acids the chocolate added to the participants' diets. These results are suggestive of a change greater would be seen from a variation HDL cholesterol of a coefficient of variation of 6.2-7.7% in a similar well controlled T2DM population (Sathyapalan, Atkin & Kilpatrick, 2008). The coefficient of variance for other lipid parameters, in this population have been reported to vary from 6.9% for total cholesterol for individuals treated with atorvastatin to nearly 20% for triglycerides in the same cohort of patients (Sathyapalan, Atkin & Kilpatrick, 2008). This is similar to the coefficients of variation seen in healthy subjects (Bookstein, Gidding, Donovan and Smith, 1990). The Data on the hospital pathology records were reviewed 6-9 months following completion of the study and showed that lipid profiles,

especially HDL cholesterol, had reverted back to pre-study levels. Although HOMA appeared to significantly improve, with the high polyphenol chocolate relative to low polyphenol chocolate (p=0.013), this was an effect of the increase HOMA following low polyphenol chocolate (increase of 1.23±1.13 following low polyphenol chocolate, compared to 0.04±1.03). Systolic blood pressure, did not change significantly following eight weeks of either chocolate, high polyphenol chocolate slightly decreased -0.88±13.5mmHg whereas following low polyphenol chocolate it slightly increased 1.55±8.19mmHg (p=0.83). A similar lack of effect was seen with respect to diastolic blood pressure, following high polyphenol chocolate an increase of 2.63±4.53mmHg, with low polyphenol chocolate having a similar effect 1.55±7.57mmHg (p=0.57). Neither the changes in HOMA or blood pressure were clinically significant.

4.2.3 - Participant Reported Outcomes

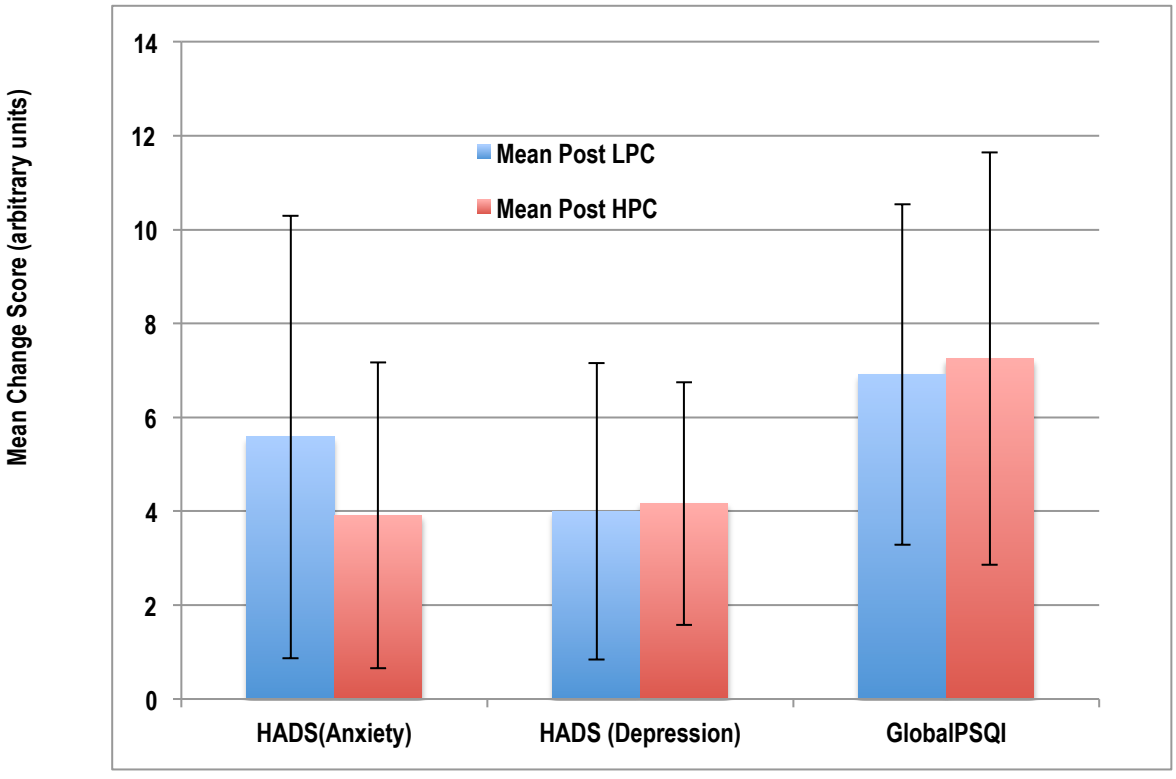


Figure 4.2.3.1: Hospital Anxiety Depression Scores for Anxiety and Depression following 8 weeks of consuming either low polyphenol chocolate and high polyphenol chocolate. The HADS(Anxiety Score) significantly decreased following 8 weeks of consuming HPC (p=0.002; baseline to 8 weeks) and was significantly different between the HPC and LPC (p=0.027). No differences were seen in

other aspects of HADS or PSQI . Analysis by 2-way ANOVA (p>0.05) with presented p values from post-hoc analysis using LSD.

The participants reported outcomes did not show any difference between or within the groups following chocolate consumption (Figures 4.2.3.1 and 4.2.3.2., with the exception of a reduced anxiety score from the HADS questionnaire following the consumption of high flavanol chocolate (p=0.002) and between the two chocolate groups following 8 weeks of daily chocolate consumption (p=0.027).

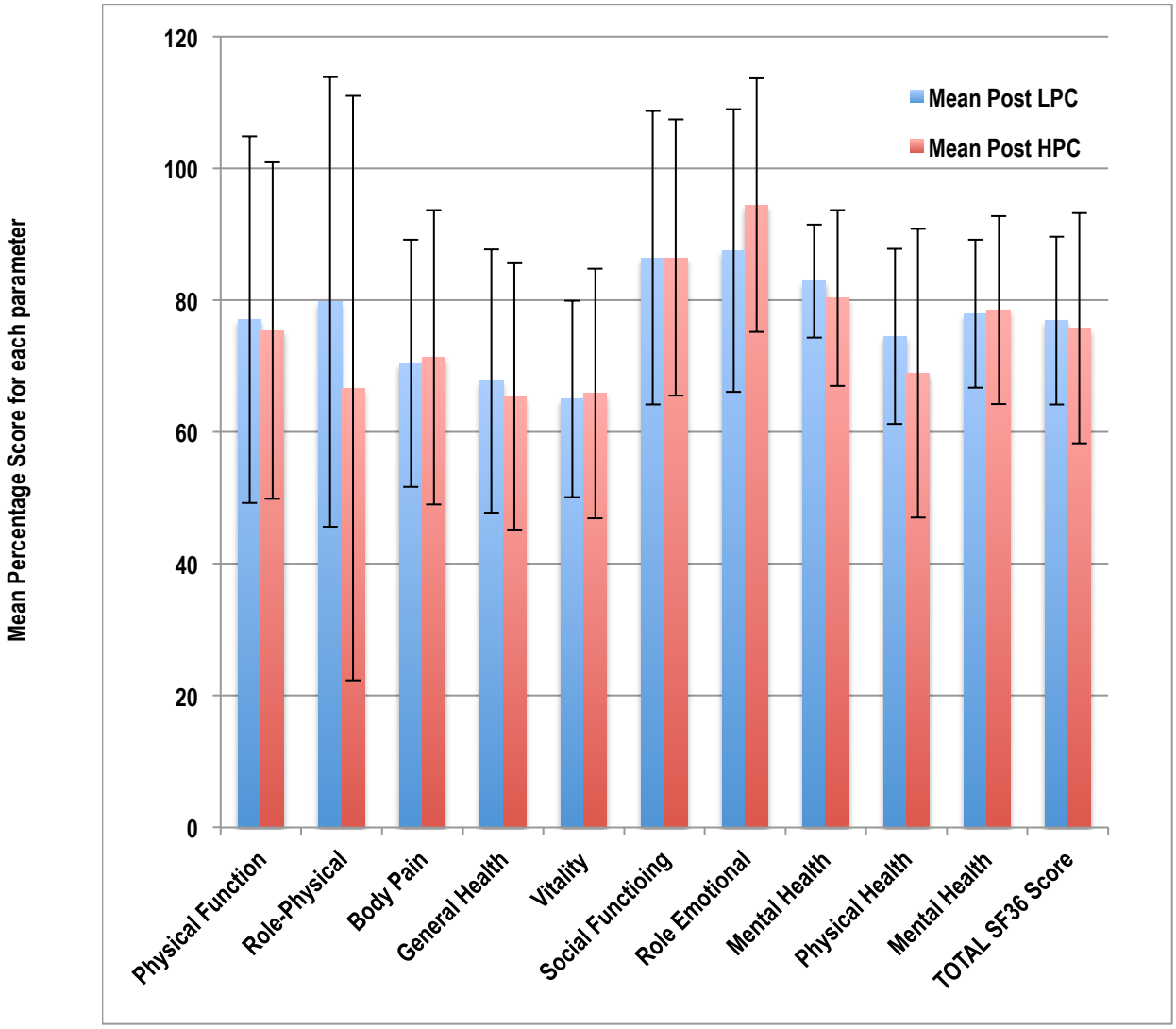


Figure 4.2.3.2: SF-36 (short form) score including the individual parameters following 8 weeks of consuming either low polyphenol chocolate and high polyphenol chocolate. No differences were seen between the arms of the study or within the study arms (2-way ANOVA)

4.3 - Discussion

This is the first study to give individuals with T2DM the equivalent of a standard chocolate bar (45g, as three 15g bars) a day chronically for 5 months (16 weeks of chocolate consumption) with minimal adverse events (two of the three adverse events occurred during the washout period), weight gain or deterioration in glycaemic control. This study was also novel because of the chronic nature of providing the chocolate over the five-month period as previous studies have been over a much shorter timeframe. With the flavanols-rich chocolate, HDL cholesterol was seen to increase significantly; an effect in accord with a study in populations without diabetes (Mursu et al., 2004) and indicating that this effect can be sustained. In addition, the reciprocal reduction in HDL: total cholesterol ratio, suggests that the high polyphenol chocolate might be a useful adjunction to lipid lowering in individuals with T2DM. This suggests that high flavanols chocolate may be of benefit in individuals who are on lipid lowering therapy, but have suppressed HDL cholesterol, and is worthy of further investigation, particularly as when the high flavanol chocolate intervention was stopped, the beneficial effects were reversed.

Previous studies have reported acute beneficial effects of chocolate supplementation on blood pressure (Grassi et al., 2005a; Taubert, Roesen, & Schomig, 2007) and insulin resistance as assessed by HOMA (Grassi et al., 2008); something which was not found in the present study. This may have been due to the more chronic nature of this study, that the participants had T2DM and raised blood pressure that can be more resistant to treatment, or were taking concurrent antihypertensive therapy that prevented further improvement. Along with a lack of effect on glycaemia there were no increases in weight despite an increase in participants' energy intake of 1Mj (320kcal) per day.

In order to detect gross changes in dietary habits, I carried out a 24-hour food and physical activity. The only difference detected was with the low polyphenol chocolate,

where portion sizes tended to decrease at meal times. In other words, participants appeared to compensate for eating chocolate by eating less at meal times. The recall, was aimed at assessing compliance, and was unable to detect whether these changes were intentional. It was a deliberately decided not to use more in-depth dietary intake methods since these are acknowledged to affect habitual food intake and might have biased the data and even result in weight loss.

Glycaemic control was not affected by chocolate intake, in agreement with the initial hypothesis, but due to the pilot nature of this study, this finding may have lacked power. In terms of glycaemia, the data showed that post-prandial peaks in glycaemia were reduced following breakfast. This finding was statistically significant for the Self Monitored Blood Glucose (SMBG) ($p=0.041$) and was mirrored by the CGMS data. High-cocoa flavanol-rich chocolate has been shown to reduce insulin resistance in participants without diabetes but this was not seen as a change in HOMA in the present study. However the reduction in post-prandial glycaemia may have been the result of improved insulin sensitivity or insulin secretion. This observation needs further work to elucidate the underlying mechanism.

This study differed from previous studies investigating cocoa flavanols in T2DM, which have used much shorter intervention periods and used only cocoa (Balzer et al., 2008) which the meta-analysis presented in Chapter Two indicated was not the ideal formulation for administering flavanols, with chocolate appearing to be more efficacious. Subsequently the work of Curtis et al. (2012), combined chocolate rich in flavanols with isoflavones in postmenopausal women with T2DM which concurred with my data suggesting a reduction in cardiovascular risk

The mechanism by which high-polyphenol chocolate improves lipid profile remains unclear. It has been suggested that the polyphenols may have either an insulin sensitizing effect (Grassi et al., 2005b), which was not seen in this study, or that there is

a reduction in the inflammation-independent action of insulin (Mursu et al., 2004). Lipid peroxidation was reduced, although this could not have been inferred from the high-sensitivity C-reactive protein (CRP) levels, which did not differ between groups. It has further been hypothesized that additional beneficial effects of flavanols on the endothelium may be mediated through increasing nitric oxide levels (Fisher, Hughes, Gerhard-Herman, & Hollenberg, 2003; Heiss et al., 2006; Persson, Persson, Hägg, & Andersson, 2011; Schmitt & Dirsch, 2009).

The small sample size of this study potentially placed the data at risk from type 1 errors (with a false positive finding), although the crossover design probably overcame this to some extent. Being a crossover study, there was concern that participants may have been able to tell the difference between the two preparations and a blinded taste study was therefore undertaken prior to the trial. Reassuringly, this showed that the participants could not tell any difference in appearance or taste between the high-polyphenol chocolate and low-polyphenol chocolate preparations. A further criticism of this study could be the lack of measurements of plasma polyphenols in this study; this was due to lack of available funds. Chocolate rich in flavanols, increase HDL cholesterol significantly based upon the reported data. It may be useful in future studies to measure epicatechin metabolites as these would be useful if the protocol aims to include an aspect of dose–response effects.

4.3.1 - Conclusion

This is the first study to investigate the potential of chocolate to reduce cardiovascular risk in individuals with T2DM. It has demonstrated that consuming 45g per day of (high flavanol chocolate over eight weeks increases plasma HDL with no detrimental effect on glycaemic control or weight gain; effects which are likely to reduce cardiovascular risk. Further work is needed to investigate the effects of chocolate and polyphenols on lipid metabolism and their potential interactions with lipid lowering medication.

Chapter Five: High-Polyphenol Chocolate Reduces Endothelial Dysfunction and Oxidative Stress during Acute Transient Hyperglycaemia in Type 2 Diabetes: A Pilot Randomized Controlled Trial

This chapter investigates the effects of high polyphenol (flavanol rich) chocolate on markers of endothelial dysfunction and oxidative stress during acute transient hyperglycaemia in individuals with type 2 diabetes mellitus (T2DM).

Aims and Scope of Chapter:

- To undertake formally a taste test trial to investigate the ability of individuals to distinguish between the study chocolates.
- To undertake a proof of concept study to investigate the ability of high flavanol chocolate to reduce oxidative stress in the presence of transient hyperglycaemia.

Table 5.0, defines the study question and PICOS approach to achieving the above aims of this chapter.

Table 5.0: Study question and PICOS for the proof for study two.

Study Question(s)	**Can chocolate rich in polyphenols administered one hour before a 75g oral glucose challenge ameliorate the postprandial increase in endothelial dysfunction and oxidative stress type 2 diabetes mellitus?**
Participants	**Ten individuals with type 2 diabetes mellitus controlled by lifestyle alone, or in combination with metformin.**
Interventions	**13.5g of chocolate rich in polyphenols (3.5% polyphenols)**
Comparisons	**13.5g of chocolate low in polyphenols (0.9 polyphenols)**
Outcomes	**Primary outcomes:** **1. Change in endothelial function** **2. Change in oxidative stress** **Secondary outcomes:** **1. Sensory evaluation of chocolates** **2. Glycaemic effects of chocolates**
Study design	**A double blind randomised controlled trial, following a seven day run in period, a familiarisation visit was undertaken prior to participants being randomly allocated the chocolates over the subsequent visits each seven days apart.**

The clinical study will be presented in five parts:

5.1 - Introduction.

5.2 - Taste trial.

5.3 - Study specific methodology.

5.4 – Endothelial Function and Glycaemia effects of chocolate prior to a 75g oral glucose load, including analysis of the familiarisation/ water control arm.

5.5 – Biochemical effects of high polyphenol chocolate compared to low polyphenol chocolate prior to a 75 g oral glucose load.

5.1 - Introduction

Type 2 diabetes mellitus is associated with increased cardiovascular morbidity and mortality via accelerated atherogenesis. However this excess cardiovascular risk cannot be fully accounted for by traditional risk factors (Barr et al., 2007), and it is proposed that this is at least partially associated with hyperglycaemia (Raz et al., 2009). Hyperglycaemia itself is likely to increase risk via a number of mechanisms that may encompass a combination of acute and chronic inflammation. An inflammation centred model suggests this risk may be driven by increased endothelial dysfunction and oxidative stress (de Jager et al., 2006).

The increase in endothelial dysfunction and oxidative stress by hyperglycaemia has been revealed by changes in functional endothelial assessment, biomarkers and metabolites of nitric oxide, in healthy individuals and populations at increased risk of cardiovascular disease and/or diabetes (Ceriello et al., 2008).

Beneficial effects of cocoa polyphenols, especially the monomeric flavon-3-ols (predominantly epicatechins), which constitute 34-37% of total flavanol content in chocolate (Langer et al., 2011; Wollgast, Pallaroni, Agazzi and Anklam, 2001) have been demonstrated in the fasting state (Grassi et al., 2005b; Faridi et al., 2008) and 2 hours following ingestion of cocoa polyphenols (Balzer et al., 2008). These effects have been seen in healthy individuals and those with T2DM. The initial mechanism was thought to be due to an antioxidant effect of these compounds; however, this has recently been disputed (Lotito and Frei, 2006; Hollman et al., 2011). Currently the favoured hypothesised mechanism is via enhancement of the bioavailable pool of nitric oxide through the up-regulation of nitric oxide synthase. This view is supported by a study of smokers with endothelial dysfunction, which reported an increase in the level

of nitric oxide metabolites and improved endothelial function following supplementation with cocoa (Heiss et al., 2005).

A recent meta-analysis suggested an acute improvement in endothelial function of 3.9% (CI 2.04-4.33%) (Hooper et al., 2012). However, it is not known whether chocolate or cocoa can negate the inflammatory effects of an oral glucose load.

It was therefore hypothesised that that ingestion of polyphenol rich chocolate, prior to an oral glucose load, would ameliorate any adverse effects of acute transient hyperglycaemia on endothelial function and oxidative stress.

5.2 - Taste Differential Panel for Chocolate

5.2.1 - Background

High polyphenol content chocolate has been shown to reduce cardiovascular risk (Grassi et al., 2005a; 2008; Hooper et al., 2012; Taubert et al., 2003; Taubert et al., 2007). But however, could pose a potential problem in that eating a large quantity of chocolate might lead to an increased energy intake and potential weight gain, although this is disputed (Golomb, Koperski & White, 2012; O'Neil, Fulgoni & Nicklas, 2011a; 2011b). Palatability may be an issue; chocolate is widely acknowledged as being a desirable food however the high flavanol chocolates can tend to be more astringent and bitter tasting. It has been reported that in one study participants preferred to take tablets rather than dark chocolate habitually (Ried, Frank & Stocks, 2009). Acticoa™ (Barry Callebaut BV, Lebbeke-Wieze, Belgium) is a specially formulated chocolate containing twice the polyphenol content of regular plain chocolate (Bernaert, 2007), thus the same polyphenol/flavanol content is achieved in a smaller volume with potentially the same health benefits. However for this option to be successful, the chocolate must be acceptable to the consumer.

It is important to the conduct of a truly double blind crossover design that the control or placebo chocolate which contains none or relatively low levels of the bitter polyphenols, is palatable and more importantly taste the same as the very high polyphenol (flavanol) chocolate. It is also important to match the bars with respect to the energy, sugar and fat content of chocolate. A simple pragmatic approach to these issues is a Sensory Evaluation II (O'Mahony & Rousseau, 2003) type of panel which aims to assess Participants' ability to distinguish between food samples. Such tests can be undertaken as triangular or same-different tests. Since the participants in the subsequent study reported in section 5.4, were likely to be naïve consumers of the type of chocolate being offered in the study, it is logical to adopt the methodology of Rousseau & O'Mahony, (2001) and use a same-different

design. This approach was also selected for its cognitive simplicity for the participants and its tendency to give to improved differential (t-D) values and greater statistical power.

5.2.1.1 - Aim of taste trial

Demonstrate a lack of taste difference between the Acticoa™ and the Acticoa™ placebo. The panel also assessed the palatability and difference (or lack of taste difference) between the ultra high polyphenol content chocolate and the Acticoa™.

5.2.1.2 - Objectives

To discover whether it is possible to distinguish the Acticoa™ chocolate from the placebo Acticoa™ chocolate.

To discover whether there is equal subject tolerability of participants to the Acticoa™, the placebo Acticoa™ and the ultra high polyphenol content chocolate.

5.2.2 - Participants and Methods

A double blind taste-testing panel methodology was adopted. Each testing panel lasted for 20-30 minutes. Participants were offered each of the 3 samples, Acticoa™, Acticoa™ placebo or the ultra high polyphenol content chocolate, in one of 3 sequences (table 5.2.2.1), which were assigned randomly prior to initiating each session.

Table 5.2.2.1: Sampling sequence for the taste trial

Test 1		Test 2		Test 3	
1	A + B	**1**	C + C	**1**	C + A
2	A + A	**2**	B + B	**2**	B + A
3	C + C	**3**	C + A	**3**	A + A
4	C + A	**4**	B + A	**4**	C + C
5	C + B	**5**	A + A	**5**	B + B
6	B + B	**6**	B + C	**6**	C + B

Key - A = 2g ultra high polyphenol content chocolate

B = 2g Acticoa™ placebo

C = 2g Acticoa ™

Participants were asked to abstain from food and all drinks, except for water for one hour prior to the test, and were asked to swill their mouths with water 3 times prior to the start. In between test pairs, participants were asked to swill their mouths out twice. When sampling pairs, no swilling of mouths with water was allowed.

5.2.2.1 - Sample size

Based on the data of Rousseau & O'Mahony, (2001), 25 volunteers were recruited from the staff of Hull and East Yorkshire Hospitals NHS Hospitals Trust. The study was not deemed to require ethical or other governance permissions, following discussion with chair of the local research ethics committee and the hospital trust research and development department. Data were analysed using Chi^2 to assess the subject opinion of the chocolate presented.

5.2.4 – Taste Trial Results

Twenty-five volunteers were recruited; one subject did not manage to consume the ultra high polyphenol chocolate due to its palatability. Generally the Acticoa™ was the preferred chocolate.

Table 5.2.4.1: Same: difference data and analysis for the tasted chocolate pairs

Chocolate Pair		Number	Significance
Acticoa™ Placebo Vs. Acticoa™ Placebo	Same	14	p=0.541
	Different	11	
Acticoa™ Placebo Vs. Acticoa™	Same	7	p=0.043
	Different	18	
Acticoa™ Placebo Vs. Ultra high polyphenol	Same	9	p=0.230
	Different	16	
Acticoa™ Vs. Acticoa™	Same	18	p= 0.043
	Different	7	
Acticoa™ Vs. Ultra high polyphenol	Same	12	p=1.000
	Different	13	
Ultra high polyphenol Vs. Ultra high polyphenol	Same	21	P= 0.001
	Different	3	

This population of untrained taste testers were better able to distinguish the Acticoa™ from the Acticoa™ placebo (p=0.043) than the Acticoa™ placebo from the ultra high polyphenol chocolate (p=0.230) (Table 5.2.4.1).

Subjective comments regarding the chocolate were collated; they mainly related to the difference in colour between the chocolates. The ultra high polyphenol chocolate was noted as being slightly harder and darker in colour than the other chocolates, with comments regarding its bitter aftertaste also being reported. A small number of participants found that this chocolate was of limited palatability, with one subject not able to complete the study after first tasting this particular chocolate. All chocolates were recognised as dark chocolate, but, apart from the Acticoa™, were reported as having a more bitter taste compared to chocolate normally consumed by the study population.

5.2.5 – Taste Trial Discussion and Conclusion

The data suggested that the three chocolates tested by the panels were well matched. There were similar perceptions of difference and similarity when the chocolates were the same as when they were different. This might be a reflection of the taste panel being untrained, or alternatively could demonstrate the care put into ensuring the flavour, texture and appearance of the three chocolates.

The participants appeared to be very capable of distinguishing the Acticoa™ from the Acticoa™ placebo. This ability was not always associated with differences in perceived taste, but more to the differences in texture. The placebo was not readily distinguished from the ultra high polyphenol chocolate, even though the taste difference in theory was greater for this combination. This might have reflected a sequencing effect of previously being exposed to the bitter chocolate, or that these two chocolates shared a common texture and mouth feel.

The presentation of the chocolate was also of note was that, it appeared to vary in tablet size or square size, which were the same for the placebo and high polyphenol chocolates, but different to the Acticoa™. It was felt from the comments made by the participants that alongside the difference in presentation, the texture of the placebo was 'foamy' and the Acticoa™ smoother than the participants were expecting in terms of mouth feel for a dark or plain chocolate.

The data from this preliminary taste trial suggested that these chocolates would be suitable for use in a double blind study. The study would need to be carefully designed to avoid bias, since the bitter nature may be perceived either as a sign of benefit or masking another ingredient of the chocolate. However, if chocolates were consumed a week apart, this was not likely to be a concern, and therefore appropriate for the design of this crossover study.

The Acticoa™ was well tolerated and found to be acceptable to the participants. The placebo (polyphenol free chocolate) was also generally acceptable to participants, however, a few were not happy with the foamy mouth feel of the chocolate. The ultra high polyphenol chocolate was acceptable to the majority of participants, especially those who were male. From the taste trial, the bitter nature of the ultra-high polyphenol chocolate could potentially have led to a 10-20% drop out rate.

5.3 - Interventional Study

5.3.1 - Study Specific Methods

Eleven individuals expressed interest in the study; one was excluded after being found to be anaemic, with ten meeting the recruitment criteria (Figure 5.3.1.1). All the participants were Caucasian with stable T2DM, treated with metformin or lifestyle alone. The age range was 40-75. Nine were male and one was a post-menopausal woman. All participants gave written informed consent and the study had full ethical and regulatory approval from the Hull and East Riding NHS Research Ethics Committee and the Hull and East Yorkshire Hospitals NHS Trust Research and Development department.

Following screening, participants underwent a two-week 'wash-out' period during which they abstained from rich sources of polyphenol, using the list of foods described in Appendix II, and refrained from consuming all types of chocolate and cocoa.

Following screening, studies attended for the first visit, which was a familiarisation visit consisting of water only before the 75g Oral Glucose Tolerance Test (OGTT) solution. Following a 12-hour fast, participants arrived for their first visit. They were asked to rest for 20 minutes in a temperature controlled (22^{o}C) low-lit room in a supine position. Reactive Hyperaemia Peripheral Artery Tonometry (RH-PAT) (EndoPAT 2000, Itamar Medical, Caesarea, Israel) was undertaken to assess endothelial function, prior to blood being withdrawn via a cannula inserted into the median-cubital vein, for glucose, insulin and endothelial serum adhesion molecules (iCAM, e-selectin, p-selectin and p-selectin glycoprotein ligand 1(PSGL-1)).

After baseline tests, participants were given 200ml of water. Sixty minutes after the ingestion of the water, further endothelial function blood tests were made prior to a 75g

OGTT solution being given. Participants then rested for a further 120 minutes after which time the assessment methods were repeated.

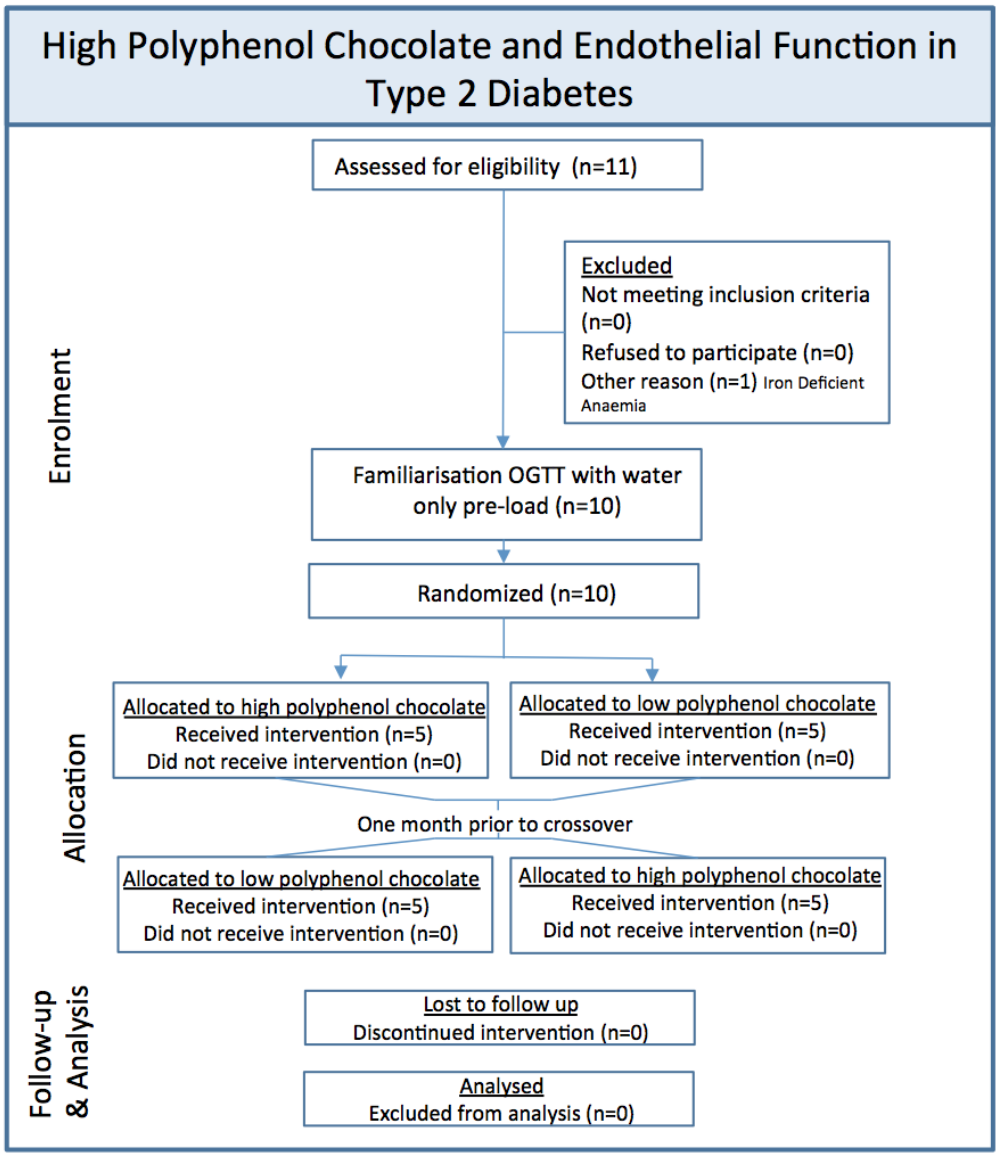


Figure 5.3.1.1: Consort flow diagram to show enrolment and randomisation. This double-blinded, randomised control trial was designed to investigate the effects of high polyphenol chocolate on endothelial function and oxidative stress in T2DM during transient hyperglycaemia.

At the second and third visits an identical protocol was followed, except that in addition to the 200ml of water, participants were given 13.5g chocolate. This was randomly assigned either to 13.5g of high polyphenol chocolate (containing 3.5% polyphenols (Folin-Ciocalteau (GA)) (Acticoa™, Barry Callebaut BV, Lebbeke-Wieze, Belgium) or to 13.5g of low polyphenol chocolate (control) (identical in formulation to the high

polyphenol chocolate except, 0.9% polyphenols, Barry Callebaut BV, Lebbeke-Wieze, Belgium). The dose of polyphenol selected approximated that used in previous work (Grassi et al., 2005b; Faridi et al., 2008). Barry Callebaut BV provided both chocolates in identical presentations, and also held the randomisation code. The study chocolate and the blood analysis was funded by Barry Callebaut BV via an unrestricted grant, but the study design and analysis were undertaken independently by the research team.

Oxidative stress was measured using 24-hour urine collections; a baseline 24-hour urine collection which was undertaken prior to the test period and a second 24 hour urine collection was timed to start with from the ingestion of the chocolate. All participants were provided with a standard lunch following the completion of their chocolate and glucose tolerance tests.

Alongside the urine collection, participants were asked to keep a food, fluid and exercise diary. A small nested cohort undertook CGMS and was asked to make at least 1 self-measured glucose reading per 24 hours. With the participants' additional consent, an Ambulatory Blood Pressure Monitor (ABPM) was fitted. However this had unfortunately to be abandoned, since other participants were either bruised following the occlusion with the blood pressure cuff during the EndoPAT assessment or had mid-upper arm circumferences too large for the cuff. Participants then returned the next working day to deliver the urine samples, food diaries and CGMS.

To assess dietary adherence and reduce potential confounding resulting from a change in background diet, food intake was recorded using 24-hour dietary recall undertaken by the study dietitian. Nutritional intake was then assessed using Microdiet (Downlee Systems Ltd., High Peak, UK).

Endothelial function was assessed using an EndoPAT 2000. Adhesion molecules (ICAM, E-selectin, P-selectin and PSGL-1) were measured using serum ELISA assays

(Bender MedSystems, Vienna, Austria). Oxidative stress was assessed by the measurement of 15-F2t-Isoprostane from the 24-hour urine collections, which were frozen in aliquots at -80^{o}C prior to analysis by ELISA (Oxford Biochemical Research, Mitchigan, USA); this was adjusted to urine creatinine concentrations.

A power calculation was undertaken based upon the data of Balzer et al. (2008) using G*Power (Faul et al., 2007), which indicated a minimum sample size of seven (based on a difference of 1.8, power=0.80 for α <0.05). However, a sample size of ten was selected to allow for attrition. Data were tested for normality (Shapiro-Wilks), and paired data were then tested between conditions by *t*-tests or Wilcoxon's signed ranks. All analysis was undertaken using PASW Statistics 18.0 (IBM, New York, NY, USA). The initial data were analysed comparing the three arms of the trial using analysis of variance (ANOVA) and where significance was found, post-hoc testing with a Bonferroni correction, which is presented in 5.4.3 and 5.4.5. However following initial peer review of draft manuscripts by an academic journal, editorial opinion was that as the study was a pilot, the small sample might lead to potential type 1 error (reporting a falsely positive finding); for that reason the results presented in section 5.5 compare the two interventional chocolate groups alone (discarding the water only arm) using *t*-tests or Wilcoxon's signed ranks depending on the nature of the distribution of the data. The trial was registered (ISRCTN35988358).

5.4 - Results

5.4.1 - Baseline Characteristics

Table 5.4.1.1: Baseline Characteristics of Participants

Characteristic and units	Mean	Standard Deviation
Age (years)	59.2	8.0
Duration of Diabetes (years)	6.3	3.3
Alcohol intake (units per week)	9.4	10
Weight (kg)	100.0	17.7
BMI (kgm^{-2})	32.5	6.0
Diabetes Medication (Metformin Only: None)	7:3	
Lipid Lowering Medication (Yes : None)	6:4	
Blood Pressure Medication (Yes : None)	7:3	
HbA1c %	6.5	0.7
Total Cholesterol (mmol/l)	3.9	1.4
Triglycerides (mmol/l)	1.7	0.7
LDL Cholesterol (mmol/l)	2.2	1.2
HDL Cholesterol (mmol/l)	1.0	0.2
Estimated Glomerular Filtration Rate (ml per min)	82	10.8
Haemoglobin (mmol/l)	14.6	0.9
Hs-CRP (mmol/l)	1.3	0.7

The study population consisted of nine males and one postmenopausal female. They were either diet controlled (three of the ten participants) or on a stable dose of metformin (seven of the ten participants) for more than three months. Of the ten subjects six were taking antihypertensive medication, seven were taking HMG-co-ase inhibitors ('statins') for dyslipidaemias and two were on aspirin as prophylactic anti-platelet therapy. All were none smokers and were moderate self-reported consumers of alcohol (less than current UK recommended upper limits for intake). Although participants varied in terms of their medication, the risk of this being a source of confounding was minimised by the study having a crossover design.

5.4.2 - Dietary Intake Data

Dietary recall data were normally distributed (assessed by Shapiro-Wilks test). Table 5.4.2.1 shows that there were no significant differences in the amount of food or the nutrient intake across the three visits of the study. Adverse events were recorded throughout the study and only one minor event was reported; a participant with weakness and light-headiness following the water-only test day.

Table 5.4.2.1: Descriptive statistics and ANOVA of 24-hour dietary intake for the run in period, 7 days prior to the chocolate phases of the studies. Data were tested for normality using Shapiro-Wilks; all data was normally distributed.

Nutrient	Study Arm	N	Mean	Std. Deviation	ANOVA
Total Weight of food consumed	Water (run in)	10	2074.7g	734.8	0.583
	High Polyphenol chocolate	10	2165.7g	709.6	
	Low Polyphenol Chocolate	10	1827.5g	792.1	
Water	Water (run in)	10	1648.2ml	710.6	0.636
	High Polyphenol chocolate	10	1730.5ml	673.7	
	Low Polyphenol Chocolate	10	1439.2ml	714.5	
Energy (Calories)	Water (run in)	10	1736.2kcal	331.5	0.588
	High Polyphenol chocolate	10	1865.6kcal	407.9	
	Low Polyphenol Chocolate	10	1675.8kcal	494.3	
Energy (Kj)	Water (run in)	10	7309.9Kj	1379.2	0.590
	High Polyphenol chocolate	10	7847.9kj	1713.8	
	Low Polyphenol Chocolate	10	7054.6kj	2077.4	
Protein	Water (run in)	10	70.8g	12.3	0.480
	High Polyphenol chocolate	10	81.8g	18.4	
	Low Polyphenol Chocolate	10	75.7g	26.9	
Total Carbohydrate	Water (run in)	10	228.2g	39.7	0.754
	High Polyphenol chocolate	10	227.4g	59.9	
	Low Polyphenol Chocolate	10	210.7g	71.8	
Sugars	Water (run in)	10	74.8g	21.5	0.215
	High Polyphenol chocolate	10	57.0g	21.3	
	Low Polyphenol Chocolate	10	56.2g	33.7	
Fat	Water (run in)	10	61.6g	24.7	0.394
	High Polyphenol chocolate	10	73.4g	21.0	
	Low Polyphenol Chocolate	10	59.0g	28.0	
Saturated Fatty Acid	Water (run in)	10	19.7g	11.3	0.134
	High Polyphenol chocolate	10	25.5g	9.3	
	Low Polyphenol Chocolate	10	16.8g	7.4	
Fibre	Water (run in)	10	15.7g	4.4	0.718
	High Polyphenol chocolate	10	14.3g	4.6	
	Low Polyphenol Chocolate	10	13.7g	7.3	
Sodium	Water (run in)	10	3114.7mg	1308.2	0.232
	High Polyphenol chocolate	10	3832.7mg	1230.3	
	Low Polyphenol Chocolate	10	28386mg	1377.2	
Iron	Water (run in)	10	10.8mg	5.6	0.417
	High Polyphenol chocolate	10	12.1mg	6.1	
	Low Polyphenol Chocolate	10	9.1mg	2.8	
Vitamin C	Water (run in)	10	74.6mg	34.9	0.200
	High Polyphenol chocolate	10	48. 5mg	29.1	
	Low Polyphenol Chocolate	10	62.mg	30.5	

5.4.3 - Endothelial Function Data

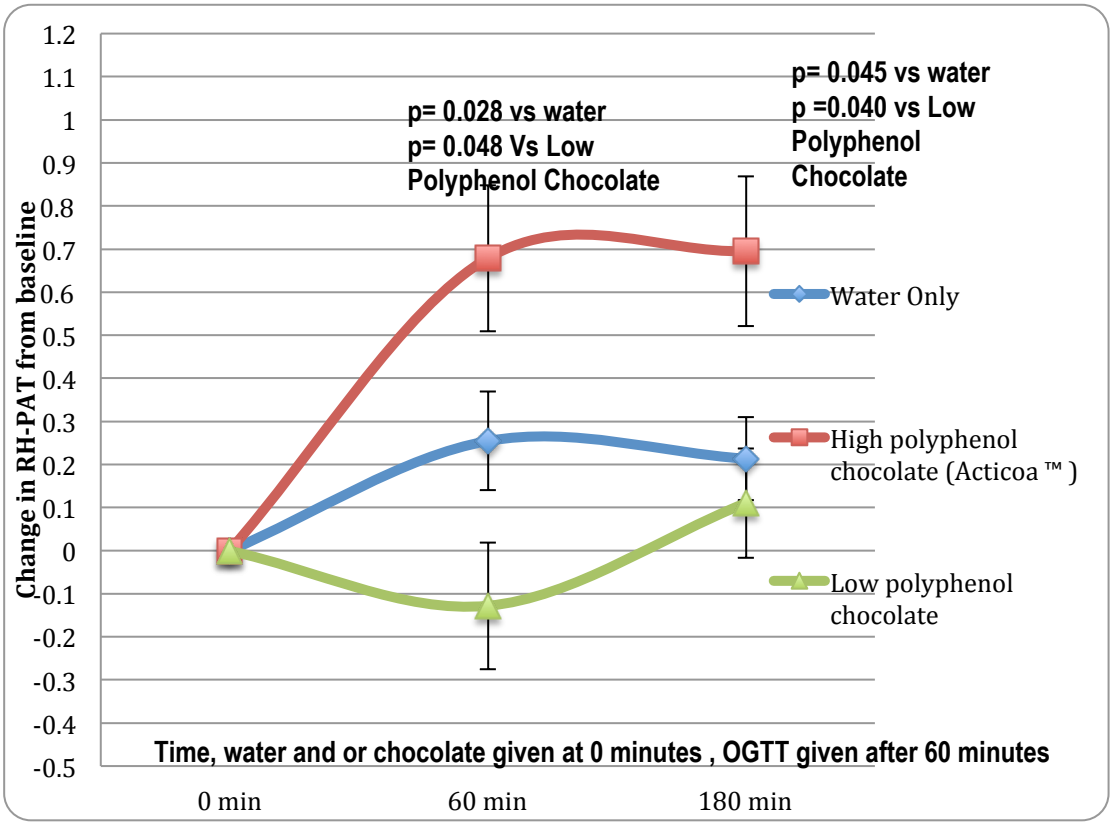


Figure 5.4.3.1: Reactive hyperaemia (RH-PAT) change in participants with Type 2 Diabetes corrected to a zero baseline, to remove the non-significant variation at baseline mean change ± SEM

Reactive hyperaemia (RH-PAT) improved significantly for high polyphenol chocolate compared to both water and low polyphenol chocolate at 60 minutes ($p<0.05$) and following the 75g glucose load (OGTT) ($p<0.05$) (Figure 3.4.3.1). Since the change in RH-PAT has arbitrary units, it is best practice to consider the percentage change rather than change in arbitrary units.

The data demonstrated a 30% improvement from baseline for the high polyphenol chocolate which was sustained throughout the oral glucose tolerance test, whereas consuming the low polyphenol chocolate resulted in a 15% reduction in endothelial function as measured by RH-PAT.

5.4.4 - Results of OGTT and Area Under the Curve (AUC) Glucose

There were no significant individual differences for fasting glucoses or glucose levels between subjects or for the groups at the end of the oral glucose tolerance test. Sixty minutes after consuming the chocolate, glucose levels were significantly higher for the high polyphenol chocolate than for the water group (p=0.01), while the increase following low polyphenol chocolate was not significant (p=0.07). The high polyphenol chocolate increased glucose significantly more than was seen with the low polyphenol chocolate.

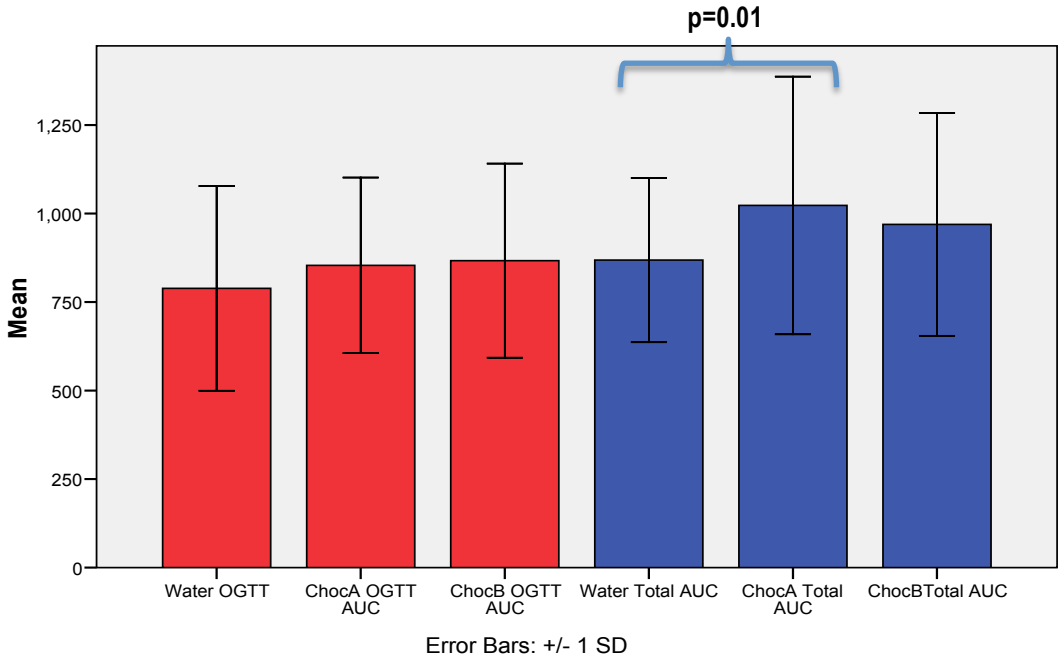


Figure 5.4.4.1: Mean Area Under the Curve (AUC) for Oral Glucose Tolerance Test (OGTT), with and without previous feeding of Chocolate 60 minutes (mmol/l/min). Red bars: the results for the OGTT only (120 minutes); Blue bars: for the duration of the test (180 minutes). Choc A – high polyphenol chocolate; Choc B – low polyphenol chocolate

No significant differences were seen between the chocolate groups or between the water and chocolate groups. The increase in total AUC may be linked to the difference in rise in the plasma glucose levels between 0 and 60 minutes.

5.5 – Biochemical Assessments

The following data are for the two arms, which included the chocolate with 200ml water, prior to the 75g glucose OGTT. The data from the familiarisation test were discarded as this required analysis of variance to assess difference between groups, which had a greater potential for error due to the small sample size.

5.5.1 Fasting Data and 120 minutes after the 75g Oral Glucose Load

No significant differences were seen in baseline fasting measures of glucose, insulin, endothelial function or oxidative stress at each visit (Table 5.5.1.1). There were also no differences in area under curve between the two groups for plasma glucose for the duration of the 75g oral glucose load (p=0.77). Following the glucose load, a significant difference was seen in the 15-F2t-Isoprostane between high polyphenol chocolate (110.4±3.0 mg/mol (mean±SEM)) and control (207.1±5.7mg/mol) (p=0.03).

The oral glucose load significantly increased glucose and insulin concentrations (Table 5.5.1.1) in both groups, but as indicated previously there were no significant differences between them. However, when preceded by high polyphenol chocolate, an improvement in endothelial function (RH-PAT) was seen (p=0.01), whilst baseline measurements differed by 0.3%, which was not significant (p=0.09). A preload of the control, resulted in a significant deterioration in oxidative stress (p=0.02) and iCAM (p=0.04).

Table 5.5.1.1: The baseline and 120 minutes following the 75g oral glucose load.

	High Polyphenol Chocolate (mean±SEM)			Low Polyphenol Chocolate (mean±SEM)		
	Fasting	120 minutes after 75g oral glucose load (180 minutes after consuming chocolate)	P value	Fasting	120 minutes after 75g oral glucose load (180 minutes after consuming chocolate)	P value
Glucose mmol/l	6.6 ± 0.1	11.8±0.3	<0.01	6.5 ± 0.1	11.6 ± 0.3	<0.01
Insulin µIU/l	16.6 ± 1.2	103.2±8.5	0.02	15.1 ± 0.8	101.6 ± 8.2	0.02
RH-PAT %	1.7 ± 0.1	2.3±0.1	0.01	2.0 ± 0.1	2.1 ±0.1	0.44
iCAM ng/ml	325.6 ± 9.0	310.0±8.4	0.20	321.1 ± 7.6	373.6 ± 10.5	0.04
e-Selectin ng/ml	111.3 ± 5.8	96.6±5.6	0.09	94.4 ± 4.0	105.8 ± 3.5	0.28
p-Selectin ng/ml	253.0 ± 14.8	235.0 ± 7.7	0.62	265 ±15.2	268.5 ± 12.4	0.92
PSGL-1 U/ml	281.9 ± 12.2	212.6 ±8 .7	0.13	262.9 ± 5.5	327.5 ± 7.3	0.09
Urinary 15-F2t-isoprostane: creatinine mg/mol	117.7 ± 4.0	116.8 ± 5.7	0.48	110.4 ± 3.0	207.1 ± 5.7	0.02

All data were tested for normality using Shapiro-Wilks. Data found to be normally distributed were analysed using paired t-test and that which violated normality were analysed using Wilcoxon's signed ranks. All P values are the difference from baseline to 120 minutes following the 75g glucose load (taken 60 minutes after the consumption of either 13.5g of high polyphenol or low polyphenol chocolate)

The percentage changes from fasting (Figure 5.5.1) showed a significant improvement in endothelial function (RH-PAT) following high polyphenol chocolate compared to the control. In addition, three of the four adhesion molecules (iCAM, e-Selectin and PSGL-1) reduced significantly following high polyphenol chocolate compared to baseline and control. Urinary 15-F2t-Isoprostane: creatinine ratio did not change with high polyphenol chocolate, whereas following low polyphenol chocolate this resulted in a significant increase in this oxidative stress biomarker (figure 5.5.1.2). Dietary analysis and assessment of physical activity levels showed no significance intra-subject differences between the two groups.

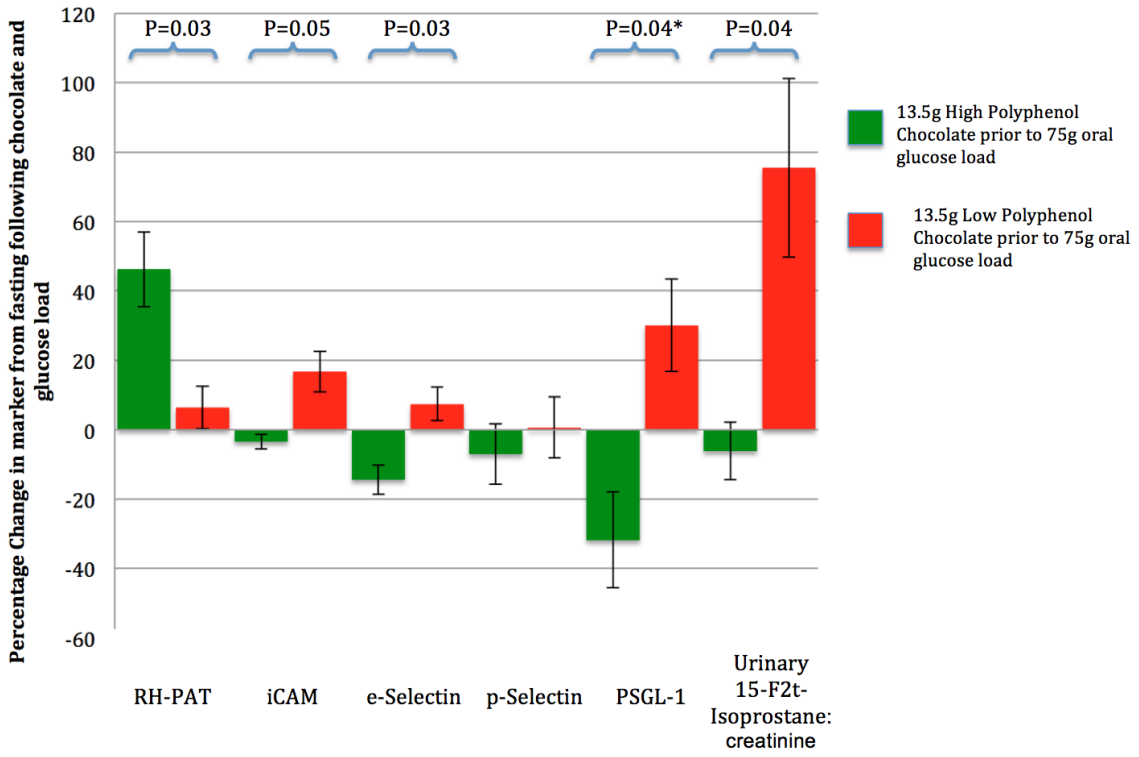


Figure 5.5.1.2: Percentage changes from baseline to 120 minutes following ingestion of 75g oral glucose load (180 minutes after ingestion of 13.5g of chocolate). Improvement in RH-PAT indicates improved endothelial function, reduction in iCAM, e-Selectin, p-Selectin and PSGL-1 indicates improved endothelial function. Urinary 15-F2t-isoprostane compared 24 hours before the consumption of the 13.5g chocolate and 75g oral glucose load and the 24 hours comencing with the ingestion of the chocolate. A decrease in urinary 15-F2t-isoprostane suggests a reduction in oxidative stress. Non-significant change for p-selectin (p=0.86). All data was tested for normality (Shapiro-Wilks) * indicates a violation of normality. Data presented as mean±SEM.

5.6 – Discussion

This is the first study showing an acute protective effect of high polyphenol chocolate on endothelial function with a concurrent reduction in an oxidative stress marker in patients with type 2 diabetes exposed to transient hyperglycaemia resulting from an oral glucose load. Previously Ceriello et al. (2008) demonstrated that periodic hyperglycaemia increases oxidative stress and endothelial dysfunction. The present data suggest that cocoa polyphenols in chocolate might mitigate some of the endothelial dysfunction and oxidative stress resulting from this hyperglycaemia.

These results could be explained via an antioxidant effect of the polyphenols (predominantly epicatechins), as there was a reduction effect of the glucose load upon oxidative stress. However, it is also plausible that modulation through multiple metabolic pathways, including nitric oxide synthase and ACE could be likely candidates (Hollman et al., 2011). The results showing improvement of functional endothelial responsiveness are in accord with those showing that cocoa ingestion resulted in an acute response of endothelial function via flow mediated vasodilatation (Balzer et al., 2008). However, this is the first study to show that the improvement in endothelial function was more pronounced following the induction of a hyperglycaemic state.

It has been demonstrated that cocoa polyphenols (predominantly epicatechins) increase nitric oxide (NO) levels, which in turn result in endothelial relaxation and a reduction in endothelial markers such as iCAM and p-selectin (Vázquez-Agell et al., 2011). This would explain why the NO dependent adhesion molecule biomarkers iCAM and p-selectin were significantly reduced within 180 minutes in this cohort. This observation is also in accord with animal data reporting that iCAM and e-selectin respond acutely, whereas p-selectin may take longer to induce (Liu et al., 1998). Therefore, the data suggest that high polyphenol chocolate has an acute beneficial and protective effect on

the endothelium in individuals with T2DM seen both functionally and through the expression of adhesion molecules in a dynamic hyperglycaemic state.

Oxidative stress is a feature of type 2 diabetes, possibly as a result of hyperglycaemia increasing levels of reactive oxygen species (ROS) (Brownlee, 2001). ROS scavenges NO to produce peroxynitrite ($ONOO^-$), reducing the NO pool and resulting in endothelial dysfunction (Hollman et al., 2011). High polyphenol chocolate may reduce ROS levels, thus improving endothelial function, whilst also enhancing NO synthesis. Our observations are supported by observations in both healthy participants and those at increased cardiovascular risk where cocoa polyphenols increase the bioavailability of NO, reflected in improved endothelial function and reduced oxidative stress, suggestive of potential cardiovascular health benefits (Pieper, 1999).

Oral feeding of a glucose load to individuals with T2DM has been shown to lead to an increase in oxidative stress and endothelial dysfunction (Ceriello et al., 2008), which was reflected in the findings for the low polyphenol chocolate group, where oxidative stress increased and the biomarkers for endothelial cell dysfunction increased. Conversely, protection was afforded by high polyphenol chocolate where oxidative stress and endothelial function markers decreased significantly. Although there were statistically significant differences in AUC for glucose for the high polyphenol chocolate, these had no adverse effects as oxidative stress or endothelial function were reduced following its consumption in this study.

Whilst the study is limited by the nature of it being a pilot and its small sample size, the crossover design and careful controlling of variables, mitigate this, in part. The study is potentially further limited by the variability in the EndoPAT 2000 measurements of endothelial function varying at baseline, although this did not differ significantly. However, the consistency of the functional assessment of endothelial function was in agreement with the soluble biomarkers, and the reciprocal reduction in oxidative stress

was highly supportive of the hypothesis that high polyphenol chocolate has acute beneficial effects in T2DM that are maintained in a hyperglycaemic state.

5.6.1 - Conclusion

High polyphenol (flavanols) chocolate has a beneficial acute effect in reducing endothelial dysfunction and oxidative stress in individuals with T2DM during acute transient hyperglycaemia. Further work is needed to investigate the potential benefits of cocoa polyphenols as modulators of post-prandial endothelial dysfunction and oxidative stress in T2DM, and the potential effects on reducing cardiovascular risk.

Chapter Six: Effects of Polyphenol Enriched Milk Chocolate in Type 2 Diabetes: A Randomised Controlled Trial

Aim and scope of chapter:

- To investigate the effects of milk chocolate rich in polyphenols upon markers of glycaemic control and cardiovascular risk in individuals with type 2 diabetes mellitus (T2DM).
- Consider the effects of acute chocolate consumption alone upon insulin and glucose responses.

To address these aims, the clinical study will be presented in four parts:

6.1 – Introduction and methodology.

6.2 – Baseline features and characteristics and post study follow up.

6.3 – Effect of chronic consumption of milk chocolate.

6.4 – Effect of acute consumption of milk chocolate.

Please note, due to contractual clauses with the sponsor of the work presented in this chapter, it is not possible to fully report all the clinical trial data. This particularly is with respect to the recipes and composition of the chocolates used beyond the data presented.

Table 6.0: Study question and PICOS for the proof for study three.

Study Question(s)	**Can milk chocolate enriched with polyphenols improve measures of insulin resistance, cardiovascular risk and glycaemic control in type 2 diabetes mellitus?** **Is milk chocolate enriched with polyphenols more effective than a dark chocolate with the same epicatechin level?**
Participants	**60 individuals with type 2 diabetes mellitus controlled by lifestyle alone, or in combination with metformin. These will be randomised to receive either a low polyphenol chocolate, a milk chocolate enriched with polyphenols or a dark chocolate rich in polyphenols**
Interventions	**20g Milk chocolate rich in polyphenols (fortified to 19.2mg epicatechin per bar) by the addition of CocoanOX 12%.**
Comparisons	**20g Dark chocolate (19mg epicatechin) as hypothesised active control** **20g Low polyphenol chocolate (2.7mg epicatechin) as hypothesised inactive control**
Outcomes	**Primary outcomes:** **1. Change in insulin resistance (HOMA)** **Secondary outcomes:** **1. Endothelial function (acute and over 12 weeks)** **2. Glycaemia (HbA1c) following 12 weeks of chocolate and glucose, both fasting and following consumption of 40g of chocolate** **3. Lipid profile (total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides)** **4. Blood pressure** **5. Weight, waist and hip circumference** **6. Inflammatory markers – hs-CRP, TNF-alpha, IL1Ra and IL6** **7. Oxidative stress, serum malondialdehyde and urinary 15f-2t isoprostane** **8. Participant reported outcome measures including attachment style, mood, fatigue and sleep.**
Study design	**A double blind randomised controlled trial following a parallel design. Following a 28 day chocolate and cocoa free run in period, participants will be attend for initial testing of biochemical and endothelial function following acute consumption of one of three randomly assigned chocolates. The effect of 40g of chocolate (2 bars of chocolate) over a three-hour period will be assessed. Participants will then be provided with 20g chocolate per day for 12 weeks before returning for a second assessment of biochemical and endothelial function.**

6.1 - Introduction and Methodology

Short term studies of chocolate in healthy volunteer studies (Grassi et al., 2005a; 2005b) and in participants with insulin resistance (Grassi et al., 2008) have shown chocolate is effective in lowering blood pressure, improving lipid profiles and reducing insulin resistance. However, there are only two trials that have reported the effect of regular chocolate consumption in diabetes (Mellor et al., 2010b, Mellor et al., 2012), as described in Chapters 3 and 4 of this thesis. Others have investigated the effects of cocoa (Balzer et al., 2008) and chocolate with and without added isoflavones (Curtis et al., 2012). Very few studies have considered the potential effects of milk chocolate on cardiovascular risk reduction.

Although initially promising, there are a number of potential considerations before chocolate might be accepted as a suitable and advantageous food for regular consumption in individuals with T2DM. The high energy content of chocolate may result in weight gain and the high sugar content of may affect glycaemia. Finally, chocolate is a rich source of saturated fatty acids, and the general consensus is a high intake of this type of fat is undesirable, having been associated with increased risk of cardiovascular disease (EFSA, 2010b). This may not be a true concern, as the predominant fatty acid in chocolate is stearate, which is considered to be benign with respect to cholesterol. Additionally, the emerging consensus is that saturated fat may not be as significant contributor to cardiovascular risk as has been historically thought (Astrup et al., 2011).

The epidemiological data does not suggest that weight gain is associated with chocolate consumption (Golomb, Koperski and White, 2012) in children and adolescents (O'Neil, Fulgoni and Nicklas, 2011b) or in adults (O'Neil, Fulgoni and Nicklas, 2011a). The last two named studies used data from the NHANES data set to show that although 'candy' or confectionary consumers (including chocolate), consumed more energy, they had a

significantly lower mean body mass index. The doubt about sugar also does not appear to be significant since chocolate has a low glycaemic index, and a low glycaemic load (Brand-Miller et al., 2009).

Initial studies focused on dark chocolate compared to white chocolate (that is cocoa butter and flavanol-free as the control) in large daily doses of up to 100g per day (Grassi (Grassi et al., 2005a; 2005b; Taubert et al., 2003). This quantity would provide up to 20% of the recommended daily energy requirements for many participants (SACN, 2011). One hundred grams of chocolate size was selected in these studies, as it provided an estimated dose of 500mg of flavon-3-ols. This is the quantity which was seen to be effective in the initial studies, and is approximately half the intake seen in the Kuna Indian populations of Panama, a population where cardiovascular disease and hypertension are extremely rare (Hollenberg, 2006). Reducing the quantity of chocolate but maintaining the flavanol content may prove challenging due to the bitter nature of these compounds, which could therefore make a chocolate less palatable and therefore unacceptable to the consumer. Evidence for this perspective was reported by Ried, Frank and Stocks (2009), who found the chocolate was less acceptable than the comparator tomato extract supplement over an eight-week period.

In the UK, market research found that 66% of adults have a preference for milk chocolate (Valued Opinions, 2011), with only 22% preferring dark chocolate; the predominant chocolate formulation demonstrating evidence of health benefits in the published literature. There are very few studies reporting the effects of milk chocolate perhaps because of concerns about milk inhibiting the bioavailability and action of flavanols. Initial research by Serafini, Ghiselli and Ferro-Luzzi (1996), suggested consuming milk with tea totally inhibited the activity of these flavonoids *in vivo*. Serafini followed this work up later (Serafini et al., 2003) to suggest that milk ingested alongside or when included as part of the manufacture process inhibited the antioxidant

activity of the epicatechins. However this position has largely been refuted by other research groups (Hollman, Van Het Hof, Tijburg & Katan, 2001; van het Hof, Kivits, Weststrate and Tijburg, 1998; Schroeter et al., 2003; Roura et al., 2007).

To date, there are only very limited data on the effects of high flavanol milk chocolate. They include a short-term (14 day) study of young healthy footballers, which demonstrated a significant reduction in both blood pressure and lipid parameters (Fraga et al., 2005). However, this study did not report parameters of glycaemia or insulin sensitivity. Two further studies used active cocoa flavon-3-ol compounds including milk. Innes et al. (2003) provided single doses of milk chocolate. No improvement in platelet function was seen with the milk chocolate, when compared to dark chocolate. The only other study, which combined cocoa with milk in a drink, was by Balzer et al. (2008). This study of participants with T2DM demonstrated improvements in endothelial function, both following acute consumption and with daily cocoa supplementation for 30 days. Although changes in lipids and glycaemic control appear to have occurred, this was not commented on. Moreover, the data were potentially confounded by the formulation that provided approximately 50% of the recommended daily sugar intake (approximately 5% of total energy from sugar) for individuals with diabetes (Dyson et al., 2011), which is the same as that recommended for the population as a whole.

The challenge of delivering an adequate dose of flavanols to be effective, without an excess of energy, sugar or fat, whilst still offering a palatable product, is clear. Despite initial work implying a lack of efficacy from the polyphenols in milk chocolate, there remains the potential for benefit, with the additional benefit of enhanced palatability and acceptability of this type of chocolate. This study aimed to investigate whether a dose of 20g milk chocolate rich in polyphenols was able to demonstrate a beneficial effect upon cardiovascular risk factors and measures of glycaemic control.

6.1.1 - Research Design and Methods

6.1.1.1 - Participants

The study consisted of sixty-one participants, 31 men and 30 postmenopausal women who were on stable medication for at least 3 months for cardiovascular risk (including for lipids and hypertension) (Figure 6.1.1.1). With respect to their T2DM, the participants were treated with either lifestyle (diet and exercise) or lifestyle in conjunction with metformin. The participants were aged 45-80 years and receiving standard UK care for their T2DM, which included structured patient education (either X-PERT, DESMOND or Living with Diabetes (a local structured diabetes programme) at least one month prior to enrolment onto the study. All participants were recruited through the local diabetes network that generated a database, from which participants were invited to participant in this study.

The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Hull and East Yorkshire NHS Research Ethics Committee. All participants were required to provide written informed consent prior to enrolling in the study.

6.1.1.2 - Study protocol

All participants underwent a 28-day run in period when they avoided polyphenol/ flavonoid rich foods, following discussion with a Dietitian (details in Appendix II). Participants were randomised to either 20g per day (40g for the acute testing) of high polyphenol milk chocolate (19.11mg epicatechin per bar) (referred to within this chapter as Milk Chocolate), low polyphenol chocolate (Control) as a control for sugar and fat content (2.71mg epicatechin per bar) or Nestlé Noir, a high polyphenol dark chocolate (Dark Chocolate) (19.0mg epicatechin per bar). The epicatechin content of the milk chocolate was matched to that of the dark chocolate. Previous studies indicated

that 20 g Nestlé Noir, the bar size used in this intervention contained 312mg of total flavonoids (Folin-Ciocaltaeu method), (Davison et al., 2012). Such data were not available for the other chocolates used in this study, as they had been specially formulated for use in this clinical trial.

Table 6.1.1.2 Overview of study protocol outlining the tests at each visit

Study plan:	*Screening*	*Visit 1 'Meal test'*	*Visit 2 'Check up'*	*Visit 3 'Check up'*	*Visit 4 'Meal test'*
Days	*0*	*28*	*58*	*86*	*114*
Medical and Social History / Subjects' demographic data	*x*				
Clinical examination, including blood pressure measurements	*x*	*x*	*x*	*x*	*x*
Questionnaires		*x*			*x*
Dispensing of study chocolate		*x*	*x*	*x*	
Fasting blood samples for screening: *HbA1c* *Lipids* *Liver and renal function*	*x*				
Fasting blood samples for: *Insulin* *Glucose* *HbA1c* *Lipid profile* *Oxidative Stress* *Inflammatory Markers*		*x*			*x*
24 urine collection		*x*			*x*
Acute feeding of 40g chocolate		*x*			*x*
Bloods at 60, 120, 180 minutes for: *Glucose,* *Triglycerides* *Insulin*		*x*			*x*
Endopat measurement of endothelial function		*x*			*x*
Treatment of 20g chocolate per day	*...*	*Chocolate Started→*	*→*	*→*	*Chocolate Ends*

Treatment was allocated in a double blind fashion, with participants being informed that they were testing the effects of one of three types of chocolate on

heart disease risk in T2DM. The sponsor prior to the enrolment of the first participant via a predefined code provided the allocation of the randomisation; this was stratified by gender in six blocks, the size the block was not disclosed to investigator prior to analysis of the data. Table 6.1.1.2 outlines the conduct of the study and procedures undertaken.

Participants were recruited from the Hull and East Riding Diabetes Network database. All individuals who consented to receive information regarding research were sent an invitation (approximate 850 from a population with diabetes of 24,000). Interested individuals then contacted the author of this thesis to discuss the study and their potential suitability. Those suitable were sent the full participant information sheet, and if still interested invited to attend a screening visit. Following attending a screening visit and their results being assessed (typically one week from this visit) participants were allocated a study code based on their initials, this was then communicated to the sponsor who allocated a study number and the code for the chocolate dispensed at the subsequent visit.

The author of this thesis enrolled the participants, with the assigning to treatment undertaken by the sponsor. The author of this thesis (except for 5% of visits undertaken by a clinical trials assistant), administered the questionnaires, undertook the follow up meetings and blood sampling. Plasma, serum and urine samples were analysed by chemical pathology laboratories of Hull and East Yorkshire Hospital for routine clinical markers, and for inflammatory markers and oxidative stress these were undertaken within the biomedical laboratories at the University of Hull (by Drs Madden and Courts).

The study was conducted between May 2009 and June 2010, participants were typically enrolled in the study for a total of 112 days (84 days of chocolate consumption) this only varied if personal reasons (business or holidays) or illness

prevented attendance on the planned days. Data was then scrutinised by the sponsor until October 2011, with the primary data analysis report released in March 2012 when the study was unblinded.

The sponsor provided the chocolates, with all three being formulated and manufactured in line with EU regulations for chocolate (European Union, 2004). Products were issued in a single batch to the research site and were tested for safety prior to delivery, and at the mid-point of the study. The dose was based on previous data on the effect of Nestlé Noir, (Flammer et al., 2007), which used a protocol based on 40g of chocolate. This was the same dose selected for use in the 'meal test' studies at the start and end of the intervention phase. For the continuous chronic feeding, a lower dose of 20g per day was provided since the aim was to keep the carbohydrate and fat in the bar below 10g, in order to provide an energy content around 420Kj (100Kcal). The chocolate was wrapped in identical white wrappers with labelled with the study identification code, the different chocolates were labelled either as A, B or C.

A three-arm study was chosen, to assess the primary comparison of a milk chocolate high in polyphenols with a low polyphenol chocolate along with a secondary comparison of milk chocolate high in polyphenols with a dark chocolate with equivalent epicatechin content. This design was chosen, in part for pragmatic reasons, to allow for two comparisons without the cost and administration of two separate clinical trials. This design also allowed for the efficacy of milk chocolate as a source of polyphenols to be assessed, with an assessment of potential additional effect compared to dark chocolate which might be an effect of either the different nutritional composition of milk chocolate including calcium and milk fats (including conjugated linoleic acid) or the effect on polyphenol metabolism of milk chocolate (Roura et al., 2007; Mullen et al., 2009, Keogh, McInerney & Clifton, 2007).

Adherence was determined through the counting of returned wrappers, on which the participants marked the time and date of consumption. The participants were advised to consume the chocolate in two 10g portions, one mid-morning and one mid-afternoon or during the evening. The chocolate was dispensed every four weeks over the 12-weeks of the intervention period.

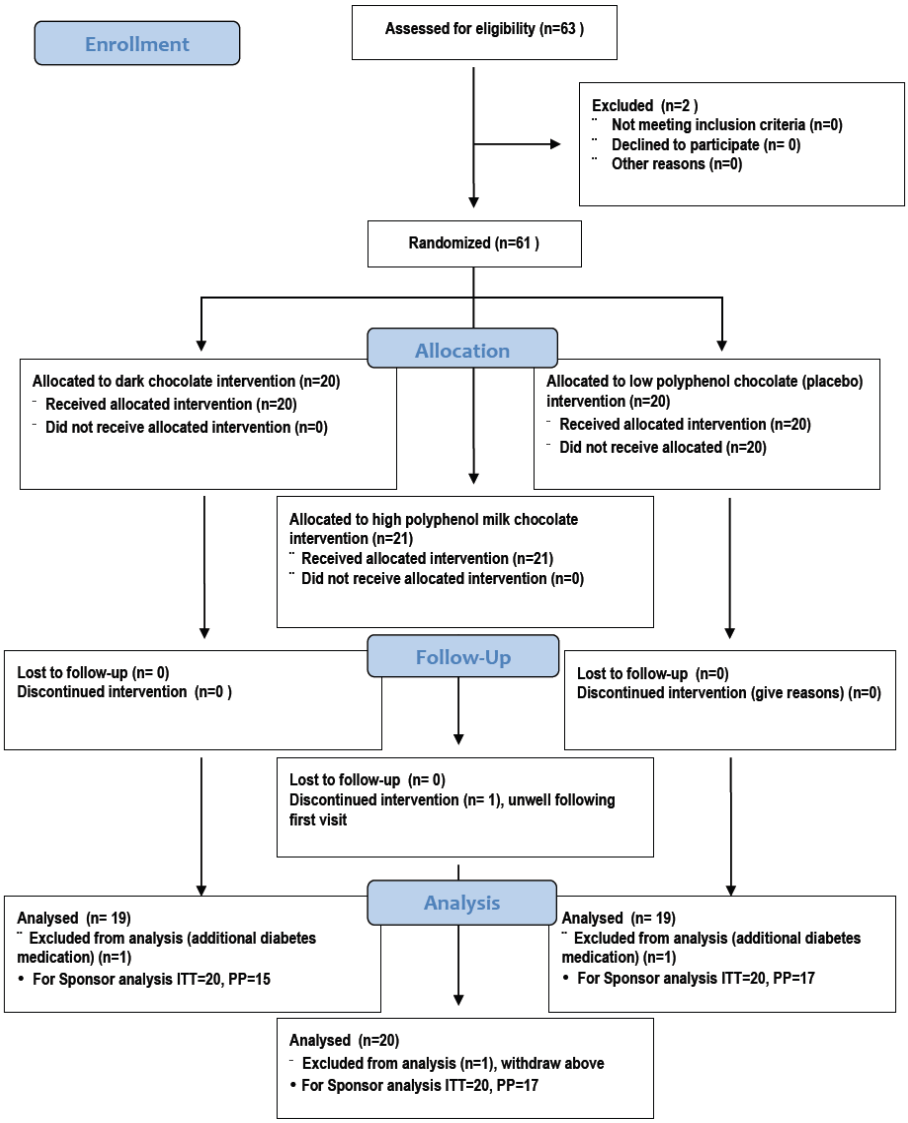


Figure 6.1.1.2: Consort flow diagram indicating the conduct of the study. A CONSORT checklist is presented in Appendix III

Nutritional and dietary compliance with the study was monitored along with any variation in energy and macronutrients by a dietitian interviewing participants and taking a 24-hour recall of intake on a four weekly basis at clinic and dispensing visits.

Following informed consent, all participants attended for fasting blood tests along with clinical and anthropometric testing. Height and weight were measured and used to calculate BMI; waist circumference was measured to assess central obesity and blood pressure measured in accordance with the protocol described in Section 2.3.

Twenty-four hour urine collections were made to measure 15-F2t-isoprostane: creatinine ratio following the protocol described in Section 2.3.2.5.2 ratio to assess oxidative stress. Bloods were also drawn to measure Hba1c, fasting glucose, fasting lipids profile, insulin and inflammatory markers as described in section 2.3 Insulin sensitivity was calculated by HOMA as described in section 2.3.2.3.4.

Four weeks later, participants returned after fasting, for a meal test. Following 15 minutes acclimatisation, participants had fasting endothelial function measured using EndoPAT 2000 (Itamar, Caesarea, Israel), in accordance with the method described in section 2.3.2.4.1. Following the assessment, participants blood was drawn the median-cubital vein for the identical assays employed at screening. This was followed by a clinical and anthropometry assessment. Participants were then given two bars of chocolate (2x20g bars, 40g in total), of the same chocolate to which the participant had been randomized for the 12-week period (taking 20g daily). Blood samples were drawn at 60, 120 and 180 minutes for insulin, glucose and triglycerides. Participants also undertook two 24-hour urine collections; one before their first dose of chocolate and one on their last day of chocolate consumption.

At the end of the assessment visit, participants were issued with enough chocolate for the following 28 days. All participants were instructed to consume their chocolate as part of their normal dietary routines, as described in section 2.3.2.8. Participants

returned for dispensing visits after 28 and 56 days to check compliance and have the anthropometric measurements repeated along with the reporting of any adverse events or changes in medication. At the end of the 86 days all participants returned for a final meal test visit which followed the identical protocol to the first meal test. Participant compliance was confirmed from the return of used packaging. Weight was also measured in 32 participants who attended a four-week post study follow-up.

6.1.2 - Study Questionnaires

At baseline all participants were screened using the Relationship Questionnaire (Bartholomew & Horowitz, 1991), this was used in order to assess if the population is typical with respect to attachment style to the general adult population and populations with diabetes. This has been hypothesised to affect engagement with service and response to a placebo. In addition, at screening and each visit (both clinical and dispensing visits), participants completed the Stanford Sleep Scale, (Hoddes, Dement & Zarcone, 1972) subjective assessment of mood (Bond & Lader, 1974) and Samn-Perlli subjective fatigue score (Samn and Perelli, 1982). These were used to assess participant reported outcomes linked with fatigue and mood; the rationale for their inclusion was following the findings of Sathyapalan et al. (2010) which suggested a beneficial effect of high polyphenol chocolate on symptoms of chronic fatigue syndrome.

6.1.3 - Study Set-up

- Group 1 (active): 20g/d of milk chocolate product, which provided 20mg epicatechin/day (high polyphenol). The milk chocolate contained approximately 1 mg/g epicatechin from the cocoa liquor, which was supplemented by the addition of the high-polyphenol cocoa ingredient, CocoanOX 12%® (Natraceutical S.A., Valencia, Spain) added to the chocolate base.
- Group 2 (control): 20g/d of product 1 mg epicatechin/day (low polyphenol). The

control was a milk-type product, which was matched to the group 1 (active) milk chocolate in terms of milk, energy, sugar and fat content.

- Group 3 (active): 20g/d of dark chocolate product giving 20mg epicatechin/day. This was a 70% cocoa commercially available dark chocolate containing cocoa liquor, sugar, cocoa butter, milk fat, lecithin and vanillin. It naturally contained approximately 1 mg/g epicatechin from the cocoa liquor. This was identical to Nestlé Noir 70%, which is freely available for sale in continental Europe.

A summary of the chocolate compositions is given in table 6.1.3 A-C.

Tables 6.1.3: A, B and C Composition of chocolate provided to participants throughout the course of the study. Further information regarding the composition and recipe for the chocolate have been restricted by the sponsor of the study.

A. Chocolate 1 Milk chocolate

Milk Chocolate with added flavanols	g	Kcal /g	Kcal/100g	EC mg/ 20g	EC mg/100g	
Cocoa liquor (55% fat)	5.5	5.6	30.8	2.71	14.91	
Cocoa butter	24.7	9.0	222.3			
Sugar	45.0	4.0	180.0			
Skimmed milk powder	14.9	3.6	53.6			
Milk fat	5.2	9.0	46.8			
Lecithin	0.41	3.6	1.5			
Vanilla	0.05	4.5	0.23			
CocoanOX (12%fat)	4.2	2.6	10.8	16.4	82.00	
						Per bar 20g
Total epicatechin (mg)	95.55			19.11		19.11
Total Kcal	546.0					109.19
Cocoa solids	34.4					6.88
Cocoa solids non fat	6.2					1.23
Milk solids	20.1					4.03
Milk solids non-fat	14.9					2.99
Total sugar	45.0					9.00
Total fat	33.8					6.77

B. Control Chocolate – iso-fat/ iso-sugar

Control iso fat/ iso sugar	g	Kcal /g	Kcal/100g	EC mg/20 g	EC mg/100g	
Cocoa liquor (55% fat)	2.1	5.6	11.7	2.71	5.69	
Cocoa butter	30.0	9.0	270.0			
Sugar	45.8	3.6	183.0			
Skimmed milk powder	15.3	9.0	54.9			
Milk fat	6.3	9.0	56.7			
Lecithin	0.50	3.6	1.8			
Vanilla	0.05	4.5	0.22			
CocoanOX (12% fat)	None					
						Per bar 20g
Total epicatechin (mg)	5.69			2.71		2.71
Total Kcal	578.4					115.68
Cocoa solids	32.1					6.42
Cocoa solids non fat	0.9					0.19
Milk solids	21.6					4.32
Milk solids non-fat	15.3					3.06
Total sugar	45.8					9.15
Total fat	38.0					7.59

Table 6.1.3.1C: Nestlé Noir Chocolate – Dark Chocolate

Dark Chocolate	g	Kcal /g	Kcal/100g	EC mg/ 20g	EC mg/100g	
Cocoa liquor (55% fat)	63.1	5.6		19	95.00	
Cocoa butter	7.4	9.0				
Sugar	26.2	3.6				
Skimmed milk powder		9.0				
Milk fat	2.9	9.0				
Lecithin	0.4	3.6				
Vanilla	0.05	4.5				
CocoanOX (12% fat)	None					
						Per bar 20g
Total epicatechin (mg)	95.0					19.0
Total Kcal	551.5					110.3
Cocoa solids						
Cocoa solids non fat						
Milk solids						
Milk solids non-fat						
Total sugar						
Total fat	44.7					8.94

6.1.3.1 - Inclusion criteria

All participants were required to comply with all the following inclusion criteria:

- T2DM managed by diet alone or diet and metformin. If metformin was used the dose should have been stable for a minimum of three months prior to the start of the study,
- HbA1c below 9.9% DCCT aligned (85mmol/mol),
- Aged between 45 and 75 years at the start of the study
- All female participants post-menopausal, based on self-reported last date of menstruation being more than 12 months prior to the start of the study
- BMI between 25 and 39 kg/m^2,
- Had attended a structured group patient education program and on stable medication for hypertension, lipids and gout for 3 months prior to entry into the study.

6.1.3.2 - Exclusion criteria

Participants representing one or more of the following criteria were excluded from participation in the study.

- Participants with concurrent illness or any changes in medication in the previous three months,
- Participants whose T2DM was managed with thiazolidinediones, DPP-IV inhibitors, GLP-1 analogues, insulin or sulphonylureas or prandial regulators,
- Participants not wishing to allow disclosure to their GPs,
- Hba1c at recruiting stage of >10.0% DCCT aligned (86mmol/mol),
- Participants who could not be expected to comply with treatment,
- Participants who were enrolled or had participated in another clinical trial during three months prior to the beginning of this study,
- Participants who consumed more than 20g/d of chocolate or had a very high polyphenol content in their diet, and were not willing to change their diet,
- Participants taking high dose antioxidant supplements including single and multivitamin preparations including/containing A, C and/ or E,
- Female participants taking HRT treatment.

6.1.4 - Statistical Methodologies

An external statistician, as specified in the research agreement, undertook the analyses on differences between the three groups. I undertook additional analyses including the analysis of the baseline data, and the nutritional intake and the post study follow up data.

6.1.4.1 - Analysis of baseline, nutrition and post study data

Data were tested for normality using Shapiro-Wilks, both for the whole sample and between groups. Data were presented as mean and standard deviations, before and after the intervention. Where data violated normality, equivalent non-parametric tests were selected. For the whole cohort and each of the three arms, paired *t*-tests were used to assess the effect of the intervention on the various parameters. To determine the difference between chocolates prior to and after feeding a repeated measures design ANOVA was used. Where significant differences were found, the absolute change in parameter was tested with a one-way ANOVA with post-hoc testing using a Bonferonni correction.

6.1.4.2 - Sample-size calculation

The calculation suggested that 48 volunteers (16 per group) were required for this study. However, a dropout rate of approximately 20% was anticipated. Thus it was decided to enrol a total of 60 volunteers (20 per treatment group) onto the study. The above calculation was implemented using N-Query software 4.0 (Statistical Solutions, Saugus, MA, USA). The calculation was primarily based on the effect of chocolate supplementation in hypertensive participants on insulin resistance as assessed by HOMA (Grassi et al., 2008; Grassi et al., 2005b) This was based on an analysis of covariance (ANCOVA) at a significance level of 5% with a statistical power of 80% assuming an estimated standard deviation of 0.9. Such power would enable a difference of $\Delta = 1.0$ in mean HOMA which was considered clinically meaningful to be detected

6.1.4.3 - Planned statistical analysis (intervention data)

6.1.4.3.1 - Primary analyses

The treatment effects were estimated using ANCOVA where milk chocolate (rich in polyphenols) and the low polyphenol control chocolate were to be compared adjusting for the effect of relevant covariates in the model such as HOMA value at baseline. Appropriate non-parametric methods

were to be applied if the data did not meet the statistical model's normality assumptions despite relevant transformation methods. The primary analysis was to be performed as both intention-to-treat (ITT) and per-protocol (PP) populations.

6.1.4.3.2 - Secondary analyses

The cardiovascular parameters; primarily endothelial function and cholesterol profile were assessed during the study as secondary outcomes. The treatment effects were estimated for the milk chocolate and the control chocolate using ANCOVA as for the primary analysis. In addition, the treatment effects on HOMA and cardiovascular parameters were estimated between milk chocolate and dark chocolate using ANCOVA as above. All secondary analyses were performed on the ITT population only.

6.1.4.3.3 - Changes from planned analysis and updated statistical analysis plan

The primary analysis was performed on the log-transformed HOMA using a robust ANCOVA model based on MM-estimators to take into account the outlying values in the data adjusting for age and prior to starting chocolate supplementation for HOMA.

Secondary endpoints: All the analyses of the secondary outcomes were based on the ITT population.

- HbA1c percentage: A similar ANCOVA model as the primary endpoint was used after log-transforming the data.
- Other secondary outcomes: A similar ANCOVA model as the primary endpoint was used after a suitable Box-Cox transformation. In case a suitable transformation was not found, Wilcoxon two-sample rank-sum test was used to test for differences between the appropriate contrasts while these differences were quantified using the Hodges-Lehmann estimate with its 95% confidence interval. For measurements done over time such as glucose, insulin, triglycerides and body weight, a linear mixed modelling approach was used.

All the statistical analyses were done in R 2.14.0; a piece of proprietary software belonging to the sponsor. The sponsor undertook the statistical analysis in line with the research agreement.

6.1.4.4 - Randomization

Participants were randomly assigned to one of the three groups using age and gender as the stratification factors. The randomization was performed using the software TRIALSYS which was developed by the sponsors.

6.1.5 - Protocol Deviations and Analysis Populations

A total of 62 participants were recruited into the trial. The intention-to-treat (ITT) set comprised of a pseudo-population (full analysis set) with all the participants who completed all visits of the trial. One participant joined another trial after recruitment while two other participants dropped out during the course of the trial. These three participants were not included in the full analysis population. The per-protocol (PP) population was based on the blind data review. All the participants who were excluded from the PP dataset and the reasons for their exclusion are given in Table 6.1.5.1. The PP dataset therefore comprised 48 participants.

Table 6.1.5.1: The participants excluded from the per protocol analysis, exclusion criteria independently assigned by the sponsor.

Subject ID	Reason	Value
042	BMI at inclusion	44.5 kg/m^2
014	Restricted medication	Simvastatin
026	Restricted medication	Simvastatin
047	Restricted medication	Simvastatin
002	Visit 4 out of window (>101 days)	102 days
008	Visit 4 out of window (>101 days)	130 days
012	Visit 4 out of window (>101 days)	104 days
020	Visit 4 out of window (>101 days)	105 days
050	Visit 4 out of window (<81 days)	77 days
056	Visit 4 out of window (>101 days)	140 days
059	Visit 4 out of window (>101 days)	107 days

6.1.6 - Demographic and Baseline Characteristics

Several demographic and baseline characteristics were recorded during the screening visit (V0). These are summarized in Tables 6.2.1. and 6.2.1.1 Overall, 31 men and 31 women were enrolled into the trial. 8% of these were smokers of which 60% were male.

6.1.7 - Compliance

Compliance with respect to chocolate intake was monitored throughout the trial. All participants were asked to keep wrappers from the consumed products, writing on them the dates when they were consumed during the entire trial and bring them back to the investigator for the intermediate and final visits.

6.1.8 – Adverse Event Reporting

Adverse events, both harms and unintended effects were assessed by asking all participants at each visit if they had any concomitant illnesses, symptoms or effects since their last visit. This included episodes of hypoglycaemia or symptoms from their diabetes. Additionally any changes to their medication were also recorded at each visit, including routine vaccinations (influenza vaccine), these were then cross referenced by discussion with the participant and medical notes to cross-reference any missed adverse events. Any severe adverse events, including hospitalisation, unplanned surgery and death would have been reported to the sponsor and ethics committee within 24 hours, in accordance with Good Clinical Practice (EMEA, 2002).

6.2 - Baseline features and characteristics

6.2.1 - Study Population

A total of 59 participants completed at least 11 weeks of daily chocolate consumption with complicance being very high (median complicance 96% of bars eaten, 97%, 95%, 94% of bars respectively for high polyphenol milk chocolate, low polyphenol and dark chocolates). Mean age of the cohort was 63.9±8.0 years with a range of 44-79 years. There were two participants who failed to complete the study. The mean duration of diabetes was 3.7±2.3 years, with no difference between groups for age or duration of diabetes. Thirty males and 29 females completed the study. Table 6.2.1, summarises the baseline demographics. Waist and hip circumferences were omitted for 3 participants who declined to have the measurements taken.

Table 6.2.1: Summary statistics for demographic and baseline characteristics following the four week run in period

	Gender	N	Mean	SD	Min	Max
Weight (kg)	**Female**	30.0	86.39	13.2	66.1	113.8
	Male	31.0	93.47	13.1	71.4	117.6
Height (cm)	**Female**	30.0	162.16	7.6	141.0	178.0
	Male	31.0	176.32	7.7	157.0	195.0
BMI (kg/m^2)	**Female**	30.0	32.86	4.6	25.1	44.5
	Male	31.0	30.05	3.8	25.0	39.3
Hip (cm)	**Female**	29.0	115.32	10.8	94.0	134.9
	Male	29.0	109.62	7.0	99.0	130.0
Waist (cm)	**Female**	29.0	103.95	10.1	86.8	122.0
	Male	29.0	105.06	10.8	85.0	127.3

Table 6.2.1.1: Summary statistics for demographic by chocolate at screening visit

Characteristic	Dark Chocolate	High Polyphenol Milk Chocolate	Low Polyphenol Chocolate
Age (Mean years ± standard deviation)	62.8±8.8	61.6±7.5	64.8±8.0
Gender ratio (Male: Female)	10:10	11:10	11:10
Duration of Diabetes (Mean years ± standard deviation)	3.9±2.7	3.5±2.1	3.6±2.4
Weight (Kg ± standard deviation)	92.4±13.9	90.2±15.2	87.8±12.1
BMI (Kgm^{-2}± standard deviation)	35.3±14.3	31.3±4.3	30.9±3.9
Oral Diabetes Medication (Metformin: None)	6:14	9:12	11:9
Lipid Lowering Medication (Yes : None)	12:8	12:9	12:8
Blood Pressure Medication (Yes : None)	16:4	11:10	13:7

There were only five participants who reported that they currently smoked at their screening visit, three were male (two were allocated to the dark chocolate and the other to the low polyphenol chocolate) and two females who were allocated to the high polyphenol milk chocolate and low polyphenol chocolate arms respectively

6.2.2 - Anthropometrics

Weight for the whole population at screening was 89.1±13.0 kg, BMI 31.2±4.8kgm^{-2}. This was found to reduce significantly in the whole study population during the four-week run-in phase to 88.5±13.4kg, BMI 30.7±4.4kgm^{-2} (p=0.01). Table 6.2.1, shows the overall population at the start of the intervention. The population was characterised by central obesity, with mean BMI, waist and waist circumference all being indicative of this feature (Klein et al., 2007; Alberti, Zimmet and Shaw, 2006). No significant effect on weight was seen during the twelve weeks of chocolate consumption (88.2±13.3kg, BMI 30.9±4.5kgm-2, visit 1 – visit 4, p=0.825). Of the participants who were followed up four weeks after discontinuing daily chocolate consumption (n=32), a notable weight increase was seen across the whole study cohort, with 4 weeks after discontinuing the study, mean weight was 86.8±12.8kg and BMI 30.9±4.6kgm^{-2} (change from visit 4 to post study monitoring, p=0.02).

6.2.3 - Dietary Intake

With the exception of the statistically significant difference at visit four between those consuming the milk chocolate and control with respect to sodium intake (p=0.028), it appeared that the milk chocolate group consumed less sodium than the control (Tables 6.2.3.1 and 6.2.3.2). No significant differences were seen between visit one and visit four.

Energy and macronutrient intakes were not found to be significantly different between the three groups. The same result was seen for fibre and vitamin C, intake suggesting that there were no changes in consumption of plant based foods or flavanols over the course of the study.

Data regarding food intake were collected at the screening visit (V0) and intermediate visits (V1 & V4): no change in energy, macronutrient or micronutrients observed at these points (data not presented).

Table 6.2.3.1: Dietary Intake of the three study groups at Visit One and Four (beginning and end of 12 weeks of chocolate consumption). P-values represent the ANOVA between groups and the overall population over time. Time group effects were not explored. All data were tested for normality using Shapiro-Wilks and found not to violate this assumption.

		Dietary Intake Visit One		Dietary Intake Visit Four		P value	
						Between Chocolate Groups	
						Between Visits	
		Mean	Std. Deviation	Mean	Std. Deviation	Visit One	Visit Four
Weight of Food	Milk	1895.2g	859.1	2012.9g	792.1	0.501	0.758
	Control	1619.6g	507.4	1869.7g	530.0	0.252	
	Dark	1751.3g	520.5	1856.9g	587.1		
Water	Milk	1538.7 ml	859.2	1644.5ml	734.7	0.399	0.664
	Control	1233.6ml	450.6	1489.6ml	461.9	0.235	
	Dark	1368.7ml	471.3	1469.8ml	525.6		
Energy (calories)	Milk	1428.7kcal	445.8	1495.0kcal	319.9	0.426	0.608
	Control	1649.7kcal	471.6	1662.3kcal	529.3	0.604	
	Dark	1554.1kcal	511.0	1623.8kcal	570.5		
Energy (Kj)	Milk	5652.0kj	2341.8	60144kj	1992.0	0.241	0.434
	Control	6921.7kj	1970.3	6982.9kj	2226.9	0.592	
	Dark	6536.1kj	2135.7	6812.3kj	2372.9		
Protein	Milk	61.1g	17.7	69.6g	14.2	0.410	0.136
	Control	71.8g	21.5	80.7g	20.1	0.158	
	Dark	66.6g	27.2	67.9g	17.9		
Fat	Milk	57.8g	25.8	60.1g	25.0	0.707	0.788
	Control	65.6g	23.1	67.3g	22.9	0.730	
	Dark	62.1g	29.5	63.7g	35.3		
Saturated fatty acid intake	Milk	18.9g	9.2	20.8g	8.3	0.822	0.949
	Control	21.0g	11.2	22.1g	10.3	0.607	
	Dark	21.0g	12.3	21.4g	13.5		
Carbohydrate	Milk	168.3g	64.83	170.3g	42.5	0.299	0.565
	Control	203.2g	70.3	192.8g	84. 0	0.852	
	Dark	188.2g	53.0	188.3g	53.7		
Sugar	Milk	52.5g	33.3	64.1g	34.4	0.353	0.939
	Control	66.0g	29.8	67.0g	37.4	0.715	
	Dark	67.7g	35.1	62.3g	38.2		
Fibre	Milk	13.2g	6.6	13.1g	8.3	0.916	0.690
	Control	13.0g	5.5	10.9g	4.8	0.640	
	Dark	12.4g	5.0	12.6g	7.9		
Sodium	Milk	2201.3mg	851.6	2099.1mg	771.5	0.152	0.028
	Control	2585.2mg	858.3	3022.4mg	1214.6	0.849	
	Dark	2873.4mg	1138.6	2707.3mg	774.3		
Iron	Milk	9.4mg	3.7	9.5mg	3.1	0.858	0.669
	Control	9.6mg	3.0	10.2mg	4.2	0.582	
	Dark	10.1mg	3.6	10.7mg	4.2		
Vitamin C	Milk	78.3mg	71.5	62.5mg	82.1	0.359	0.666
	Control	61.2mg	32.7	48.9mg	39.2	0.760	
	Dark	51.9mg	43.9	68.1mg	44.1		

Table 6.2.3.2: Differences in dietary Intake of the three study groups at Visit One and Four (beginning and end of 12 weeks of chocolate consumption). P-values represent the ANOVA between groups and the post-hoc analysis (least significant difference) for the primary and secondary comparisons. All data were tested for normality using Shapiro-Wilks and found not to violate this assumption.

		Change in intake between Visit One and Visit Four		ANOVA P value	Post Hoc Analysis (LSD) Between Chocolate Groups	
		Mean	Std. Deviation		High Polyphenol Milk Chocolate Vs Low Polyphenol Chocolate	High Polyphenol Milk Chocolate Vs Dark Chocolate
Weight of Food	**Milk**	126.1g	845.2	0.895	0.719	0.930
	Control	213.3g	598.8			
	Dark	105.6g	462.3			
Water	**Milk**	119.7 ml	796.2	0.844	0.645	0.933
	Control	224.7ml	556.2			
	Dark	101.09ml	440.1			
Energy (calories)	**Milk**	46.9kcal	418.1	0.864	0.709	0.873
	Control	-8.5kcal	458.7			
	Dark	69.6kcal	326.1			
Energy (Kj)	**Milk**	322.0kj	1750.3	0.831	0.577	0.939
	Control	-25.8kj	1892.6			
	Dark	276.2kj	1346.9			
Protein	**Milk**	8.3g	22.3	0.675	0.800	0.389
	Control	6.2g	20.3			
	Dark	1.3g	25.0			
Fat	**Milk**	0.9g	18.7	0.996	0.970	0.929
	Control	1.2g	27.7			
	Dark	1.7g	25.5			
Saturated fatty acid	**Milk**	1.3g	6.6	0.968	0.855	0.807
	Control	0.7g	13.2			
	Dark	0.5g	8.5			
Carbohydrate	**Milk**	1.0g	64.2	0.775	0.521	0.965
	Control	-12.1g	63.2			
	Dark	0.1g	34.5			
Sugar	**Milk**	7.9g	34.2	0.390	0.288	0.204
	Control	-3.2g	33.9			
	Dark	-5.4g	17.5			
Fibre	**Milk**	-0.2g	7.5	0.449	0.329	0.834
	Control	-2.3g	3.8			
	Dark	0.2g	4.7			
Sodium	**Milk**	-15.6mg	969.3	0.284	0.252	0.701
	Control	451.7mg	1041.0			
	Dark	-166.1mg	1211.0			
Iron	**Milk**	0.9mg	3.5	0.932	0.709	0.853
	Control	0.4mg	3.0			
	Dark	0.6mg	4.0			
Vitamin C	**Milk**	-2.6mg	101.2	0.600	0.754	0.456
	Control	-10.8mg	37.7			
	Dark	16.3	53.1			

6.2.4 - Baseline Characteristics – Attachment Type

There were no significant differences in attachment style (how individuals relate to individuals or potentially services) between groups; also, attachment style did not predict a positive outcome. Eighty five percent of the participants who consented to take part in the study had a secure attachment style. A Chi^2 test of independence, demonstrated that this was significantly different to that published for the general population (55-65%) and in populations of people with T2DM (30-40.2%) (Ciechanowski et al., 2004; Ciechanowski, Hirsch, & Katon, 2002) ($p<0.001$).

6.2.5 - Interrelationships between Clinical Measures at Baseline

The published literature regarding the effects of cocoa flavanols and the six markers of diabetes control or cardiovascular risk were explored in the whole cohort of 60 participants at baseline. A multiple regression analysis using a step 1 model and step 2 model, suggested that the change in endothelial function appeared to account for 19.1% of the variance in HDL cholesterol ($F=0.001$); the addition of systolic blood pressure to endothelial function appeared to account for 26.4% of the variation in HDL cholesterol. The correlation between endothelial function (assessed by RH-PAT) and HDL cholesterol can be seen in figure 6.2.5.1 for the whole cohort and in figure 6.2.5.2 for the 28 participants who were not taking lipid-lowering therapy. In this group, endothelial function appeared to account for 32.6% of the variation in HDL cholesterol. This suggested that in the absence of statin therapy, the influence of endothelial function upon HDL cholesterol was greater than seen in those treated with statins. However, this might have been limited by the sample size.

These analyses of variance were highly significant, supporting the hypothesis that there is an inter-relationship between these features of cardiovascular risk as discussed in

Chapter One, and shown to be moderated by chocolate consumption in Chapters Four and Five.

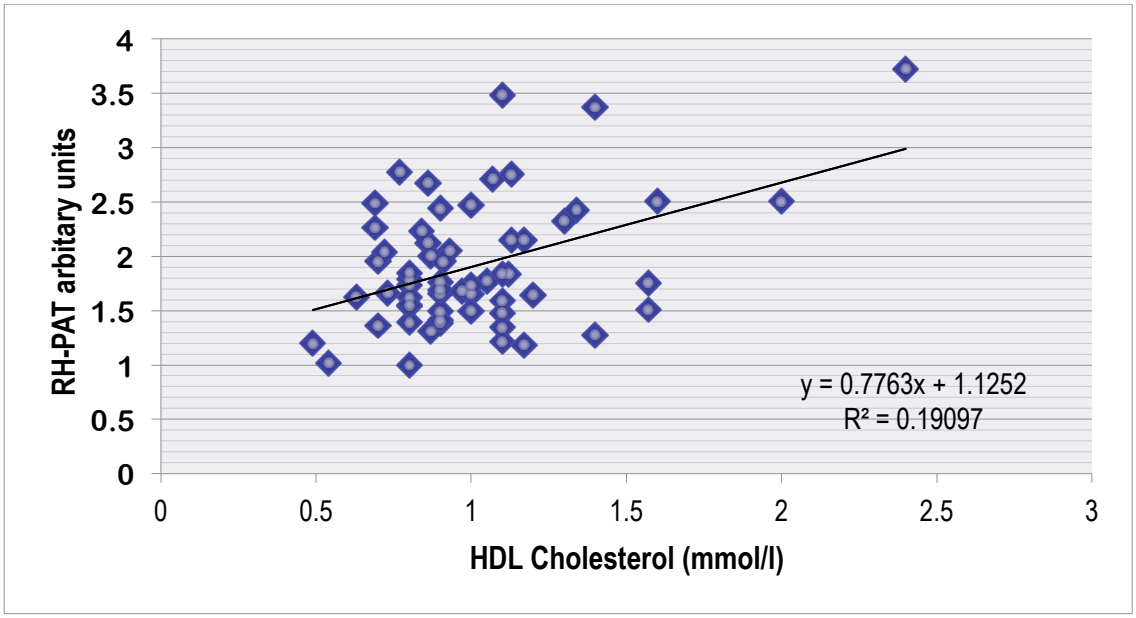


Figure 6.2.5.1: Correlation between endothelial function and HDL cholesterol at baseline across the whole study population (n=60)

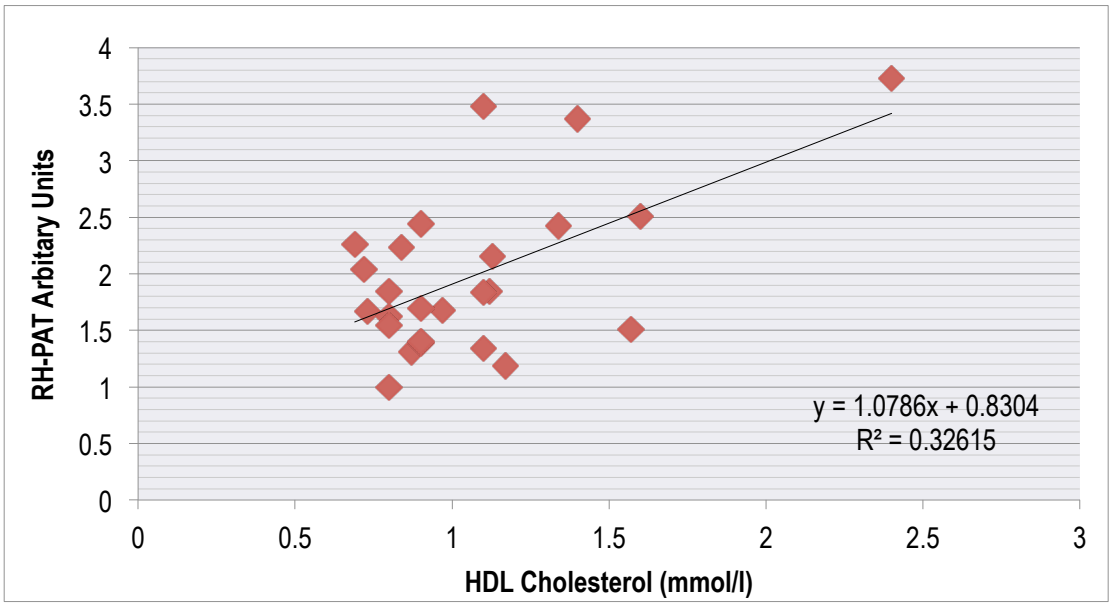


Figure 6.2.5.2: Correlation between endothelial function and HDL cholesterol at baseline in the study population not treated with HMG-Co-ase reductase inhibitors (statins) **(n=28, 26 on no treatment and 2 on ezetimide)**

6.2.6 - Adverse Events and Concurrent Medication

The study was partly undertaken during the winter 2009 during the Swine Flu epidemic (Donaldson et al., 2009). It was noted that a number of the participants may have demonstrated an acute phase response indicated by a CRP >8mg/l, a potential indicator of acute infection. Thirty-one participants were taking metformin, 34 were on lipid medication (2 ezetimibe, managed with at least a statin) and 25 on blood pressure medication. Two participants had their medication adjusted by their general practitioner and were therefore removed from the analyses undertaken by the author, the only changes in the robust ANCOVA model used by the sponsor are reported in table 6.1.5.1. A full summary of the adverse events (table 6.2.6), show that upper respiratory tract infections were the most common event, occurring more frequently in the dark chocolate and low polyphenol groups. This was unlikely to be an effect of the chocolates, but could have acted as confounding and lead to changes in inflammatory markers in these groups and possibly insulin resistance.

Table 6.2.6: Summary frequencies of adverse events separated by the type of chocolate

Adverse Event	Dark Chocolate	High Polyphenol Milk Chocolate	Low Polyphenol Chocolate	Association with chocolate
Upper Respiratory Tract Infections (including Influenza)	5	1	6	Unlikely
Diarrhoea and vomiting	2	1		Unlikely
Nausea and Vomitting			1	Unlikely
Depression and anxiety			1	Unlikely
Dizziness and postural hypotension	1			Possible, participant required a reduction in blood pressure medication
Road Traffic Accident			1	Unlikely, no link of accident to diabetes/ hypoglycaemia
Increase/ Change medication	1	1		Unlikely as both participants demonstrated a reduction in Hba1c during the study

6.2.7 - Post Study Effects on Weight

No effect on weight was seen during the course of the study (Sections 6.3 and 6.4). However, figure 6.2.7.1 shows that during the run-in period participants (n=60) appeared to lose weight (p=0.001). The cohort of 32 participants who returned four weeks after completing the study had significantly gained weight since their last study visit (p=0.002, PP analysis).

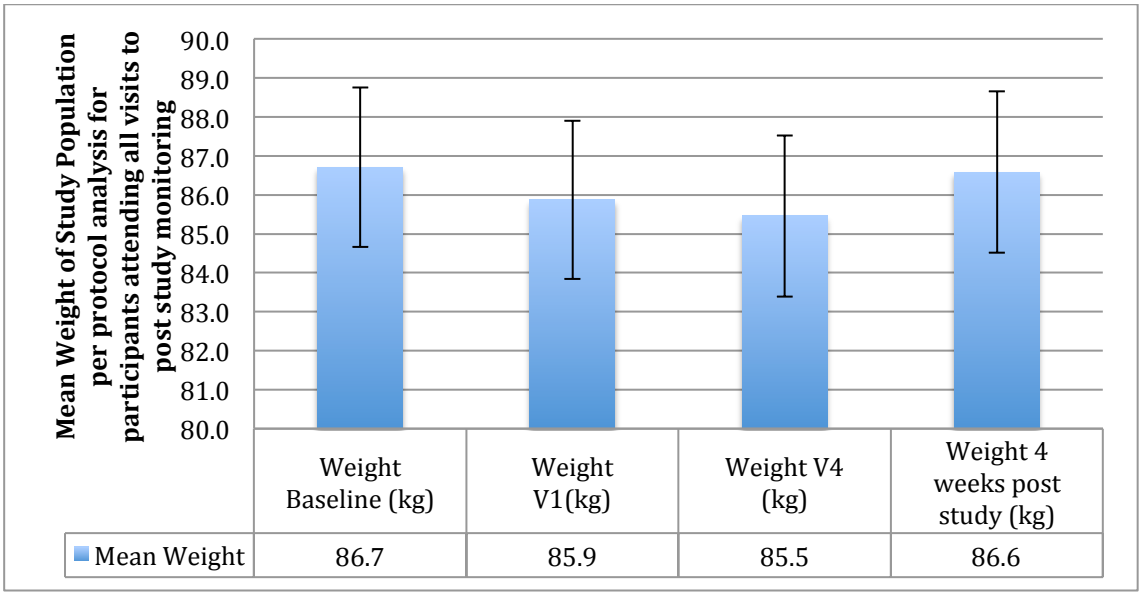


Figure 6.2.7.1: Change in weight over the total course of the study from screening (-28 days), chocolate feeding (0-84 days) and post study monitoring (112 days). **Weight screening to starting chocolate feeding (v1) p=0.001 with a mean weight loss of 0.75±0.17kg, weight was stable through the 20g (approx. 100kcal per day stage) 0.15±0.20kg weight gain (p=0.483) and with weight gain following the end of the study 0.70±0.18kg(p=0.002). Data presented as mean weight± standard error of mean (kg).**

6.3 - Chronic Effects of Chocolate Consumption

The data were tested to explore changes from baseline across the whole cohort and within groups (Table 6.3.1). This suggested that within the whole population fasting insulin fell significantly, in both the milk and control chocolate groups, but not in the dark chocolate group. There was also an increase in total cholesterol and HDL cholesterol across the whole study population. This was also seen in the control group for total cholesterol, and in the dark chocolate group for HDL cholesterol. This did not suggest a change compared to control. These relationships were further explored using the ANCOVA model (Section 6.3.3.2).

6.3.2 - Primary Outcome

The primary outcome for this study was the changes in homeostatic model assessment (HOMA) between visit one and visit four. Geometric means for HOMA at initiation of chocolate supplementation (Visit One -V1) and the end of the chocolate supplementation (Visit Four-V4) and their 95% confidence limits are given in Table 6.3.2.1 for each treatment group. These geometric means with the upper and lower quartiles are also displayed in Figure 6.3.2.1. On average, the high polyphenol milk chocolate induced the highest decline in HOMA values while the dark chocolate had the lowest.

Table 6.3.1: Baseline and twelve-week biochemical data for whole study cohort and for each of the three chocolate intervention groups. HPMC – High Polyphenol Milk Chocolate, HPDC – High Polyphenol Dark Chocolate and LPC – Low polyphenol intake. All data tested for normality using Shapiro –Wilks and then difference from baseline calculated using paired t-test. α indicates p<0.05 and β indicates p<0.01.

	Whole Cohort		Milk Chocolate		Dark chocolate		Control	
	Visit One	Visit Four	Visit One	Visit Four	Visit One	Visit Four	Visit One	Visit Four
HOMA	*6.4±4.5*	*3.4±2.8 β*	*8.1±5.7*	*3.4±2.8 α*	4.5±2.7	3.5±2.7	*6.1±3.8*	*3.2v3.0 α*
QUICKI	*0.51±0.12*	*0.59±0.13 β*	*0.48±0.8*	*0.58±0.11 β*	0.58±0.18	0.59±0.15	*0.50±0.8*	*0.61±0.15 β*
Insulin mU/l	*20.5±13.7*	*10.1±8.0 β*	*24.7±16.7*	*9.0±5.7 β*	15.4±9.4	11.2±9.1	*20.5±12.5*	*10.4±9.4 β*
Glucose Mmol/l	7.1±1.4	7.3±1.6	7.5±1.7	7.9±1.4	6.8±0.8	7.0±1.3	6.9±1.2	6.9±1.3
HbA1c mmol/mol	47.3±10.0	*48.5±11 α*	49.8±13.4	51.4±15.1	44.2±6.8	44.7±6.5	47.0±7.5	48.5±7.5
Cholesterol mmol/l	*3.99±1.00*	*4.27±0.95 α*	4.14±1.06	4.37±0.87	3.63±0.91	4.12±0.78	*4.13±1.01*	*4.29±1.17 α*
HDL Cholesterol mmol/l	*0.98±0.03*	*1.06±0.26 α*	*0.99±0.24*	*1.09±0.25*	0.98±0.31	1.09±0.35	0.98±0.27	1.02±0.20
LDL Cholesterol mmol/l	2.32±0.84	2.48±0.90	2.34±0.78	2.52±0.81	2.06±0.78	2.30±0.69	2.46±0.93	2.58±1.14
Triglycerides mmol/l	1.62±0.82	1.69±0.81	1.66±0.77	1.65±0.82	1.78±0.93	1.50±0.59	1.41±0.77	1.88±0.96
hsCRP mg/l	1.81±1.53	1.92±1.76	2.03±1.62	2.11±2.228	1.36±1.63	1.67±1.59	1.90±1.36	1.93±1.26
15-F2t-isoprostane	196.6±235.9	147.7±69.0	170.0±113.6	150.0±59.3	189.3±138.6	173.0±94.0	229.0±361.7	130.1±54.6

The sponsor of the study analysed this data using a Box-Cox transformation and a robust ANCOVA model. Table 6.3.2 shows the summary statistics for the primary and secondary clinical and biochemical outcome measures. The contrast estimate is the estimate of the difference of the active compared to the control. The control is corrected to 1.0 and then an estimate of the difference is made following the active intervention, e.g. for HOMA in table 6.3.2, the contrast estimate for high polyphenol milk chocolate compared to dark chocolate was 0.61, implying a 39% reduction following high polyphenol milk chocolate. However this did not reach significance as the 95%CI 0.35-1.05, resulting in p=0.0754.

Table 6.3.2: The summary descriptive and inferential statistics of primary and secondary clinical measures. Data presented as contrast estimate based on the robust ANCOVA model. The sponsor of the study produced data presented.

	Comparison of interventions between Visits 1 and 4	Contrast Estimate	Lower 95%CI	Upper 95%CI	p-value
HOMA (ITT)	High Polyphenol V Low Polyphenol	1.18	0.71	1.96	0.5309
	High Polyphenol V Dark	0.61	0.35	1.05	0.0754
HOMA (PP)	High Polyphenol V Low Polyphenol	0.85	0.43	1.69	0.6439
	High Polyphenol V Dark	0.55	0.30	1.00	0.0518
HbA1c	High Polyphenol V Low Polyphenol	1.00	0.97	1.03	0.8621
	High Polyphenol V Dark	1.00	0.98	1.03	0.8518
Fasting Glucose	High Polyphenol V Low Polyphenol	1.08	0.99	1.18	0.0795
	High Polyphenol V Dark	1.07	0.98	1.17	0.1096
Insulin	High Polyphenol V Low Polyphenol	0.98	0.86	1.46	0.9243
	High Polyphenol V Dark	0.82	0.56	1.20	0.3134
Total Cholesterol	High Polyphenol V Low Polyphenol	1.03	0.95	1.12	0.4335
	High Polyphenol V Dark	1.04	0.96	1.13	0.3572
HDL Cholesterol	High Polyphenol V Low Polyphenol	1.01	0.86	1.18	0.9066
	High Polyphenol V Dark	1.03	0.91	1.16	0.6179
LDL Cholesterol	High Polyphenol V Low Polyphenol	1.04	0.98	1.16	0.5000
	High Polyphenol V Dark	1.03	0.91	1.16	0.6595
Triglycerides	High Polyphenol V Low Polyphenol	0.91	0.69	1.21	0.5276
	High Polyphenol V Dark	0.90	0.68	1.19	0.4530
RH-PAT Endothelial Function	High Polyphenol V Low Polyphenol	2.72	-5.20	10.64	0.5010
	High Polyphenol V Dark	3.16	-4.84	11.15	0.4391
hsCRP	High Polyphenol V Low Polyphenol	1.06	0.84	1.35	0.6047
	High Polyphenol V Dark	0.53	0.34	0.80	0.0028
IL-6	High Polyphenol V Low Polyphenol	1.20	0.78	1.86	0.4113
	High Polyphenol V Dark	0.76	0.49	1.20	0.2419
IL1Ra	High Polyphenol V Low Polyphenol	1.02	0.79	1.31	0.8835
	High Polyphenol V Dark	0.94	0.69	1.29	0.7083
MDA	High Polyphenol V Low Polyphenol	1.55	0.85	2.84	0.1536
	High Polyphenol V Dark	0.93	0.54	1.62	0.8071
Urinary Isoprostane	High Polyphenol V Low Polyphenol	1.16	0.90	1.48	0.2543
	High Polyphenol V Dark	1.05	0.82	1.35	0.6807
Body Weight	High Polyphenol V Low Polyphenol	1.29	-7.25	9.82	0.7675
	High Polyphenol V Dark	-2.10	-10.60	6.39	0.6274

Table 6.3.2.1: Summary statistics for log transformed data for HOMA at Visit 1 and Visit 4

	Visit	Geometric mean	95% Confidence Intervals
Dark Chocolate			
	Visit 1	4.45	3.19-6.21
	Visit 4	2.75	1.72-4.42
Milk Chocolate			
	Visit 1	6.50	4.14-10.21
	Visit 4	2.82	1.93-4.14
Control			
	Visit 1	4.63	2.77-7.74
	Visit 4	2.21	1.50-3.26

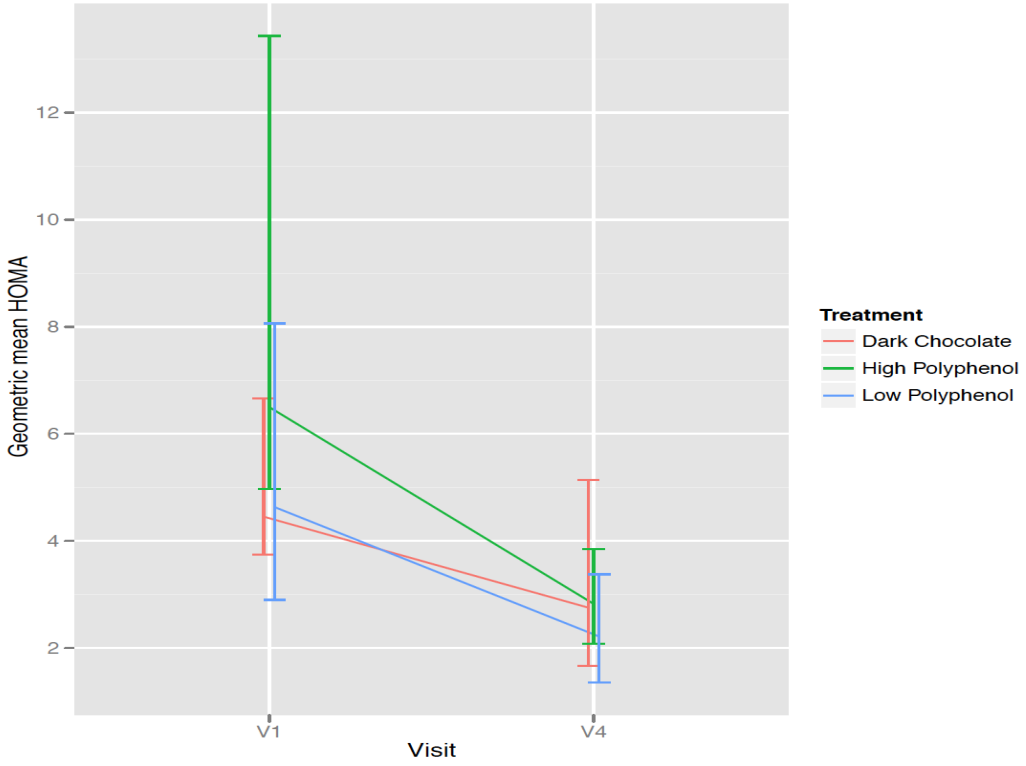


Figure 6.3.2.1: HOMA measurements by chocolate, mean values ±95% Confidence Intervals

These data were log-transformed as suggested by Box-Cox transformation to reduce the degree of skewness. The data then appear to support the raw data analysis that there was a reduction in insulin resistance seen across the study population. It is unclear if changes in weight may have acted as confounders, masking the effects of the flavanols on insulin resistance.

6.3.2.1.1 - Intention-to-treat (ITT) analysis, (n = 59)

The ITT analysis comprised all the study participants who were enrolled into the trials and had measurements taken at any visit up to and including visit four. The analysis for this endpoint was based on the ANCOVA model using the methods of moments (MM)-estimators. The estimated treatment ratios are given in Table 6.3.2.1. The estimated ratio between high and low polyphenol milk chocolate (primary objective) with respect to HOMA was 1.18 indicating an 18% increase. However, this increase was not statistically significant (p = 0.5308). Similarly, high polyphenol milk chocolate showed a reduction in HOMA compared to dark chocolate even though this reduction was not significant (p = 0.0754).

6.3.2.1.2 - Per-Protocol (PP) analysis, (n = 48)

The ANCOVA model fitted to the full analysis dataset was also fitted to the PP dataset defined in Section 6.1.5. The results suggested there was no difference between the high and low polyphenol milk chocolate (p=0.6439) with a 45% reduction in HOMA compared to the dark chocolate, which showed a trend towards, but also failed to reach, significance (p=0.0518) as the estimated 95% confidence intervals was too close to 1.

6.3.3 - Secondary Outcomes

6.3.3.1 - Glycaemia

Changes in glucose will be considered as part of the acute effect of chocolate as part of the meal test in section 6.4.

6.3.3.1.1 - HbA1c

HbA1 c was the key secondary endpoint for this trial. Due to the skewness of the data, a log-transformation was applied as suggested by the Box-Cox transformations. Table 6.3.3.1.1 shows the geometric means of HbA1 c percentage and their 95% confidence intervals across the treatment groups at each visit. On average, both low and high polyphenol milk chocolate induced an increase in the levels of HbA1c. The levels of HbA1 c under dark chocolate appeared to be constant over the duration of the study.

Table 6.3.3.1.1: Summary log transformed statistics for HbA1c at visit 1 and visit 4.

		Geometric mean	95% Confidence Intervals
Dark Chocolate (DCCT %)			
	Visit1	6.33	5.91-6.78
	Visit4	6.32	6.00-6.65
Milk Chocolate (DCCT %)			
	Visit1	6.52	6.06-7.01
	Visit4	6.68	6.12-7.30
Control (DCCT %)			
	Visit1	6.49	6.08-6.93
	Visit4	6.62	6.20-7.06

The estimate of effect suggested no difference between the dark chocolate and the milk chocolate (p=0.8621) or between the milk chocolate and the control (p=0.8518)

6.3.3.2 - Lipid profile

Total cholesterol appeared to increase in all treatment groups with the highest increases observed with dark chocolate and high polyphenol milk chocolate. However, treatment comparisons based on the log-transformed levels of total cholesterol are shown in table 6.3.3.2.1 and there were no statistically significant differences between the treatments in terms of the levels of total cholesterol. Treatment comparison data for HDL cholesterol were also log transformed. The levels of HDL cholesterol appeared to increase in all treatment groups but there were no statistically significant differences between the groups (Table 6.3.3.2.1).

Summary statistics for LDL cholesterol levels are summarized in Table 6.3.3.2.1, with geometric means and 95% confidence intervals for each treatment group at each visit. On average, LDL cholesterol levels appeared to increase under high polyphenol milk chocolate and dark chocolate while remaining relatively constant under low polyphenol milk chocolate in the duration of the trial but the differences were not statistically significant.

Table 6.3.3.2: Summary statistics of lipid profile and p value from robust ANCOVA model for the three chocolates. Note data is log transformed and therefore do not match the absolute values.

		Geometric mean	95% Confidence Intervals	
Total Cholesterol (mmol/l)				
Dark Chocolate	**Visit1**	3.72	3.3-4.2	P-value Vs. Milk chocolate =0.3572
	Visit4	4.03	3.7-4.4	
Milk Chocolate	**Visit1**	4.05	3.5-4.7	
	Visit4	4.31	3.9-4.8	
Control	**Visit1**	4.03	3.6.4.5	P-value Vs. Milk Chocolate = 0.4335
	Visit4	4.08	3.6-4.6	
HDL Total Cholesterol (mmol/l)				
Dark Chocolate	**Visit1**	4.45	3.2-6.2	P-value Vs. Milk chocolate =0.6179
	Visit4	2.75	1.7-4.4	
Milk Chocolate	**Visit1**	6.50	4.1-10.2	
	Visit4	2.82	1.9-4.1	
Control	**Visit1**	4.63	2.8-7.7	P-value Vs. Milk chocolate =0.9066
	Visit4	2.21	1.5-3.3	
LDL Total Cholesterol (mmol/l)				
Dark Chocolate	**Visit1**	2.10	1.8-2.5	P-value Vs. Milk chocolate=0.6595
	Visit4	2.22	1.9-2.6	
Milk Chocolate	**Visit1**	2.20	1.8-2.7	
	Visit4	2.41	2.0-2.9	
Control	**Visit1**	2.32	2.0-2.7	P-value Vs. Milk chocolate =0.5000
	Visit4	2.33	1.9-2.8	

6.3.3.4 - Oxidative stress

15-F2t-isoprostane: creatinine ratios (mg: mol) measured in the urine at visit one and four are given in Table 6.3.3.4.1 (geometric mean with lower and upper 95% confidence limits). Urinary 15-F2t isoprostane appeared to decrease under dark chocolate and low polyphenol milk chocolate while high polyphenol milk chocolate seemed to induce an increase. There were no statistically significant differences between the treatments in terms of urinary 15-F2t isoprostane (estimate milk compared to control 1.16; 95% confidence interval 0.90, 1.38; p=0.2543; high compared to dark p=0.6807).

Table 6.3.3.4.1: Summary log transformed statistics for 15-F2t-sioprostane: creatinine ratios at visit 1 and visit 4.

		Geometric mean	95% Confidence Intervals
Dark Chocolate (mg: mol)			
	Visit1	150.13	115.2-195.6
	Visit4	134.70	106.6-170.2
Milk Chocolate (mg: mol)			
	Visit1	139.90	105.4-185.8
	Visit4	145.07	116.8-180.2
Control (mg: mol)			
	Visit1	123.53	96.0-159.0
	Visit4	108.74	84.2-140.5

No statistically significant differences were seen for malondialdehyde.

6.3.3.5 - Inflammatory markers

No significant changes were seen for the interleukins (IL1Ra and IL6). TNF-α was not measurable in 90% of cases using the high sensitivity ELISA kit available.

High sensitivity-C-Reactive Protein (hs-CRP) significantly decreased following milk chocolate consumption compared to dark chocolate, with an estimated 47% reduction (95% confidence interval 0.35, 0.80; p=0.0028) (figure: 6.3.3.5.1). There was no effect when comparing milk chocolate with control. Summary statistics for hs-CRP are shown in table 6.3.3.5.1.

Table 6.3.3.5.1: Summary for Hs-CRP at visit 1 and visit 4.

		Geometric mean	95% Confidence Intervals
Dark Chocolate (mg/l)			
	Visit1	1.57	0.85-2.88
	Visit4	1.81	0.96-3.38
Milk Chocolate (mg/l)			
	Visit1	1.70	1.06-2.74
	Visit4	1.42	0.85-2.37
Control (mg/l)			
	Visit1	1.84	1.12-3.03
	Visit4	2.24	1.22-4.12

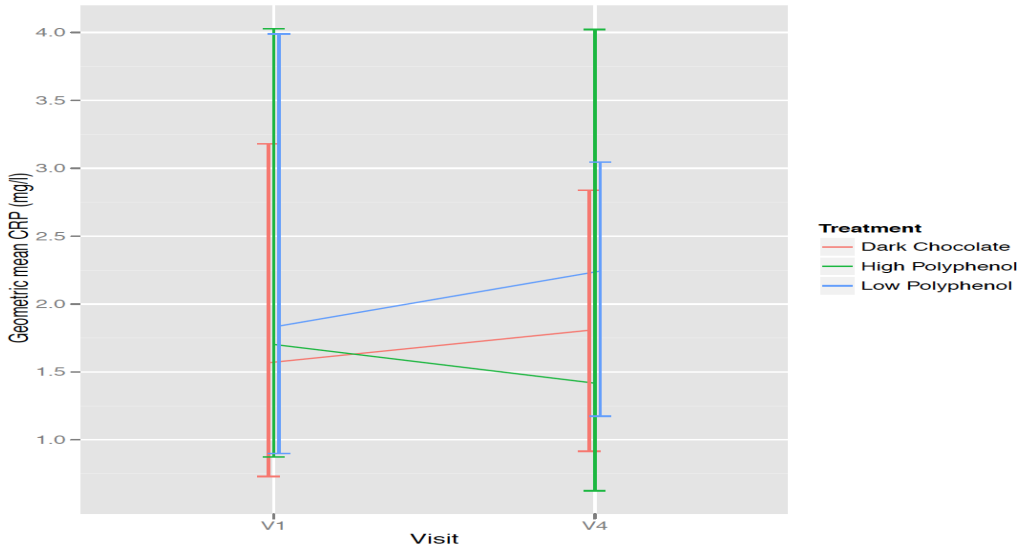


Figure 6.3.5.5.1: Hs-CRP measurements by chocolate, mean values ±95% Confidence Intervals

6.3.3.6 - Body weight

Body weight decreased during the run in phase and increased again after chocolate supplementation ceased (Section 6.2.7).

Weight appeared to be stable during the chocolate supplementation phases of the study. Figure 6.3.3.6.1 shows body weight by chocolate group and figure 6.3.3.6.2 shows individual weight changes over the course of the 12-week study. It is notable that in the dark chocolate arm, two participants lost greater than 5% of bodyweight during the trial despite no recordable change in energy intake.

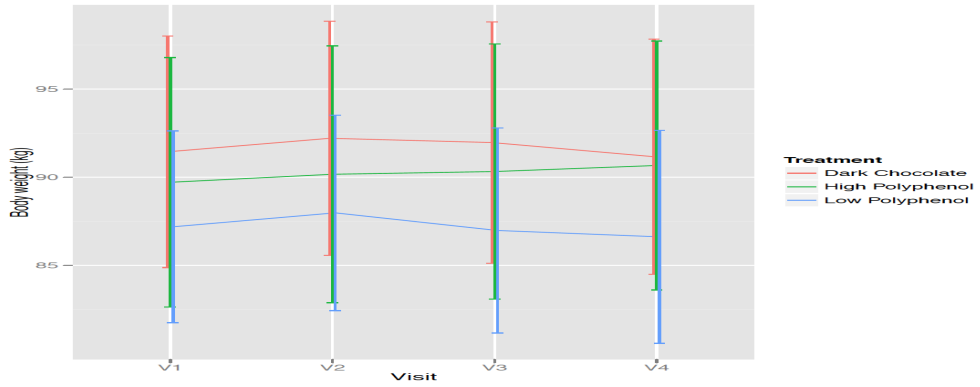


Figure 6.3.3.6.1: Body weight over time (mean and 95% confidence intervals) by chocolate. Weight at the screening visit (V0) was not included in the analysis due to protocol restrictions.

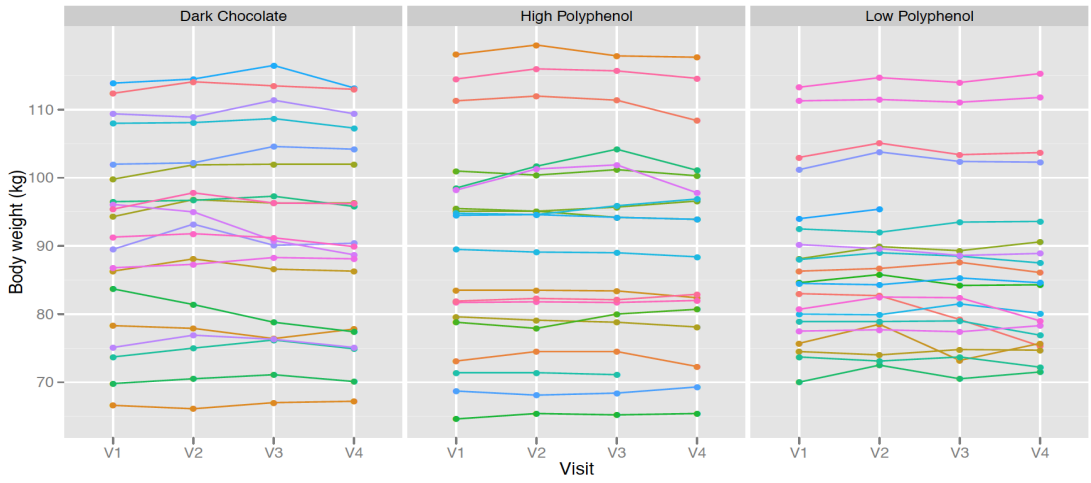


Figure 6.3.3.6.2: Individual body weight over time by individual participant. Weight at the screening visit (V0) was not included in the analysis due to protocol restrictions.

There were no statistical differences between the chocolate groups with respect to body weight during the intervention.

Table 6.3.3.6.1 Estimated treatment differences in terms of body weight

	Estimate	Lower	Upper	p-value
Milk Vs. Control	1.29	-7.25	9.82	0.7675
Milk Vs. Dark	-2.10	-10.60	6.39	0.6274

6.3.3.7 - Participant reported outcomes

No significant differences were seen between the three groups either at visit one or four for any of the questionnaire scores. There were no significant changes in questionnaire scores from visit one to four between groups, descriptive statistics for the questionnaires are presented in table 6.3.3.7.

Table 6.3.3.7: Summary descriptive statistics from the participant reported outcomes.

	Scales	Treatment	n	Geomean	Lower	Upper		n	Geomean	Lower	Upper
V1							V4				
	Alert	Dark Chocolate	20	17.5	11.8	25.9		19	29.6	19.6	44.5
		High Polyphenol	20	19.5	11.9	31.8		20	13.6	8.5	21.9
		Low Polyphenol	20	19.3	13.9	26.8		19	15.6	9.2	26.4
	Amicable	Dark Chocolate	20	13.1	8.7	19.7		20	8.3	3.6	19.2
		High Polyphenol	20	9.3	4.9	17.8		20	8.5	5.5	13.0
		Low Polyphenol	21	13.1	7.0	24.6		21	8.0	3.7	17.5
	Attentive	Dark Chocolate	20	19.3	12.9	28.7		19	20.9	14.3	30.6
		High Polyphenol	20	15.7	8.6	28.6		20	14.1	8.4	23.5
		Low Polyphenol	20	16.3	11.3	23.5		18	14.4	8.9	23.4
	Calm	Dark Chocolate	20	12.6	9.0	17.6		19	13.6	10.0	18.4
		High Polyphenol	20	9.0	5.4	15.0		20	10.1	6.7	15.2
		Low Polyphenol	20	14.1	10.2	19.4		19	11.6	7.5	18.0
	Clear-headed	Dark Chocolate	20	19.9	13.8	28.8		19	23.0	16.1	32.8
		High Polyphenol	20	18.4	11.8	28.8		20	12.0	8.3	17.5
		Low Polyphenol	20	19.3	13.0	28.8		19	18.2	12.7	26.2
	Contentedness	Dark Chocolate	20	12.9	9.3	18.0		20	10.7	5.2	22.2
		High Polyphenol	20	9.7	5.6	16.7		20	6.7	4.6	10.0
		Low Polyphenol	21	10.5	5.3	21.0		21	6.9	3.1	15.1
	Energetic	Dark Chocolate	20	29.0	21.4	39.4		19	30.3	22.1	41.6
		High Polyphenol	20	25.6	16.5	39.5		20	16.5	10.9	25.2
		Low Polyphenol	20	35.2	25.7	48.3		19	23.8	16.9	33.6
	Gregarious	Dark Chocolate	20	21.5	14.9	31.0		19	23.8	17.4	32.7
		High Polyphenol	20	18.0	11.8	27.4		19	12.9	8.4	19.9
		Low Polyphenol	20	25.6	18.5	35.5		19	25.8	17.9	37.3
	Happy	Dark Chocolate	20	13.0	8.8	19.1		19	17.1	11.2	25.9
		High Polyphenol	20	10.6	6.6	17.2		19	8.6	5.1	14.7
		Low Polyphenol	20	12.2	8.0	18.6		19	12.4	8.2	18.8
	Interested	Dark Chocolate	20	13.2	8.5	20.3		19	15.3	9.6	24.3
		High Polyphenol	20	9.5	5.9	15.2		20	7.6	4.6	12.5
		Low Polyphenol	20	12.2	7.8	19.2		19	11.7	7.4	18.4
	Proficient	Dark Chocolate	20	23.0	17.0	31.2		20	14.8	7.5	29.3
		High Polyphenol	20	13.4	6.7	27.0		20	11.2	7.0	17.8
		Low Polyphenol	21	17.5	9.1	33.9		21	10.3	4.5	23.7
	Relaxed	Dark Chocolate	20	15.2	10.9	21.1		20	13.7	7.2	26.1
		High Polyphenol	20	11.7	6.0	22.9		20	11.5	7.9	16.7
		Low Polyphenol	21	14.0	7.4	26.3		21	10.1	4.5	22.7
	Strong	Dark Chocolate	20	17.9	12.0	26.9		19	23.0	15.9	33.3
		High Polyphenol	20	14.6	9.0	23.8		20	12.0	7.8	18.6
		Low Polyphenol	20	22.9	16.4	32.0		19	13.5	8.4	21.6
	Tranquil	Dark Chocolate	20	17.7	12.4	25.1		19	21.7	15.1	31.3
		High Polyphenol	20	11.2	7.7	16.5		20	11.7	7.8	17.4
		Low Polyphenol	20	19.6	13.7	28.0		19	16.7	11.9	23.6
	Well-coordinated	Dark Chocolate	20	14.5	10.1	20.8		19	18.7	12.8	27.4
		High Polyphenol	20	12.6	8.0	19.9		20	9.8	6.5	14.7
		Low Polyphenol	20	12.4	9.0	17.2		19	10.6	6.8	16.6
	Witted	Dark Chocolate	20	22.7	16.3	31.7		20	20.2	10.2	40.3
		High Polyphenol	20	14.2	6.8	29.7		20	14.1	8.8	22.8
		Low Polyphenol	21	20.8	10.8	40.3		21	12.0	5.2	27.9
	Samn-Perelli questionnaire score	Dark Chocolate	20	13.8	6.0	20.0		20	12.4	0.0	18.0
		High Polyphenol	20	13.3	6.0	20.0		20	12.0	2.0	20.0
		Low Polyphenol	20	12.6	2.0	18.0		19	13.7	7.0	17.0
	Stanford sleepiness scale score	Dark Chocolate	20	1.8	1.0	3.0		19	2.1	1.0	6.0
		High Polyphenol	18	1.8	1.0	3.0		20	2.3	1.0	5.0
		Low Polyphenol	20	1.8	1.0	3.0		19	1.8	1.0	3.0

6.4 – Acute Feeding Effects of Chocolate

6.4.1 - Acute Consumption of Chocolate

6.4.1.1 - Endothelial function

6.4.1.1.1 - Reactive hyperaemia index

Reactive Hyperaemia Index (RHI) also defined as Reactive Hyperaemia-Peripheral Artery Tonometry (RH-PAT) was measured at visits one (V1) and four (V4), in each case, both before the consumption of 40g of chocolate and 180minutes later.

As a whole cohort, an improvement of endothelial function prior at V1 following eating 40g of chocolate was apparent (1.855±0.510 compared to 2.146±0.585 3 hours later, p=0.007) with the trend repeated at V4 at the end of the 12-week period of 20g of chocolate daily (1.906±0.510 compared to 2.092±0.468 3 hours later, p=0.060), but at visit four this trend did not reach significance (n=47). No difference was seen in arterial stiffness as measured by augmentation index.

The ANCOVA model did not show any significant effects of time on endothelial function, although the milk chocolate did show the lowest changes in RH-PAT (table 6.4.1.1.1).

Table 6.4.1.1.1: Treatment contrast estimates for RH-PAT on the robust ANCOVA

	Estimate	Lower	Upper	p-value
Milk Vs. Control	-0.15	-0.62	0.31	0.5166
Milk Vs. Dark	-0.21	-0.64	0.23	0.3509

6.4.2.1 - Glucose

Glucose measurements (mmol/l) were taken at visit 4 on four occasions (0, 60, 120 and 180 minutes). These are summarized in Table 6.4.2.1.1. Figure 6.4.2.1.1, shows the profiles of the measurements for each participant. On average, the highest glucose concentrations were observed with milk chocolate. The peak concentrations appeared at 60 minutes on average.

Table 6.4.1.2.1: Summary log transformed statistics for insulin at visit 4

	Treatment	Geometric mean	95% Confidence Intervals
0 mins.			
	Dark Chocolate	6.76	6.20-7.40
	High Polyphenol	7.59	6.75-8.50
	Low Polyphenol	6.72	6.03-7.50
60 mins.			
	Dark Chocolate	7.59	7.02-8.20
	High Polyphenol	8.87	7.92-9.90
	Low Polyphenol	7.74	6.96-8.60
120 mins.			
	Dark Chocolate	6.77	6.23-7.40
	High Polyphenol	8.00	7.04-9.10
	Low Polyphenol	6.99	6.31-7.80
180 mins.			
	Dark Chocolate	6.26	5.84-6.70
	High Polyphenol	6.59	5.79-7.50
	Low Polyphenol	5.94	5.39-6.50

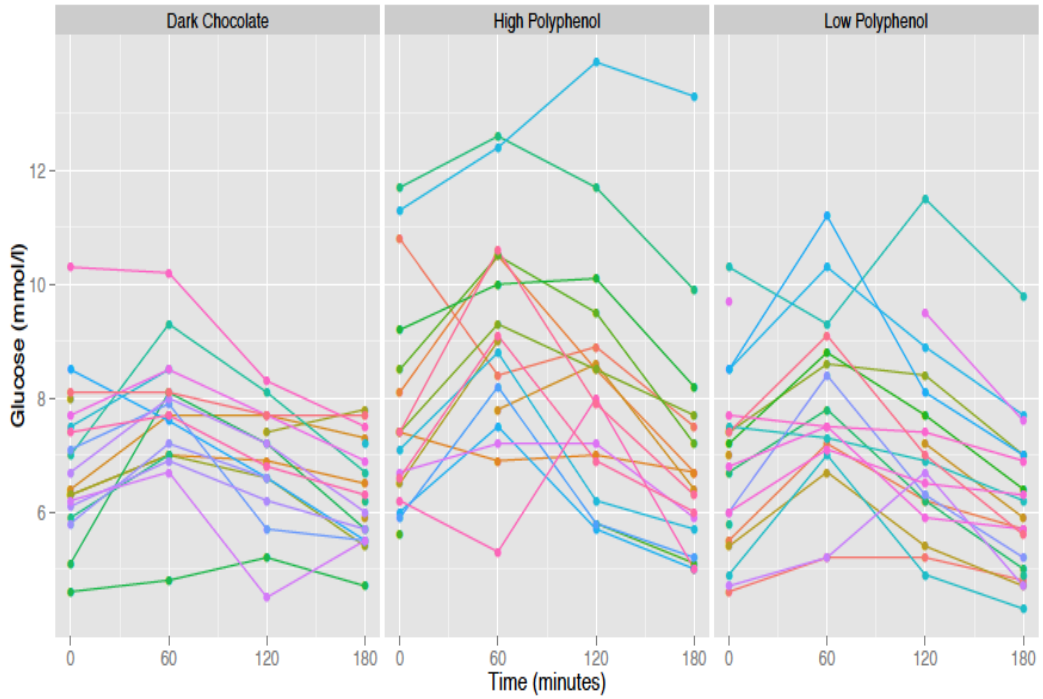


Figure 6.4.1.2.1: Individual glucose over time at visit 4 following feeding 40g of chocolate at 0 minutes. High polyphenol chocolate refers to the milk chocolate, which contains 20mg epicatechin; dark chocolate also contained 20mg epicatechin. The low polyphenol chocolate contained 1mg epicatechin.

Table 6.4.1.2.2: Estimated treatment ratio differences in terms of plasma glucose over the course of the 180 minutes following consuming 40g chocolate at visit 4.

	Estimate	Lower	Upper	p-value
Milk Vs. Control	1.08	0.99	1.18	0.0795
Milk Vs. Dark	1.07	0.98	1.17	0.1096

A linear mixed model with treatment, linear and quadratic effects of time as fixed effects was used while adjusting for age and glucose measurements at baseline. A random subject intercept was also included in the model to account for the variability at time zero. Glucose measurements were log-transformed to correct for the distributional assumptions. The estimated overall treatment ratios are given in Table 6.4.1.2.2. Overall, no statistically significant differences were observed between the treatment comparisons of interest. The 95% confidence interval for the estimated ratio between the glucose levels under milk chocolate and control chocolate was [-1.1%, 18%] while that between milk chocolate and dark chocolate was [-2%, 17%].

6.4.1.3 - Insulin

At visit one and four, insulin (μU/l) measurements were taken on four occasions (0, 60, 120 and 180 minutes), at the same time as the glucose measurements. These are summarized in table 5.4.1.2.1. All the treatment groups exhibited an increase in insulin levels, which peaked after 60 minutes before decreasing gradually. The rates of change in insulin levels over time seemed to be similar in the three treatment groups. A linear mixed model with treatment, linear and quadratic effect of time as fixed effects while adjusting for age and glucose measurements at baseline was used.

Table 6.4.1.3.1: Summary log transformed statistics for insulin at visit 4

	Treatment	Geometric mean	95% Confidence Intervals
0 mins.			
	Dark Chocolate	9.18	5.94-14.20
	High Polyphenol	7.73	5.25-11.40
	Low Polyphenol	7.43	5.15-10.70
60 mins.			
	Dark Chocolate	23.42	14.90-36.80
	High Polyphenol	19.35	11.51-32.50
	Low Polyphenol	17.87	11.38-28.10
120 mins.			
	Dark Chocolate	18.12	13.56-24.20
	High Polyphenol	15.00	9.56-23.50
	Low Polyphenol	11.02	6.61-18.40
180 mins.			
	Dark Chocolate	9.67	6.45-14.50
	High Polyphenol	11.37	8.03-16.10
	Low Polyphenol	8.21	5.23-12.90

A random subject intercept was also included in the model to account for the variability at time zero. A natural log transformation was applied to the insulin measurements to correct for the distributional assumptions. The estimated overall treatment ratios in terms of insulin levels are given in Table 6.4.1.3.2. Overall, no statistically significant differences were observed between the treatment groups.

Table 6.4.1.3.2: Treatment contrast estimates for insulin at visit 4 on the robust ANCOVA

	Estimate	Lower	Upper	p-value
Milk Vs. Control	0.98	0.66	1.46	0.9243
Milk Vs. Dark	0.92	0.56	1.20	0.3134

6.4.1.4 - Triglycerides

A linear mixed model with treat and linear effect of time as fixed effects of time (hours) while adjusting for age and triglyceride levels at visit one was applied to the log-transformed triglyceride levels at visit four. A random subject intercept and slope was also included in the model. The estimated overall treatment ratios are given in Table 6.4.1.4.1. No significant differences were seen between the treatments.

Table 6.4.1.4.1: Treatment contrast estimates for triglycerides on the robust ANCOVA

	Estimate	Lower	Upper	p-value
Milk Vs. Control	0.91	0.69	1.21	0.5276
Milk Vs. Dark	0.90	0.68	1.19	0.4530

6.5 – Discussion

This is the first report using a pragmatic study design of the effect of chocolate consumption (as defined by EU regulations (European Union, 2004), including milk chocolate, given for a period of longer than two months, on a range of biomarkers in individuals with T2DM. The chocolate consumption in this study was approximately compatible with nutritional guidelines in terms of sugar and fat.

The data provide evidence that milk chocolate can reduce inflammatory markers with a significant reduction in hs-CRP ($p=0.0028$). This effect did not appear to be linked to insulin mediated mechanism since HOMA did not significantly improve (although a trend was observed $p=0.0754$ ITT analysis, and $p=0.0518$ PP analysis).

Curtis et al. (2012) using a cohort females alone with medicated T2DM, demonstrated modest changes in lipid parameters, followed by improvements in cardiovascular risk as estimated using the UKPDS risk engine. Curtis et al. (2012) employed a longer intervention period of 12 months, compared to the 12 weeks in the present study and the 'active' chocolate also contained 100mg of isoflavones, and therefore not comparable with the study here. However, the present study confirms that normal (in terms of sugar) chocolate, although in this study did not demonstrate a beneficial effect in individuals with T2DM, in accord with the other experimental work presented in this thesis showed no negative effects on body weight or glycaemia. This has not previously been demonstrated other than in my previous published work (Mellor et al., 2010b; 2012).

Limitations of this data are the small sample size and the potential confounding of apparent acute phase responses associated with frequent upper respiratory tract infections seen during the study. This may have resulted in the dark chocolate not showing any effect on insulin resistance or on lipid profiles; contrary to other reports in participants with T2DM following chocolate consumption (Curtis et al., 2012; Mellor et al., 2010b).

A key strength of this work is that it is one of very few studies since that of Fraga et al. (2005), to investigate the health effects of milk chocolate, and the first in a population with the significant cardiovascular risk of T2DM. The lack of studies using milk chocolate formulations may be due to the influence of Serafini et al. (2003; 1996), who suggested that milk had an inhibitory effect upon flavon-3-ol metabolism; a position since widely disputed as described in section 1.3.5.1. This is important, since products based upon milk chocolate are more palatable to consumers in the UK, potentially extending benefits of chocolate to everyone who enjoys the product.

The apparent universal effect of chocolate on insulin resistance is of interest since it mainly occurs through a reduction in fasting insulin levels, although this is less clear when an ANCOVA model is applied. Hooper et al. (2012) in a meta-analysis reported that flavanol rich chocolate reduced HOMA significantly by 0.67. In the present study there was no effect of dark chocolate on HOMA, though in the per protocol analysis there was a trend towards a reduction in insulin resistance with milk chocolate ($p=0.0518$). The data suggest that regular consumption of chocolate for twelve weeks has no negative effects on the health of individuals with T2DM and may have the potential to reduce insulin resistance.

It is possible that the lack of any clear outcomes in respect to insulin resistance, may be a reflection of the relatively low epicatechin and flavanol levels, since the chocolates contained significantly less than the 200mg cocoa flavanols recently been accepted by EFSA (2012a) as having health benefits on endothelial function. This observation may explain the lack of a clear effect on insulin resistance and endothelial function in this study. It is possible that any positive effects seen, e.g. in terms of the general improvement in HOMA, could be attributed to a placebo effect.

This is the first study to provide evidence of an ability of milk chocolate to reduce inflammation as measured by hs-C-Reactive Protein following 12 weeks of

supplementation. This effect was not seen with dark chocolate with the same epicatechin content, and it is possible this effect might not be associated with cocoa content but reflect the effects of the milk content. Further work with higher flavanol content milk chocolate is required to explore whether a synergistic effect of milk with cocoa flavanol compounds exists. However the present results may be a reflection of the adverse event rate (upper respiratory tract infections) and represent type 1 error.

The findings of this study indicate that modest reductions in insulin resistance and cardiovascular risk across all groups are apparent following 12 weeks of chocolate supplementation with no evidence of harm. More research is required to explore if this effect of chocolate is independent of the flavanols, as proposed by Mursu et al. (2004) or due to a placebo effect. Supplementation with milk chocolate enriched with flavanols showed trends towards additional effects beyond those seen for the control or dark chocolate; there was a trend seen towards an improvement for HOMA for milk chocolate compared to dark chocolate ($p=0.0754$) in the ITT analysis. This trend was further supported by the PP analysis ($p=0.0518$).

This study also highlighted the potential challenges of working with an industrial sponsor, as this set limits with respect to the products used and the type of analysis conducted. Although, this was necessary to allow the work to take place, it could have resulted in the findings of the study not being as fully explored as there was a tendency for a robust but cautious approach to the analysis of data. Also unlike the initial work of Taubert et al. (2003) and Grassi et al. (2005a; 2005b; 2008) it attempted to use a smaller chocolate bar with a lower dose of polyphenols as this was considered to be commercially and poltically more acceptable as a health message than the studies which used 100g per day, which ultimately could have accounted for the lack of positive findings.

6.5.1 - Conclusion

The dose of epicatechin and hence flavanols used in this study was considerably lower than that accepted by EFSA to improve endothelial function and lower than in my previous work (Mellor et al., 2010b; 2012). This may explain the lack of a clear effect of the flavanol rich chocolate (both milk and dark formulations) in the present study although there was a trend towards a benefit of milk chocolate enriched with flavanols and no evidence of harm. The work highlights the challenge of providing an efficacious dose of epicatechins and flavanols in a product palatable to the consumer (Ried et al., 2010a), but not in the form of a calorific formulation as in the early studies of the health benefits of chocolate (Taubert et al., 2003; Grassi et al., 2005a; 2005b; 2008). Whilst at the same time suggesting that there may be potential for benefit of milk chocolate enriched with flavanols for individuals with T2DM, and at the very least it does not cause harm.

Chapter Seven: General Discussion and Conclusions

7.1 – Primary Findings of this Thesis

Nutritional management is viewed as a key intervention in the management and prevention of type 2 diabetes mellitus (T2DM) (Dyson, et al., 2011; Franz, et al., 2008). Over the past decade there has increasingly been a demand for an evidence-based approach to healthcare, including dietary approaches in diabetes. In the UK, this has been acknowledged by the Diabetes UK Nutrition Working Group (of which I was a member) in their most recent nutrition guidelines (Dyson, et al., 2011). Dyson et al. (2011) used a rating system based on the hierarchy of evidence presented in figures 1.4.1 and 2.2.1.1, using the scale of Petrie, Grimshaw and Bryson (1995). This scale gave an 'A' rating for guidelines which were based on either data from meta-analyses of randomised controlled trials (RCTs) or at least one RCT (Petrie, Grimshaw & Bryson, 1995), down to a 'D' rating for guidance based on expert or consensus opinion. For the 16 recommendations for T2DM and management of cardiovascular risk, only half claimed to be linked to the strongest experimental evidence and given an 'A' grade. In the 1990s the dietetic profession considered that chocolate should be avoided or if consumed, limited to a post-meal snack to moderate the glycaemic response. However, the data presented in this thesis provides potentially 'A' grade evidence for health benefits of flavanol rich chocolate for individuals with T2DM. It is suggested that the data presented in this thesis are based on more scientifically sound data (albeit limited by the study population size) of the published nutritional recommendations for T2DM (Dyson et al., 2011).

7.1.1 – Original Nature of Work

This thesis represents the first systematic study of the effects of chocolate containing cocoa polyphenols in individuals with T2DM. The inspiration for this work came from

the comments of Cesar Fraga (2005), who posed the question 'cocoa, diabetes and hypertension: should we eat more chocolate?' a question suggested without the support of any experimental data on the effects of cocoa or chocolate in diabetes! This thesis represents the sum total of the published data investigating the effects of chocolate (not as cocoa or without additional isoflavones) in individuals with T2DM to the end of 2012.

7.1.2 - Chocolate or Cocoa

The exploratory review with meta-analysis reported in Chapter Three explored the beneficial effects of chocolate identified in the literature review of Chapter One, when compared to cocoa supplementation as the optimal matrix for delivery of epicatechins. It was found that chocolate had greater beneficial effects upon blood pressure and fasting glucose than cocoa. The positive effects of chocolate and cocoa supplementation with respect to blood pressure and endothelial function were in accordance with other recent meta-analyses (Hooper, et al., 2012; Shrime et al., 2011). However, these findings need to be treated with caution due to the limitations of the search strategy and statistical methodology.

7.1.3 – Beneficial Effects in Continuous and Acute Feeding of Chocolate

The work presented in Chapters Four and Five, provide evidence for the beneficial effects of chocolate consumption in reducing markers of cardiovascular risk. The proof of concept work (Chapter Four) demonstrated an improvement in HDL cholesterol (5.9% or 0.07mmol/l, p =0.04) and HDL cholesterol: total cholesterol ratio (-9.8 % or -0.3, p =0.03) following eight weeks of consuming 45g of high polyphenol (flavanols) chocolate containing 55.3mg of epicatechins and 783mg of total flavanols. These beneficial effects of continued consumption over a two-month period extend the work of Mursu et al. (2004), on healthy individuals to those with T2DM; a population group with greater cardiovascular risk than used in their study.

In the second clinical trial (Chapter Five), a single bar of chocolate containing 472.5mg of flavanols administered 60 minutes prior to a 75g oral glucose load, ameliorated the increase in oxidative stress ($p = 0.02$) and endothelial dysfunction ($p = 0.01$) which had been reported following acute transient hyperglycaemia (Ceriello et al., 1998; 2008; Wright, Scism-Bacon, & Glass, 2006). These data extend the previous work of Balzer et al. (2008), in terms of demonstrating the beneficial effects on endothelial function following a significant carbohydrate load and in the ability to link an improvement in endothelial function to a reduction in urinary 15-F2t-isoprostane (oxidative stress).

The work in Chapter Five was accepted for publication at the same time as a similar study by Grassi et al. (2012), but went further as this group who fed a larger weight (100g) of chocolate for three days, limited their study population to 12 healthy young adults. Grassi et al. (2012) also undertook the assessments from a fasting baseline, so no chocolate was given immediately before the 75g Oral Glucose Tolerance Test Solution. As the work presented in Chapter Five, was in subjects with T2DM, my data demonstrated a maintained effect of chocolate flavanols at a greater degree of hyperglycaemia in subjects at a greater cardiovascular risk and hence endothelial dysfunction. The findings of both pieces of work imply that further investigation of how dietary factors, including cocoa flavanols might modulate postprandial metabolism stress is required.

7.1.4 – Dose Required for Effect and Potential Health Claim for the Role of Chocolate in T2DM?

Chapter Six investigated whether the beneficial effects of chocolate flavanols observed with dark chocolate formulations extended to milk chocolate, but failed to show any clear beneficial effects, either with dark chocolate containing 19mg epicatechin, or milk chocolate containing 19mg epicatechins. The lack of efficacy seen in this study may be due to the relatively low levels of epicatechins, although Taubert et al. (2007)

demonstrated a significant reduction in blood pressure in untreated pre-hypertensive elderly participants with a chocolate containing only 5.1mg of epicatechins. It is plausible that having T2DM or taking medication including antihypertensive agents may require a higher dose of epicatechins to demonstrate efficacy.

With regard to the beneficial effects of cocoa and chocolate, the Dietetic products, Nutrition and Allergies (NDA) Committee of the European Food Safety Agency (EFSA, 2012a) provided a positive opinion for an Article 13(5) claim by Barry Callebaut BV. Potentially, this could mean chocolate being labelled with a health claim. This may present set precedence, for a future Article 14 claim linked to modification of disease risk associated with T2DM using the findings of this thesis. Such a claim would require further work including dose response data from people with T2DM. Data from Chapter Six suggested that 60mg polyphenols per daily dose (estimated from the 19mg of epicatechin) was insufficient to show a significant effect in individuals with T2DM. This dose was also far less than the 783mg (55.4mg of epicatechins) and 472.5mg of total polyphenols provided as supplements in Chapters Four and Five, which demonstrated positive outcomes.

7.2 - Similarities Between Studies

Overall only three participants across the three studies (which were made up of 83 participants and totalled 17.56 participant years of daily chocolate intake) withdrew; two because of being unable to consume the chocolate or feeling weak and a third on the advice of their general practitioner.

7.2.1 - Chocolate is Not Harmful in Type 2 Diabetes

In the final study there were a number of adverse events, all of which were expected in view of the population. The most common of these were upper respiratory tract infections and gastroenteritis. In terms of concomitant medication, over 80% of participants reported being given the influenza vaccine and over 50% had the swine flu vaccine due to the time when the study was undertaken (2009-10).

One participant following having had their diabetes medication increased by their general practitioner was advised that the study chocolate was having a detrimental effect on their diabetes. This was despite a 0.2% decrease in their HbA1C whilst they had been consuming chocolate and was withdrawn. This highlights a common perception within both the general population and healthcare communities that diabetes is a condition of 'sugar' as discussed in Chapter One, and the work Peters et al. (1990). They suggested that isocalorific substitution of potato with chocolate cake results in no significant variation in postprandial glycaemia. This has been extrapolated and interpreted as meaning that high sugar foods should only be consumed as part of a starch rich meal. This ignores subsequent work that has defined the concept of glycaemic index and has been extended to include the idea of a glycaemic load.

The combination of the concepts of carbohydrate 'quality' as defined by rate of absorption and uptake and 'quantity' means that it is possible to predict the glycaemic

response of a food portion consumed, and in the present work, there were no adverse effects on acute glycaemia or longer term assessments in the form of HbA1c.

7.2.2 - Weight

Weight was deemed a key outcome measure, both in the proof of concept study (Chapter Four) and the parallel study (Chapter Six) which involved at least three months of eating chocolate on a daily basis. Both studies had at least 95% compliance. For the proof of concept study, that meant that an extra 240 kcal of energy per day, could, if not compensated for in terms of change in diet or physical activity, result in a 3.84kg weight gain the five months of the study. For the final parallel study participants were asked to consume an additional 110 kcal per day, which gave a potential risk of participants gaining 1.32kg over the 12 weeks of the study.

The studies presented in Chapters Four and Six did not demonstrate any weight gain, in accordance with the data of O'Neil, Fulgoni and Nicklas (2011a; 2011b) and the meta-analysis data presented in Chapter Three (Figure: 3.3.7.1). Notably, there was a significant reduction in weight between the screening visit and the first administration of chocolate at visit one, which appeared to be a type of 'Hawthorne effect', with the reduction in weight appearing to be an effect of mere participation in the study. This was despite participants being asked not to make any changes to their eating or other lifestyle behaviours, apart from abstaining from chocolate or cocoa, which according to their dietary recalls resulted in no difference in energy or macronutrient intake.

7.3 - Limitations

There were a number of critical limitations to the exploratory review and meta-analysis which need to be revised should this work be further developed with a view to publish. The search strategy for the exploratory review was limited, in its completeness and ability to restrict to randomised studies, in part this was corrected by a hand search and review of clinical trial registries, but any future study should address its literature searching strategy. This should include the correct use of indexing to reduce missed studies. The search strategy would then be developed using Boolean operators, and then test words and synonyms (including truncation e.g. diabet* or $ depending on the database). Finally limiters would be introduced to refine the search to randomised clinical trials in humans. The search strategy would be trialled and reviewed to see if it could be enhanced. The meta-analysis was limited by the data selected, only using published numerical data and not using graphical data and contacting authors. Further work should attempt to include this data and contact authors to verify aspects of study design and missing data. Where this is not possible, the estimates of variance of mean difference could be refined to include aspects of the paired nature of the data e.g. correlations to allow for the use of the sum of variance law 2.

The lack of measurements of nitric oxide metabolites is one that future studies should consider. This would help to try and identify the potential mechanism of the chocolate, which although it has been widely described in healthy volunteer studies, has not been assessed in T2DM. This has made it necessary to assume that the mechanism seen in healthy individuals also occurs in T2DM. This would be important in studies exploring the effects of milk chocolate compared to dark chocolate, as there may be subtle differences in time of absorption (Mullen et al., 2009).

The formulation is a potential issue; the funding of the studies meant that although one corporation provided the chocolate for two studies, in fact all three studies used different chocolates. This also meant that as found in Chapter Three, and commented upon by Cooper et al. (2008), there was a lack of consistency of chocolate formulation and assessment of polyphenol, flavanols or epicatechin content. Although ideal, it is unlikely due to commercial and intellectual property interests that a common study formulation of chocolate, with a known nutritional composition in terms of energy, macronutrients and flavanols will be made available. This is perhaps the only way to define precisely the physiological effects of chocolate and its components within a multi-centre randomised trial. However, although a criticism of having to use different chocolates in each study, it is also a potential strength that there were positive findings seen in the same population group with different formulations of chocolate. This might suggest a greater degree of robustness and generalizability of the findings to clinical practice.

Despite these limitations a number of reviews have complimented the quality of the study design (Shrime et al., 2011) and conduct of the study reported in Chapter One and included the data in reviews and meta-analyses (Ellinger et al., 2012; Hooper et al., 2012; Shrime, et al., 2011; Tokede, Gaziano, & Djoussé, 2011)

7.4 - Implications

Chocolate appears to be the most biologically effective way of delivering cocoa flavanols and potentially polyphenols, although its energy content needs to be considered before it can be widely recommended as part of a healthy diet by nutrition professionals. Further work needs to be undertaken to assess the most effective chocolate formulations for the delivery of flavanols. The dose of flavanols appears to be critical; a total polyphenol content of 500mg or 55mg of epicatechins per day were shown in this thesis to produce positive benefits for individuals with T2DM.

The data presented in this thesis suggest that beneficial effects may be seen across a range of cardiovascular risk factors, including individuals receiving medication. This may imply that the effects on endothelial function and HDL cholesterol may be at least in addition to the effects of pharmaceutical agents and could potentially be synergistic. This avenue would benefit from further investigation both in *in vitro* and *in vivo* studies.

It is likely that many individuals with T2DM would benefit from the consumption of flavanol rich chocolate. The prior belief held by many health professionals was that the consumption of chocolate is harmful in terms of weight or glycaemic control and should therefore be excluded from the diet of individuals with T2DM. This belief has failed to stand up to the scrutiny of the RCTs presented in this thesis.

The findings of this thesis should be considered, interpreted and then if meeting the critical scrutiny requirements of the profession, added to nutritional guidelines. If accepted, such recommendation would probably be included with the caution and caveats stated above, to avoid excessive consumption of chocolate. Then subsequent research, as part of clinical practice, would be required to assess the best way of incorporating flavanol rich chocolate in an individual's dietetic care plan.

7.5 - Directions for Future Work

The evidence from previously published epidemiological and clinical studies of healthy participants is that cocoa flavanols can have health benefits. Potentially, the key question is what is the best way to consume these health maintaining and potentially disease modifying compounds? Thus, leading to the question what formulation is required to achieve the optimal biological activity of cocoa flavanols? The requirement is for a palatable product, which is not too bitter nor too energy dense or high in carbohydrates, especially sugar (Faridi et al., 2008), with the caveat that too little sugar may reduce bioavailability and uptake in the gut.

The next steps therefore is to:

1. Develop formulations which are palatable and have the optimal sugar and energy content either as cocoa drinks or chocolate;
2. Undertake pharmacokinetic and pharmacodynamics studies to assess bioavailability;
3. Undertake further clinical trials to assess the optimal formulation or matrix for the delivery and effect of cocoa flavanols.

Considering chocolate is a food containing potentially multiple biologically active compounds, it is possible that the epicatechins and flavanols are not the only 'actives', which are responsible for the biological effects reported in this thesis. For example, there is potential for the peptides in chocolate to have beneficial biological effects, especially those found in milk chocolate (Erdmann, Cheung, & Schröder, 2008). A further possibility is that 100g dark chocolate can provide 115mg magnesium, over a third of the Reference Nutrient Intake (Department of Health, 1991), which a recent meta-analysis has linked to reducing the risk of developing diabetes (Dong, Xun, He, & Qin, 2011). Finally, the manufacturing of the chocolate starting with the agricultural origin of the cocoa (Caligiani et al., 2007) and how its processed (Jolić et al., 2011)

could also be significant since Barry Callebaut BV, claim to have increased the flavanol content of the Acticoa™ used in Chapter Five (Bernaert, 2007) by altering how it is processed.

Future work should therefore attempt to clarify what the active compounds are, and if a number of biological relevant compounds are identified, whether these compounds have synergistic effects. These should then be investigated in combination with common medications (both *in vitro* and *in vivo*), as knowledge of drug-nutrient interactions is very limited (Hu, 2007; Boullata & Hudson, 2012; Heuberger, 2012). This is of vital importance if the initial findings of this work are to be extended to the wider population of people with diabetes and so to avoid the risk of adverse interactions with medication.

Learning Points from thesis:

If type of work were to be revisited or undertaken again, the following should be considered during the planning phases and conduct:

1. For the meta-analysis, work as part of a team which includes an experienced reviewer. Insure at all points, search strategy, study selection, data extraction and data analysis is initially for the first four undertaken separately prior to cross-checking and then the data analysis checked for accuracy. To broaden the available data, enough time and finance for this would be required along with the contacting of authors to minimise the need to undertake estimate of mean change and variance of change. This could include use of different approaches to estimating the variance of the change, and using sensitivity analysis to assess the effects.
2. It is necessary to fund the clinical trials, this means working with industry. A carefully designed contract needs to be drawn up, otherwise there is a potential risk to the researcher (including potentially their degree if they are a student).

This should include an agreement regarding allowing the researcher to independently analyse their data.

7.6 – Key Questions

Following the work presented in this thesis three key questions appear to still require investigational work. These being:

1. *What is the optimal dose of cocoa polyphenols needed to demonstrate improved endothelial function in T2DM?*

   Aim: To define the optimal polyphenol/ epicatechin dose in a chocolate bar for individuals with T2DM with respect to improved endothelial function.

   Although it is plausible that the health effects resulting from supplementation with cocoa polyphenols are not solely due to the effect of epicatechins, this is the molecule which has been identified post ingestion and linked to beneficial effects *in vivo* and *in vitro*, it is logical that this should be level assessed in chocolates developed to provide potential health benefits to individuals with T2DM. The work presented in Chapter Six, suggested about 20mg epicatechin was not enough to produce an effect, where as the 55mg used in Chapter Four appeared to have demonstrated beneficial effects.

   This work would require three phases;

   I. Product development, to develop a 15-20g chocolate bar containing approximately 100kcal and less than 10g of sugar containing <2mg, 20mg, 40mg, 60mg and 80mg of epicatechin. These will be matched for macronutrients, taste and appearance.

   II. Taste test, using the approach reported in Chapter Five.

   III. A clinical trial would then be undertaken in a group of medically stable individuals with T2DM using a crossover design. The

work presented in Chapter Five and Balzer et al. (2008) suggests a sample size of 10 should be adequate. The work of Grassi et al., (2012) and the data in this thesis suggest that both acute and three day feeding of chocolate can improve endothelial function.

Following screening individuals and baseline assessment of endothelial function, will be randomised to one of the five chocolates for three days, then after three days, endothelial function will be reassessed. Following one weeks wash out, individuals will return for baseline assessment (this will allow a true assessment of change and more robust analysis of the crossover study), this will be followed by three days of chocolate and reassessment of endothelial function. This will be repeated until all the chocolates had been tested. As there are five arms, around 20 individuals with T2DM would need to be recruited to allow for group out and concurrent illnesses that could act as a source of confounding.

2. *Can supplementation with cocoa polyphenols lead to drug nutrient interactions?*

   Aim: To investigate the potential of cocoa polyphenols as inhibitors of CYP4a and its potential effect on drug pharmacokinetics.

   Although there is an increasing body of evidence to support a potential health benefit of cocoa and chocolate in healthy individuals, to the extent that the NDA of EFSA (2012) have approved at Article 13.5 health claim. The potential problem of supplementation with polyphenols, including those from chocolate is that they may lead to food/nutrient- drug interactions. These have been poorly defined. It has been suggested that epicatechins and other flavanols can act on

cytochrome P450, including CYP3A4 (Muto et al., 2001). These are responsible for the conjugation of many organic compounds including a wide range of drugs, thus any compounds altering the action of this and similar enzyme systems can affect circulating levels of drugs, their half-life and ultimately increase risk of side effects.

The methodology would be based upon the methods initially described by Lown et al. (1997) and Bailey et al. (2003) who undertook pharmokinetic studies of felodipine. Although this work initially aimed to investigate the effects of alcohol upon drug metabolism, it discovered grapefruit juice, which contain a number of polyphenolic compounds including naringin and hesperidin increased the AUC for the drug. As such interactions can lead to serious complications including torsades des pointes affecting cardiac function, it is vital for the ageing populations in Westernised countries who are increasingly on chronic prescriptions of multiple drugs that we gain a better understanding of how polyphenols may interact with common drugs.

In a group of 12 healthy mean would be used as part of an oral pharmacokinetic study of felodipine and dehydrofelopidine (its primary metabolite). In a crossover design, an initial study will be undertaken with felodipine and water followed by felodipine with 250ml of grapefruit juice. These will act as control and a demonstration of an interaction effect. This will be followed in a randomised designed study where 60mg of epicatechins/500mg of polyphenols (or a low polyphenol placebo) are given as a cocoa drink. A drink would be used, as solid chocolate may influence pharmacokinetics by altering gut transit time and not being comparable to the juice. This would then be with the alternative cocoa. This should provide evidence to suggest whether cocoa polyphenols can have an effect upon drug metabolism and thus, should the

supporting evidence for their efficacy in populations with chronic diseases be accepted, mean that advice to include cocoa polyphenols as part of their diet, along with their potential to interact with cardiovascular drugs.

3. *Which dietary interventions can demonstrate a reduction postprandial dysregulation, including endothelial dysfunction and oxidative stress?*

   Aim: Can a protocol used to investigate the acute effects of high polyphenol chocolate in T2DM be adapted to assess the efficacy of other nutritional interventions on improving postprandial dysregulation.

   Following on from the findings of Chapter Five, it was demonstrated that cocoa polyphenols in the form of chocolate could ameliorate postprandial oxidative stress and endothelial dysfunction. This was shown to be both very acutely in this study, and following three days consumption of chocolate by healthy individuals (Grassi et al., 2012).

   This protocol was based on the work of Ceriello et al., (1998; 2008) who demonstrated that varying plasma glucose could adversely affect oxidative stress and endothelial function. This is an area where a range of dietary interventions can be further investigated. This forms the basis for a number of proposals I am currently developing. These range from the effect of different macronutrient restrictions on postprandial metabolism to other dietary sources of polyphenols. The first step is to use a standard mixed meal test to investigate if the combination of macronutrients which Ceriello et al. (1998) demonstrated a metabolic disturbance, then following a crossover study design investigate if cocoa polyphenols can ameliorate these negative effects.

7.7 - Conclusion and Summary

The data presented in this thesis provides evidence to support the statement of Cesar Fraga (2005) suggesting a beneficial effect of chocolate in individuals with diabetes with the addition of two key findings. Firstly, that chocolate may provide a better formulation than cocoa and secondly, that the health benefits reported in healthy volunteers, can be demonstrated in individuals with T2DM. This aspect was further extended by the study reported in Chapter Five, where additional metabolic stress was induced using a 75g oral glucose load and the endothelial dysfunction and oxidative stress often seen in the postprandial state were successfully ameliorated by pre-feeding chocolate rich in flavanols. It was not possible to demonstrate an extension of these benefits to milk chocolate enriched with cocoa flavanols, highlighting the challenge to chocolatiers of producing a palatable chocolate with enough flavanols, but not too much fat and sugar, to have health benefits. It will also be important to overcome the widespread notion that chocolate is 'naughty but nice' when moderate amounts of chocolate are consumed as part of a sensible nutritional management plan for individuals with diabetes is clearly not harmful and may be beneficial.

So, for dietitians working in clinical practice, the data means that advice regarding the consumption of chocolate by individuals with T2DM can now be balanced with an experimental evidence base. Too high an intake may lead to positive energy balance and worsening glycaemic control, whereas smaller amounts of flavanol rich chocolate (although hard to identify from shop bought brands) may have beneficial effects in reducing cardiovascular risk.

To paraphrase Hippocrates, 'let food of the Gods (*Theobroma cacao*) be thy medicine', but only in moderation.

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Appendix I – Search Terms and Strategy for Review and Meta-Analysis (Including Harvard style citations for papers used in review).

Medline (PubMed), EMBASE, Cochrane and WHO international trials database search strategy run to June 2012.

1. Chocolate
2. Cocoa
3. Cacao
4. Flavanols
5. Randomised controlled trial OR RCT
6. Cardiovascular disease OR diabetes OR hypertension OR blood pressure OR dyslidaemia OR cholesterol OR endothelial dysfunction OR oxidative stress OR inflammation
7. 5 AND 1 OR 2 OR 3 OR epicatechin
8. Controlled trial OR 5 AND 1 OR 2 OR 3 OR epicatechin
9. Placebo AND 7
10. 8 and Human NOT animal
11. 10 and 6

Studies were checked along with systematic reviews and meta-analysis published up until June 2012 to check for the completeness of the review

The addition of the WHO international trials database was to screen for potential studies, which have reported a study design, which met the criteria for publication to search for early reports/ articles in press.

Full Search Strategy for Medline (Ovid)

Wolters Kluwer Health | OvidSP

My Account | My PayPerView | Support & Training | Help | Logoff

Search | Journals | My Workspace

Search History (23 searches) *(Click to close)* — Remove Duplicates | View Saved

	# ▲	Searches	Results	Search Type	Actions
☐	1	(cacao$ or cocoa$ or chocolat$).mp.	14410	Advanced	Display More »
☐	2	cardiovascular disease {Including Limited Related Terms}	7263	Basic	Display More »
☐	3	cardiovascular mp. {Including Limited Related Terms}	8290	Basic	Display More »
☐	4	randomized controlled trial {Including Limited Related Terms}	6612	Basic	Display More »
☐	5	controlled clinical trial {Including Limited Related Terms}	8006	Basic	Display More »
☐	6	placebo {Including Limited Related Terms}	8036	Basic	Display More »
☐	7	placebo.tw.	243369	Advanced	Display More »
☐	8	randomized.tw.	482988	Advanced	Display More »
☐	9	or/2-3	14391	Advanced	Display More »
☐	10	or /4-8 {Including Limited Related Terms}	8075	Basic	Display More »
☐	11	hypertension or blood pressure {Including Limited Related Terms}	7890	Basic	Display More »
☐	12	glucose or glycaemia {Including Limited Related Terms}	10697	Basic	Display More »
☐	13	diabetes {Including Limited Related Terms}	23934	Basic	Display More »
☐	14	dyslipidaemia or cholesterol {Including Limited Related Terms}	6835	Basic	Display More »
☐	15	endothelial $function {Including Limited Related Terms}	12165	Basic	Display More »
☐	16	oxidative stress {Including Limited Related Terms}	5283	Basic	Display More »
☐	17	inflammation {Including Limited Related Terms}	5738	Basic	Display More »
☐	18	(epicatechin or flavanol or flavanoid).mp.	2007	Advanced	Display More »
☐	19	or/11-17	67644	Advanced	Display More »
☐	20	animal/ not (humans/ and animals) {Including Limited Related Terms}	6898	Basic	Display More »
☐	21	19 not 20	67336	Advanced	Display More »
☐	22	1 or 18	15919	Advanced	Display More »
☐	23	22 and 21 and 19	393	Advanced	Display More »

Remove Selected | Save Selected | Combine selections with: And | Or | RSS

Save Search History

Note: For future systematic reviews, the limitation and logic of this search strategy need to be considered, including use of indexing and limiting terms (e.g. randomised controlled trials).

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	111 – Refers to Exploratory Systematic Reviewrather than
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Not done as part of chapter, referred to in thesis abstract – 13-14
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	111-120
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Table 3.0: 112
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	Not done as part of thesis
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	121-122
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	123
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	123 and Appendix I 302-303
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	123-124
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	123
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	122
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	122-124
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	125
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	125

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097.

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	124
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	125
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	126-129
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	130-134
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	134-135
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	137-156
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	137-156
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	134-135
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	137-156
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	As part of the discussion 157-160
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	160
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	161
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	None define, done as part of PhD studies

doi:10.1371/journal.pmed1000097

Harvard Style References supporting Table 3.1.1

Lead Author	Year	Harvard Style Reference
Allgrove	2011	(Allgrove et al., 2011)
Almoosawi (overweight)	2012	(Almoosawi, Tsang, Ostertag, Fyfe, & Al-Dujaili, 2012)
Almoosawi (obese)	2012	(Almoosawi et al., 2012)
Baba	2007	(Baba et al., 2007)
Balzer	2008	(Balzer et al., 2008)
Davison, K.	2008	(Davison, Coates, Buckley, & Howe, 2008)
Davison, K.	2010	(Davison et al., 2010)
Davison, G.	2012	(Davison et al., 2012)
Engler	2004	(Engler et al., 2004)
Faridi (Chocolate)	2008	(Faridi, Njike, Dutta, Ali, & Katz, 2008)
Faridi (Cocoa)	2008	(Faridi et al., 2008)
Farouque (Long-term)	2006	(Farouque et al., 2006)
Farouque (acute)	2006	(Farouque et al., 2006)
Flammer (acute)	2011	(Flammer et al., 2011)
Flammer (Long-term)	2011	(Farouque et al., 2006)
Flammer	2007	(Flammer et al., 2007)
Fraga	2005	(Fraga et al., 2005)
Grassi	2005a	(Grassi et al., 2005a)
Grassi	2005b	(Grassi et al., 2005b)
Grassi	2008	(Grassi et al., 2008)
Heiss	2005	(Heiss et al., 2005)
Monahan	2011	(Monahan et al., 2011)
Muniyappa	2008	(Muniyappa et al., 2008)
Murphy	2003	(Murphy et al., 2003)
Mursu	2004	(Mursu et al., 2004)
Njike	2011	(Njike et al., 2011)
Sathyapalan	2010	(Sathyapalan et al., 2010)
Shiina	2009	(Shiina et al., 2009)
Taubert	2003	(Taubert, Berkels, Roesen, & Klaus, 2003)
Taubert	2007	(Taubert et al., 2007)
Tzounis	2011	(Tzounis et al., 2011)
Wang-Polagruto	2006	(Wang-Polagruto et al., 2006)
Westphal	2011	(Westphal & Luley, 2011)
Wiswedel	2004	(Wiswedel et al., 2004)

Appendix II– Dietary Advice Provided to Participants

Concomitant diet and treatment

Permitted concomitant diets/treatments/medications

During the study participants will be asked to refrain from altering their intake of polyphenol rich foods including:

- Black or green tea
- Coffee
- Onions
- Apples,
- Cabbage
- Wine
- Cocoa products.

Just before and during the blood test and other investigations, participants will be asked to abstain from these foods all together.

Diet was monitored in each of the three studies, in Chapter Four, by the study dietitian and in Chapters Five and Six by dietary recall. A conscious decision was made, not to use a food diary as this might alter food intake and behaviour.

Unauthorized concomitant diets/treatments/medications

More than the dose of supplied chocolate

A very high polyphenol content in diet (from recall or dietetic assessment)

High dose antioxidant supplements including single and multivitamin preparations including A,C,E.

Appendix III– Consort Checklist (Non-pharmacological Trial) of Study Reported in Chapter Six

Checklist of Items for Reporting Trials of Nonpharmacologic Treatments*

Section	Item	Standard CONSORT Description	Extension for Nonpharmacologic Trials	Reported on Page No.
Title and abstract†	1	How participants were allocated to interventions (e.g., "random allocation," "randomized," or "randomly assigned")	In the abstract, description of the experimental treatment, comparator, care providers, centers, and blinding status	Included in abstract 13-14
Introduction				
Background	2	Scientific background and explanation of rationale		206-208
Methods				
Participants†	3	Eligibility criteria for participants and the settings and locations where the data were collected	When applicable, eligibility criteria for centers and those performing the interventions	209
Interventions†	4	Precise details of the interventions intended for each group and how and when they were actually administered	Precise details of both the experimental treatment and comparator	215-218
	4A		Description of the different components of the interventions and, when applicable, descriptions of the procedure for tailoring the interventions to individual participants	215-218
	4B		Details of how the interventions were standardized	210
	4C		Details of how adherence of care providers with the protocol was assessed or enhanced	213-215
Objectives	5	Specific objectives and hypotheses		205, 212

Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (e.g., multiple observations, training of assessors)		205
Sample size†	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules	When applicable, details of whether and how the clustering by care providers or centers was addressed	220
Randomization– sequence generation†	8	Method used to generate the random allocation sequence, including details of any restriction (e.g., blocking, stratification)	When applicable, how care providers were allocated to each trial group	211, 222
Allocation concealment	9	Method used to implement the random allocation sequence (e.g., numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned		211
Implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups		211
Blinding (masking)†	11A	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment	Whether or not those administering co-interventions were blinded to group assignment	211
	11B		If blinded, method of blinding and description of the similarity of interventions†	215

Statistical methods†	12	Statistical methods used to compare groups for primary outcome(s); methods for additional analyses, such as subgroup analyses and adjusted analyses	When applicable, details of whether and how the clustering by care providers or centers was addressed	220-222
Results				
Participant flow†	13	Flow of participants through each stage (a diagram is strongly recommended)--- specifically, for each group, report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome; describe deviations from study as planned, together with reasons	The number of care providers or centers performing the intervention in each group and the number of patients treated by each care provider or in each center	220, 213
Implementation of intervention†	New item		Details of the experimental treatment and comparator as they were implemented	217-218
Recruitment	14	Dates defining the periods of recruitment and follow-up		211
Baseline data†	15	Baseline demographic and clinical characteristics of each group	When applicable, a description of care providers (case volume, qualification, expertise, etc.) and centers (volume) in each group	224-225
Numbers analyzed	16	Number of participants (denominator) in each group included in each analysis and whether analysis was by "intention-to-treat"; state the results in absolute numbers when feasible (e.g., 10/20, not 50%)		222
Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group and the estimated effect size and its precision (e.g., 95% confidence interval)		235

Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those prespecified and those exploratory		235-247
Adverse events	19	All important adverse events or side effects in each intervention group		231
Discussion				
Interpretation†	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes	In addition, take into account the choice of the comparator, lack of or partial blinding, and unequal expertise of care providers or centers in each group	248-249
Generalizability†	21	Generalizability (external validity) of the trial findings	Generalizability (external validity) of the trial findings according to the intervention, comparators, patients, and care providers and centers involved in the trial	Study had no definitive results 250
Overall evidence	22	General interpretation of the results in the context of current evidence		250-251

*Additions or modifications to the CONSORT checklist. CONSORT = Consolidated Standards of Reporting Trials.

†This item was modified in the 2007 revised version of the CONSORT checklist.