Effects of Exercise and Cocoa Flavanol Supplementation on Cardiometabolic Function

Kade Davison

School of Medical Sciences

Department of Physiology

University of Adelaide

PhD Theses

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Declaration

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The following works are published or in press as they appear in this thesis:

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Chapter 6 – In Press

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The following peer reviewed conference presentations have come from the work within this thesis:

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Acknowledgements

Dedication

This thesis is dedicated to the memory of my grandfather, Francis William Davison (AM) and the enormous contribution he made to my life and the lives of so many others. His inspiration lives within me always.

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Abstract

The link between excess adipose tissue and an increased risk of developing various pathologies, including cardiovascular (CV) diseases and type 2 diabetes, is now well accepted. There is increasing evidence supporting impaired arterial function (AF) as a causal link in this process. It can therefore be postulated that interventions aimed at improving AF may attenuate or prevent this progression from overweight/obesity to CV and metabolic disease.

Exercise has been shown to improve AF in various pathologies. A limitation in the evidence for exercise or increased fitness in an obese population is the differentiation of effects of obesity and fitness or exercise on CV function and risk. To investigate this, a cross sectional study was done to compare cardiorespiratory fitness (CRF; predicted VO_2max) and markers of AF (brachial flow mediated dilatation (FMD), arterial compliance (AC) and blood pressure (BP)) in sedentary obese (N=27) and sedentary lean (N=26) volunteers. The obese group had more whole body fat and abdominal fat (43.5 ± 1.2% vs. 27.2 ± 1.6%; $p<0.001$ and 48.6 ± 0.9% vs. 28.9 ± 1.8%; $p<0.001$ respectively) and lower FMD (3.2 ± 0.4% vs. 5.7 ± 0.7%; $p<0.01$) than the lean subjects but there was no difference in AC. FMD correlated with whole body fat ($R = 0.28$; $p<0.05$) and abdominal fat ($R = 0.34$; $p<0.05$) but not with CRF. By comparison AC in large arteries was positively associated with CRF ($R = 0.5$; $p<0.01$) but not with fatness. These results suggest a differential influence of fitness and fatness on AF.

Flavanols from cocoa have been shown to positively influence AF in a variety of CV risk groups, however this effect has not been investigated in obese subjects or in long term studies. This question was addressed by conducting a 2x2 factorial, randomised controlled trial comparing a high flavanol (HF; 902mg/day) and low flavanol (LF;

36mg/day) cocoa drink, with or without a moderate exercise program for 12 weeks in 49 overweight/obese volunteers. Compared with LF, HF increased FMD acutely (2 hrs post dose) by 2.42% ($p < 0.01$) and chronically (over 12 weeks; $p < 0.01$) by 1.63% and reduced insulin resistance by 0.31 ($p < 0.05$), diastolic BP by 1.57mmHg and mean BP by 1.17mmHg ($p < 0.05$), independent of exercise. Regular exercise increased fat oxidation during exercise by 0.10g.m^{-1} ($p < 0.01$) and reduced abdominal fat by 0.92% ($p < 0.05$), independent of cocoa consumption. This study provides the first evidence for a sustained improvement in CV and metabolic function with HF cocoa in an overweight/obese population. To expand upon these potential benefits of cocoa in overweight/obesity a further study was conducted to compare the acute effects of HF (701mg) to LF (22mg) cocoa on BP during exercise in 21 overweight/obese volunteers. Results of this study showed a significant attenuation of the exercise induced rise in BP with the HF compared to LF cocoa.

The antihypertensive effects of cocoa flavanols were further examined by 24 hour ambulatory monitoring in 52 untreated hypertensive volunteers consuming 1 of 4 doses (state doses) of cocoa flavanols for 6 weeks. There were significant reductions in 24-hour systolic (5.3 ± 0.8 mmHg; $p = 0.001$), diastolic (3 ± 0.7 mmHg; $p = 0.002$) and mean arterial blood pressure (3.8 ± 0.8 mmHg; $p = 0.0004$) at the highest dose only (1052 mg/d). No reduction in BP was seen at any other dose. These results support the previous evidence for a BP lowering effect of HF cocoa but indicate that the effective dose may be higher than previously indicated. Collectively these results provide considerable support for the potential use of cocoa flavanols to resist the progressive decline in AF leading from obesity to metabolic syndrome.

Chapter 1

Introduction and Background

Introduction

The link between excess adipose tissue and an increased risk of developing various pathologies, including cardiovascular diseases (CVD) and type 2 diabetes (T2D) is now well accepted [1-3]. Treatment and prevention of obesity has attracted the interest of health policy makers as a vehicle by which to significantly reduce the financial and morbid consequences of CVD and T2D. To date there is no clear strategy for achieving the goal of successfully treating or preventing obesity. The many contributing factors that have resulted in the present record levels of overweight and obese children and adults in the developed world are largely ingrained in today‟s society. Two key contributing factors in this category are the ready access to energy dense foods and a reduction in the basal level of physical activity required to perform everyday functions such as transportation and occupational duties [4].

Beyond this clear association between obesity and, cardiovascular and metabolic diseases, there is growing evidence of a causal effect of excess adiposity, and of the potential mechanisms underlying this effect [5]. Identification of these mechanistic links affords the opportunity to provide therapeutic intervention(s) that may interfere with the disease progression. Much of this research has centred around the identification of a common clustering of cardiovascular and metabolic pathologies termed the Metabolic Syndrome (MetS) [6]. Whilst there are differing specific definitions of MetS, it is generally described as the presence of obesity and/or insulin resistance with dylipidaemia and/or hypertension. MetS is associated with an increased risk of development of CVD and T2D (not including where either was pre-existing) and CVD related mortality [7]. Obesity

appears to be causal in the development of the other MetS defining pathologies and within the MetS each pathology appears to contribute to the progression of the others [8]. The mechanism(s) behind this feed-forward type effect are of great interest as potential therapeutic targets [9].

A likely contributor to the development and progression of MetS and the related pathologies is what is broadly termed arterial dysfunction [10, 11]. In this context arterial function encompasses the functional state and integrity of the vascular endothelium as well as the overall degree of elastic compliance in the vessel wall. Multiple mechanistic processes have been demonstrated by which obesity and the MetS pathologies can disrupt arterial function. Furthermore, the development and progression of arterial dysfunction is causative in the development and progression of insulin resistance, dyslipidaemia and hypertension [11]. We can therefore hypothesise that therapeutic strategies aimed at enhancing arterial function may interrupt the progression from obesity to MetS and further disease.

Lifestyle based strategies aimed at modifying the energy balance are largely seen as the first line of prevention and treatment for obesity and the associated health risks [4]. This involves increasing energy expenditure in the form of increase physical activity (or exercise) and reducing energy intake. This combined strategy can be effective in directly treating obesity in the short term but it appears to be difficult for people to maintain the modified dietary and physical activity patterns and typically weight loss is regained over the proceeding few years [12]. It is consistently estimated that to sustain weight loss with physical activity requires between 60 and 90 minutes of moderate intensity exercise per day [13-16].

Beyond the direct effects on energy balance it is clear that exercise has the capacity to impact on cardiovascular function and ensuing risk [17]. Exercise interventions have been shown to improve vascular function and estimates of total physical activity participation as well as an assessment of exercise capacity are independent predictors of cardiovascular mortality [18]. The mechanistic links between the effects of exercise on arterial function and long term risk reduction are not yet clear. Furthermore, the common association between reduced physical activity, reduced exercise capacity and obesity makes it difficult to distinguish the potential effect of each component. The first experiment in this thesis endeavours to differentiate the influences of exercise capacity and obesity on arterial function to address this issue.

An extension of the dietary component of the lifestyle based approach includes the manipulation of nutrient intake to incorporate what are known as functional foods. Functional foods are those that may impart a health benefit beyond the basic nutritional value they provide. Many foods have evidence to support this type of effect and in a large number of cases this effect is thought to be as a result of the food containing one or more of the identified flavoniod compounds. These compounds are present in various forms and quantities in the majority of plant derived food types. One such food is the cocoa beans from the theobroma cacao tree, which provide a rich source of the flavoniod sub-category know as favanols. Powder and resin from cocoa beans are the fundamental ingredients in the production of chocolate. Interest in the potential bioactive effects of cocoa has grown over the past decade and clear evidence has emerged for a beneficial effect on vascular function. Despite the growing interest and evidence to this end, a number of questions remain to be answered to facilitate the therapeutic use of cocoa flavanols in the prevention or treatment of vascular dysfunction.

A major limitation in the interpretation of the evidence supporting the use of cocoa flavanols is the relatively short duration of the interventions previously undertaken. The longest intervention assessing arterial function published prior to the commencement of the intervention detailed in Chapter 4 of this thesis was 15 days [19, 20]. The results presented herewith (as detailed in chapter 4) remain the longest intervention in which arterial function was directly assessed. Furthermore, prior to the experiments presented in chapter 4 the effects of cocoa flavanols had not been specifically evaluated in an overweight or obese population. In order to determine the potential for cocoa flavanols to in some manner interfere with the proposed progression from increased adiposity to cardiovascular and metabolic dysfunction and disease it is critical to assess the effects in this population.

An additional limitation with the present evidence around the benefits of cocoa flavanol consumption concerns the food matrix most often used in the research. The majority of interventions assessing the effects of cocoa flavanols have used cocoa rich (typically called dark) chocolate and compared it to a cocoa solid free (white) chocolate that is void of flavanols [21]. Whilst there is merit in evaluating flavanol consumption in the context of the established food product that offers the greatest dietary contribution, there are methodological concerns with this approach. The first concern with this protocol is the relatively poor matching of the control product (white chocolate) to the active (dark chocolate). Ideally a control product should be closely matched to the test product for macro and micro nutrient content and differ only in the presence or absence of the active ingredient. The dark vs. white chocolate protocol fails in this regard with differing content of methyxanthines, certain minerals and often fatty acid profiles. This makes it difficult to conclude causality in any outcome measures based on the assumed active

ingredient (flavanols) alone. A further limitation to the experimental integrity of using chocolate is the inability to blind study participants from the treatment and subsequent lack of double-blinding to any of these studies. This limitation has been identified as a possible explanation for a discrepancy in the effects of cocoa when administered in a chocolate food matrix to those that have used a non-chocolate cocoa compound that allows better matching of control products [22]. In addition to the concerns with blinding posed by the use of chocolate, a further consideration in the high energy content in the quantities of chocolate typically used. The high fat and sugar content of chocolate combined with large daily serves poses significant limitation in its potential use as a therapeutic food supplement, particularly when considering overweight and obese individuals where energy balance is already of concern.

The work presented within this thesis is intended to address these shortfalls and discrepancies in the data. The first experiment aims to compare the effects of cardiorespiratory fitness (exercise capacity) and obesity on markers of arterial function. These observational results inform the design of the intervention studies by confirming measurable deficit in Arterial Function with obesity and proposing potential mechanism of pathological progression associated with both obesity and low fitness. The second experimental chapter details the first intervention which aims to assess the efficacy of a reduced fat cocoa flavanol product in combination with moderate exercise on arterial function and body composition in overweight and obese individuals. The third experimental chapter expands upon the functional application of cocoa flavanol supplementation by investigating the effect of flavanols on the cardiovascular response to exercise. This experiment proposes a novel means of assessing arterial function under physiological load and the potential for flavanols to impact upon this. The final study

specifically addresses the discrepancy in the literature regarding the efficacy of non-chocolate cocoa flavanol product on blood pressure. It provides the first specific evaluation of non-chocolate cocoa on blood pressure and the first dose comparison of flavanols on blood pressure. Collectively these results provide the most valuable information on the potential use of cocoa flavanols to resist the progressive decline in arterial and metabolic function associated with obesity to date. Furthermore they provide the most robust evaluation of the effects of cocoa flavanols on blood pressure, a significant risk factor for cardiovascular mortality.

Background

1.1 Obesity and Cardio-metabolic function

1.1a - Obesity

Obesity broadly describes a state of excess adiposity and is now classified by the world health organisation (WHO) as a as a global epidemic [23]. Obesity is most commonly defined by way of a mass to height ratio known as the body mass index (BMI). A BMI in excess of 25 kg/m^2 is defined as overweight and $30kg/m^2$ as obese with various subcategories of obesity defining the degree to which $30kg/m^2$ is exceeded. Obesity is also commonly classified by way of a measurement of abdominal girth referred to as waist circumference (WC). This is often termed central obesity because it is more directly indicative of abdominal (or visceral) adipose tissue accumulation than the BMI classification. Current cut off values for central obesity differ between genders and consider a circumference greater than 94cm to be overweight and greater than 102cm to be obese for males and 84cm to be overweight and 88cm to be obese in females [23]. Both BMI and WC are indirect measures of adiposity and are insensitive to specific tissue composition. More direct measures of subcutaneous and deeper adiposity are possible using a range of technologies. At the simpler and more widely accessible end of these are skin fold measures and bioelectrical impedance, and at the more complex end are medical imaging techniques of computer topography and dual energy X-ray absorptiometry (DEXA). The former two while relatively inexpensive and simple to operate have not been widely applied to the measurement of obesity. While they may take greater account of tissue composition than the BMI or WC methods they are dependent upon certain assumptions and are subject to large inter-trial and inter-operator variability, diminishing

any potential advantage over the simpler classifications [24-26]. The medical imaging technologies provide a superior assessment of adiposity but cost and access make them prohibitive for risk identification and stratification across a population. Comparative studies between medical imaging and the simpler classifications of BMI or WC have demonstrated good agreement between them, indicating that these simple techniques are adequate to identify obesity [27]. Where cost and access allows the more advanced techniques are beneficial for research purposes to provide greater account of the impact of adiposity and evaluation of strategies to treat obesity [27].

Prevalence of obesity in the developed world is continuing to increase and in Australia rates for overweight and obesity in 2001 were estimated to be 39.0% and 20.8% respectively, defined by BMI, and 30.5% and 25.5% by waist circumference [1]. More recent estimates suggest the prevalence of obesity may be closer to 25% and is expect to continue to increase [28]. The financial burden of obesity in Australia is significant with an estimated cost in 2008 of $2 billion to the health system alone for and total estimated cost including lost productivity, carer costs, etc of over $8 billion [28]. A number of factors are thought to contribute to the increase in the prevalence of obesity observed over the last two decades. There has been an increase in the per capita average energy supply worldwide, and the trend for the inclusion in modern diets of food with greater saturated fat and sugar content leads to increased energy intake [4]. Lifestyle changes, including a greater amount of time spent in sedentary activities due to changes in working and leisure time demands, are also contributing factors [4]. The increasing reliance on motorised transport relative to cycling and walking is another potential factor. There is also strong evidence for a hereditary predisposition of susceptibility to the above factors [4].

The classification of obesity as an epidemic is due to its proposed indirect impact on morbidity and mortality. Obesity is associated with an increased prevalence of many diseases including osteoarthritis, type 2 diabetes (T2D), cardiovascular diseases and some cancers [29, 30]. Causality for CVD and T2D is widely accepted [29, 30] and based on estimates contained in a recently revised report commissioned to estimate the growing cost of obesity, in 2008, 242 033 Australian adults had T2D as a direct consequence of obesity (costing Australia $8.3 billion) and 644 843 people had CVD as a direct result of obesity (costing Australia $34.6 billion) [28]. The growing prevalence of obesity and the causal role in CV and metabolic disease development support the notion of an obesity epidemic. This causal link however also presents a potential to reduce the prevalence of CV and metabolic disease through the direct treatment of obesity or by further elucidating the causal links between obesity and disease development. A better understanding of these mechanisms may allow the development of novel therapeutic targets to interrupt this process.

1.1b - The Metabolic Syndrome

The strong association between obesity and risk of insulin resistance/T2D and CVD has led to the classification a specific „syndrome" where evidence of clustering of these pathologies appears. Originally termed *Syndrome X* and then the *Insulin Resistance Syndrome* the broadly accepted title at present is the *Metabolic Syndrome* (MetS) [2]. Despite the adoption of a consistent title, there remain multiple defining criteria for MetS as displayed in the table below (refer table 1.1) [7]. The primary difference being whether insulin resistance is considered central as in the World Health Organisation (WHO) and European Group for the Study of Insulin Resistance (EGIR) definitions or simply one of the possible defining criteria. The International Diabetes Federation (IDF) criteria has

been recently revised to more closely match that of the Cholesterol Education Program Adult Treatment Program III (ATPIII). in that the presence of any three of the five factors results in classification as MetS [31]. Previously they required central obesity plus two of the remaining 4. Additionally it should be noted that three of the four refer specifically to central obesity as defined by WC and the EGIR 1999 excludes those with a diagnosis of Diabetes. The specific inclusion of central obesity is due to evidence that central but not total obesity was independently predictive of death from CVD [32]. There remains some debate about the most effective classification of obesity for use in general population screening. Recent evidence has shown central obesity as measured by waist to hip ration to be predictive of CV mortality independently of BMI [33], however another recent study suggests that central or total obesity are equally associated with insulin resistance and both classifications are highly predictive of the other [34].

WHO 1999	EGIR 1999	ATPIII 2001	IDF 2005
Diabetes or impaired glucose tolerance [a] or insulin resistance	Insulin resistance [b] or hyperinsulinaemia (only non-diabetic subjects)	Three or more or the following 1. Central obesity: Waist circumference >102cm (M), >88cm (F) 2. Hypertrigycerideraemia: Trigycerides ≥1.7 mmol/L 3. Low HDL-C <1.03mmol/L (M), 1.29 mmol/L (F) 4. Hypertension: Blood pressure ≥ 130/85mmHg or medication 5. Fasting plasma glucose ≥6.1mm/L	Three or more or the following: 1. Central obesity: Waist circumference (ethnicity specific) [c] 2. Raised triglycerides ≥1.7 mmol/L or specific treatment for this abnormality 3. Reduced HDL-C <1.03 mmol/L (M) 1.29 mmol/L (F) or specific treatment for this abnormality 4. Hypertension: 130/85 mmHg or medication 5. Fasting plasma glucose ≥5.6mmol/L or previously diagnosed type 2 diabetes
Plus two or more of the following: 1. Obesity BMI >30kg/m2 or Waist Hip ratio >0.9 (M), >0.85 (F) 2. Dyslioidaemia: Triglycerides ≥ 1.7mmol/L or HDL-C <0.9 mmol/L (M) <1.0mmol/L (F) 3.Hypertension: Blood pressure ≥ 140/90 mmHg or medication 4. Microalbuminuria: Ablumin exertion ≥ mg/mmol (M) and ≥ 3.5 mg/mmol (F)	Plus two or more of the following: 1. Central obesity: Waist circumference ≥94cm (M), ≥80cm (F) 2. Dyslipidaemia: Triglycerides >2.0mmol/L or HDL-C <1.0mmol/L 3. Hypertension: Blood pressure ≥140/90 mmHg or medication 4. Fasting plasma glucose ≥6.1mmol/L		

Table: 1.1: Current definition of the Metabolic Syndrome (Adapted from: Cameron et al [7])

Abbreviations: WHO, World Health Organization; EGIR, the European Group for the Study of Insulin Resistance; ATPIII, National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III); IDF, International Diabetes Federation; F, female; and M, male.
a Defined as the lowest quartile of HOMA-S.
b Defined as the top quartile of fasting insulin in the non-diabetic population.
c IDF recommends the use of ethnicity-specific waist circumference cut points based on language spoken at home rather than country of birth. Waist circumference cut points for Europids, Sub Saharan Africans, Eastern Mediterranean and Middle East (Arab) populations: M ≥94 cm, F≥80 cm; For South Asians, Chinese, Japanese, ethnic South and central Americans: M ≥90 cm, F ≥80 cm.

In 2007 a prediction of prevalence in Australia was published based on a national cross sectional, population based survey of 11,247 adults, aged >25 years conducted in 1999–2000 known as the AusDiab study [7]. The prevalence (95%CI) of the MetS, using the ATPIII, WHO, and IDF 2005 definitions was 22.1% (18.8, 25.4), 21.7% (19.0, 24.3), and 30.7% (27.1, 34.3), respectively. Among the non-diabetic population, the prevalence of the MetS using the EGIR definition was 13.4% (11.8, 14.9). The American Diabetes

Association recently recommended that the lower threshold for fasting glucose be reduced from 6.1 to 5.6 mmol/L as is now the case with the revised IDF criteria [35]. When this modification is applied to the ATPIII definition, the prevalence increased from 22.1 to 26.1% [7]. It can be expected that this would be the case for the revised IDF guidelines due to the similarity of these two classifications [31]. The only area of difference would be the impact of the ethnicity specific waist circumference criteria, the precise implication of this has not yet been identified in the Australian population.

Much controversy surrounds the MetS as a distinct pathological phenomenon and as a predictor of cardiometabolic risk. This controversy is mainly concerned with the question of whether the risk afforded by MetS is greater than the sum of its parts. Irrespective of the particular definition of MetS used, the defining criteria are largely well established independent risk factors for either or both of CVD and T2D. There is little doubt from the evidence that MetS is associated with increased risk of CVD and CVD related mortality [2]. Furthermore, evidence suggests this remains the case when T2D or Coronary Heart Disease is already present [6]. With current MetS definitions however, it has not been conclusively established whether the clustering of these risk factors affords a greater risk than the additive influence of each individually. A recent meta-analysis found the risk of CVD is still increased by about 50% in people with MetS after controlling for individual risk factors [36]. Other studies, however still suggest that this is not the case [37]. While this matter continues to be tested, the MetS remains an apparently valuable definition of a particular clustering of pathological processes that plays a significant role in the identification of cardiovascular and metabolic risk.

Of arguably greater significance than the classification of pathological clustering is the possibility of an intrinsic mechanistic link contributing to the prevalence and associated

cardiometabolic risk of MetS [10]. As potential causative mechanisms continue to be elucidated, it is becoming evident that certain preclinical pathological processes are implicit in the progression of each of the risk factors thereby supporting an underlying mutual progression and propagation of the associated pathologies. A likely contributor to this process is the underlying arterial function, incorporating both vascular endothelial function and arterial compliance [11].

1.1c - Vascular endothelial function

Originally thought to be no more than a semi-permeable membrane barrier between the blood and the fibrous structure of the tunica intima, the vascular endothelium has now been identified as the largest autocrine, paracrine and endocrine organ in the body [38]. It has also been identified as a key component in normal vascular function and in the pathological processes associated with most manifestations of CVD [38]. Endothelial cells play a crucial role in the regulation of vascular tone, platelet activation, monocyte adhesion, thrombogenesis and vessel growth and remodeling and it is largely via a breakdown in these processes that many of the markers of CVD exert their harmful effects [38].

This regulatory function is mediated through the production and release of various substances by the endothelial cell including the vasodilators nitric oxide (NO), prostacyclin and endothelium derived hyperpolarising factor (EDHF) and the vasoconstrictors endothelin I, angiotensin II and thromboxane [39]. Of all of these substances, NO has attracted the most interest because of its relative abundance and its multiple roles in the maintenance of vascular health. NO is produced by the oxidation of

the guanidino nitrogen atom of L-arginine by endothelial nitric oxide synthase (eNOS). Phosphorylation of eNOS to its active form is mediated through the activation of any one of a number of membrane receptor pathways by circulating insulin, bradykinin, acetylcholine, substance P, thrombin, serotonin, or beta agonists; or shear stress sensitive glycoproteins [40]. The vasodilatory action of NO occurs through its release from the endothelial cell to act in a paracrine manner on adjacent vascular smooth muscle cells. NO activates cyclic guanosine monophosphate (cGMP) dependent protein kinases that increase cGMP formation, reduce intracellular calcium concentration and calcium dependent phosphorylation of smooth muscle myosin regulatory light chains. These cellular changes also open potassium channels causing hyperpolarisation and relaxation [41, 42]. In addition to this vasodilatory role of NO, it also provides anti-atherosclerotic and anti-thrombotic effects locally [39]. The anti-atherosclerotic property of NO comes from its ability to reduce intracellular oxidative stress as well as inhibit early atherogenesis signalling processes [43]. Inhibition of these signalling processes leads to a down-regulation of oxidative enzymes, reduced leukocyte accumulation and an inhibition of vascular smooth muscle cell proliferation and migration. NO can reduce oxidative stress by several mechanisms including direct scavenging of superoxide anions and the termination of the lipid peroxidation initiated by oxidised low density lipoprotein and intracellular generation of oxygen-derived free radicals [43]. The anti-thrombotic property of NO is mediated through its inhibitory effect on platelet adherence and aggregation. This is facilitated by the stimulation of intra-platelet cGMP activity and subsequent phosphorylation of proteins that regulate platelet activation and adherence [43, 44].

Because of the many and varied actions of the vascular endothelium it is not possible to effectively test its functional capacity in its entirety. Subsequently, many tests have been developed to provide feedback on various markers of endothelial function. Both invasive and non-invasive assays have been widely used to measure proposed markers of endothelial function including Endothelin-1, intracellular and vascular cell adhesion molecules ICAM, von Willebrand factor and various markers of NO production and utilisation [45]. Exactly how these markers represent endothelial function and how they are influenced by other metabolic and pathological processes is not clearly understood however, making interpretation of the results difficult [45].

A non-invasive technique for the assessment of endothelial function uses ultrasound imaging to record the endothelium dependent vasodilatation of the brachial artery to an induced local hyperaemic event. This technique, referred to as „flow-mediated dilatation" (FMD), has been widely accepted and implemented as a reliable and valid method of assessing endothelial function [46]. In addition to this, numerous studies have reported FMD measured in the brachial artery to be a relatively accurate indirect predictor of coronary artery disease compared to other non-specific tests such as electrocardiogram stress tests and myocardial perfusion imaging [46, 47]. In the absence of established CVD, FMD is considered sub-clinical and as such it is not yet clear how directly it may impact upon risk of a coronary event [48]. The use of FMD has also proven valuable for identifying changes to endothelial function over time in response to various interventions [49]. Numerous studies have been able to show significant improvements in FMD in response to various exercise, dietary and pharmacological interventions [50-53].

1.1d – Arterial compliance

Structural changes in the vascular tissues in response to disease (including the processes discussed above) or aging can reduce the elastic compliance of arteries. As with the progression of endothelial dysfunction loss of arterial compliance is both contributive to and a consequence of cardiovascular and metabolic pathologies [54]. Traditional CV risk factors such as aging, [55] [56] hypertension, [55] diabetes mellitus, [57] dyslipidemia, [58] smoking [59] and sedentary lifestyle [60] promote an increase in arterial stiffness. Arterial stiffness can be separated into large (conduit or central) and small (resistance or peripheral) arteries, and there are differences between the causes and consequences of each.

Large arteries, such as the aorta, function as a conduit and damping mechanism to deliver continuous and steady blood flow into the arterioles and capillaries. The conduit function of larger arteries is dependent upon mean blood pressure (MBP), blood flow and the relation between them. The elastic properties of these large arteries absorb some of the pressure during systole thereby lowering peak systolic pressure and augmenting diastolic pressure downstream to achieve a more constant distribution of pressure across the cardiac cycle [61]. Mechanical properties of arterial walls also determine the dynamics of the propagation and reflection of pressure waves along the arterial tree [61]. Ventricular ejection generates a primary (or incident) pressure wave that moves away from the heart at a finite speed. Stiffening of the central arteries reduces the damping of this pressure resulting in faster transmission and higher peripheral pulse pressure. This direct systolic pressure wave is reflected at any point of structural or geometric discontinuity of the arterial tree, generating a reflected pressure wave that travels backward toward the ascending aorta. Higher pulse wave velocity resulting from this central stiffening can alter

the timing of these waves such that the reflected wave becomes superimposed upon the direct wave, augmenting it and increasing left ventricular afterload during systole.

Stiffness in the smaller resistance vessels tends to be more related to endothelial dysfunction and atherosclerosis through enhancement of vasoconstrictive pathways and loss of the NO dependent vasodilatory capacity [62]. This leads to a chronic state of constriction, increasing peripheral vascular resistance resulting in hypertension, increased magnitude of pressure wave reflections and impaired blood flow to multiple tissues [62].

Development of CVD and Type 2 Diabetes continues to impact back on arterial stiffness. Hypertension causes structural alterations through repeated cycles of arterial distension and recoil and hyperglycaemia leads to the accumulation of advanced glycation end products in the arterial wall [63]. These advanced glycation end products promote the development of arterial stiffness through impairment of endothelial function [63] and promotion of inflammation [64].

1.1e – Obesity and arterial function

Understanding of the link between excess adiposity and arterial dysfunction has evolved in recent years to provide a clearer picture of some of the mechanistic pathways that drive the progression from overweight/obesity to CVD and T2D. The MetS remains a key representation of the link between metabolic and cardiovascular dysfunction, and as a progression from this, the term *cardiometabolic syndrome* has emerged to highlight the integral nature of this relationship.

The development of atherosclerosis in obesity stems from a constellation of inter-related pro-atherogenic mechanisms. It is well established that higher BMI is associated with

subclinical inflammation, reflected in increased C-reactive protein levels [65, 66], and increased systemic oxidative stress independently from blood glucose and diabetes [67]. Endothelial dysfunction, involved in the pathogenesis of cardiovascular events, has also been demonstrated in obese patients with invasive techniques requiring artery catheterisation as well as non-invasively using FMD [68]. Adipocytes synthesise and secrete biologically active molecules implicated in cardiovascular pathophysiology, able to modify CVD risk. These mediators or adipokines include adiponectin, resistin, leptin, plasminogen, tumor necrosis factor alpha (TNF-α) and Interleukin 6 (IL-6) [69-72]. Evidence suggests that adipokines play a pivotal role in body metabolism homeostasis but are also actively implicated in the atherosclerotic process [72, 73]. Proinflammatory adipokine expression is elevated in obese humans and animals with excess adiposity, and a reduction in fat mass is strongly correlated with a decrease in circulating proinflammatory adipokines levels [73]. Visceral fat seems to be more active than other body fat in producing a variety of these adipokines [73]. Leptin, an adipokine implicated in the regulation of appetite, has been recently shown to enhance cellular immune responses [74], and to increase blood pressure [75, 76]. Furthermore evidence suggests that leptin increases sympathetic nerve activity, stimulates generation of reactive oxygen species, induces platelet aggregation and promotes arterial thrombosis [77]. Clinical studies show that leptin is an independent coronary heart disease risk factor and a potentially useful biomarker in CVD [76]. Adiponectin, the most abundant adipose tissue-derived peptide, has insulin sensitising properties and is down regulated in obesity [78]. Firm evidence suggests that adiponectin has many anti-inflammatory and anti-atherogenic effects on the vascular wall [79].

Proinflammatory cytokines increase vascular reactive oxygen species (ROS) production and activate redox-sensitive intracellular pathways (like activator protein- 1 and nuclear factor-kappa-ß) that up regulate pro-atherogenic genes expression. This includes increased expression of adhesion molecules on endothelial cells surface like intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (VCAM-1) which promote monocytes infiltration into subendothelial space [73]. Increased vascular oxidative stress leads to oxidative degradation of tetrahydrobiopterin, a critical eNOS co-factor, and increased asymmetrical dimethyl-arginine (ADMA) levels, an endogenous eNOS inhibitor, inducing eNOS uncoupling and endothelial dysfunction [80, 81]. Therefore an imbalance between vasodilatory and vasoconstrictive agents is favoured, and NO beneficial anti-inflammatory, antioxidant and antithrombotic effects are lost [81]. Refer to figure 1.1 for a simplified representation of the pathway from obesity to arterial dysfunction and consequential MetS and disease.

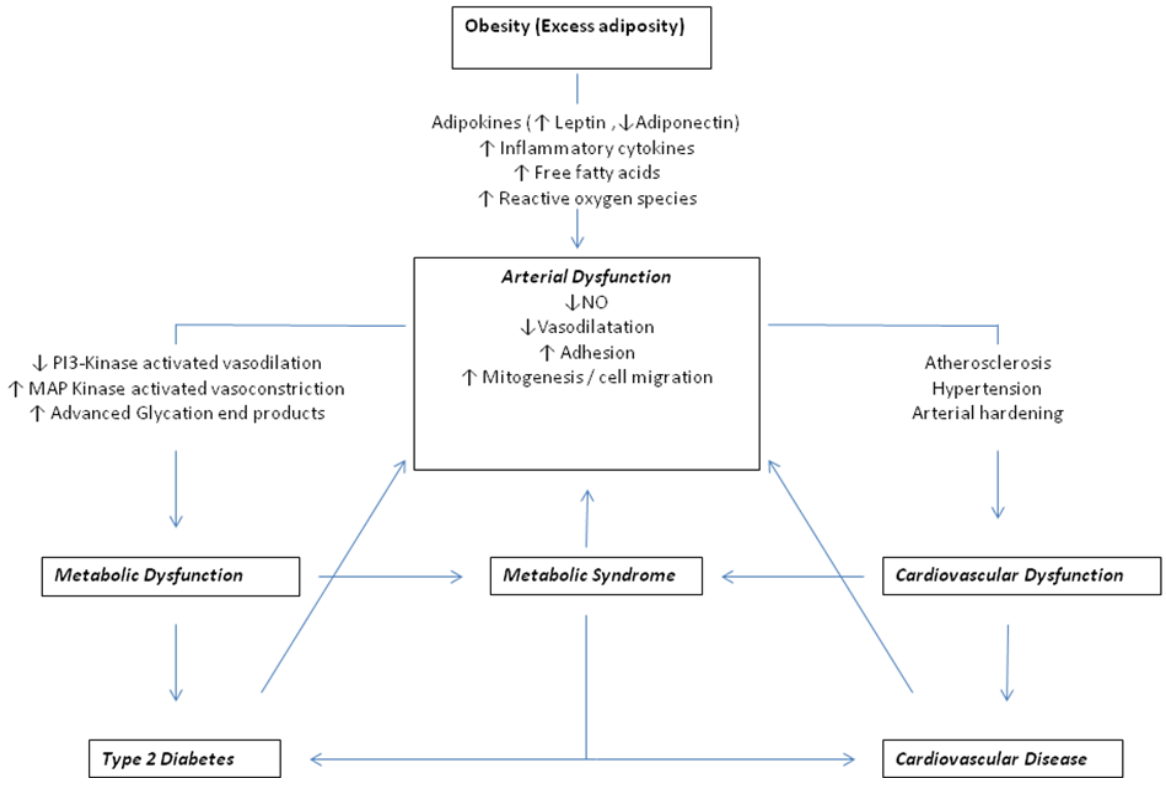

Figure 1.1: Proposed mechanistic pathway for obesity initiated cyclic progression to cardiometabolic dysfunction and disease.

1.1f – Arterial function and metabolic function

Insulin binding to its receptor at the cell surface activates two major branches of an insulin signal transduction network [82]. Like the metabolic insulin-signalling pathways in skeletal muscle and adipose tissue, insulin signalling pathways regulating endothelial production of NO are Phosphoinositide 3-kinase (PI3K) dependent [82]. Insulin resistance is characterised by pathway-selective impairment in PI3K-dependent signalling in both metabolic and vascular insulin target tissues [83, 84]. The mitogen-activated protein kinase (MAPK) pathway is associated with mitogenic effects, production of proinflammatory molecules (e.g. VCAM-1), mediation of extracellular matrix production, and vasoconstriction [82, 85]. In the setting of insulin resistance, insulin has reduced effects on PI3K-mediated pathways, while maintaining MAPK activity. This pathway-specific impairment in PI3K-dependent insulin signalling contributes to reciprocal relationships between insulin resistance and endothelial dysfunction that foster the clustering of metabolic and cardiovascular diseases in insulin-resistant states [82].

The insulin mediated, NO dependent vasodilatation plays a key role in blood glucose regulation. Local intra-arterial infusion of insulin results in a 25% increase in capillary blood volume in the deep flexor muscles of the human forearm [86]. Similarly, an hour after a mixed meal, microvascular volume in the human forearm increases by approximately 45% [87] . Thus, physiologic concentrations of insulin rapidly enhance skeletal muscle capillary recruitment. These vascular actions play an important role in augmenting the delivery of insulin and glucose to skeletal muscle. Glucose delivery to

skeletal muscle is dependent on muscle blood flow, as well as vascular capillary surface area and permeability. After a mixed meal, an oral glucose load, or infusion of insulin, recruitment of capillaries expands the capillary surface area and increases muscle blood flow, which together substantially increase glucose and insulin delivery [86, 87] This enhances direct effects of insulin to stimulate glucose uptake and utilisation in skeletal muscle. The time course for insulin-stimulated capillary recruitment approximates the time course for insulin-mediated glucose uptake in skeletal muscle [86-88]. Furthermore, inhibitors of eNOS that block insulin-mediated capillary recruitment cause a concomitant 40% reduction in glucose disposal [89]. Thus, PI3K-dependent metabolic actions of insulin directly promote glucose uptake in skeletal muscle by stimulating translocation of insulin responsive glucose transporters (GLUT4). At the same time, PI3K-dependent vascular actions of insulin to increase blood flow and capillary recruitment substantially contribute to promoting glucose disposal under healthy conditions and help to couple metabolic and hemodynamic homeostasis.

In a prospective study of the children and spouses of children of the Framingham Heart Study cohort, elevated circulating plasma markers of endothelial dysfunction (PAI-1 and von Willebrand factor) increased the risk of developing diabetes independent of other risk factors for diabetes, including obesity, insulin resistance, and inflammation [90]. Similarly, in a large, study of postmenopausal women, higher levels of circulating E-selectin and ICAM-1 were consistently associated with increased risk of developing diabetes [91]. These studies support a potential causal role for endothelial dysfunction in insulin resistance. Conversely, consequences of insulin resistance such as hyperglycaemia and dyslipidaemia are known to impact upon endothelial function [68]. This potential

reciprocally causal relationship supports the notion of a mechanistic link within the MetS as discussed previously.

Studies investigating the association between the MetS and arterial stiffness have consistently shown increased arterial stiffness in individuals with the MetS or with increasing number of traits of the MetS [92, 93]. In addition, prospective studies have shown that the increase in arterial stiffness with age is greater in individuals with the MetS as compared with those without. The chronic effects of insulin resistance on arterial stiffness have also been examined. In healthy individuals, a positive association between insulin-mediated glucose uptake and arterial distensibility was observed, although this effect was confined to the femoral artery and was more pronounced in women [93]. In a large population-based study, insulin concentrations were associated with carotid artery stiffness, and this association was also stronger in women than in men [94]. Importantly, because arterial stiffness is highly dependent on blood pressure and hypertension itself affects the stimulation of glucose uptake by insulin, it is noteworthy that the studies mentioned above have shown insulin resistance to be associated with estimates of arterial stiffness even after adjustments for mean arterial pressure levels.

A longitudinal study that has addressed the individual and combined effects of raised blood pressure and raised glucose levels on the progression of arterial stiffness found that, the estimated rate of increase in arterial stiffness was higher in individuals with both abnormalities than in those with either abnormality alone [95] . Furthermore, persistence of both abnormalities synergistically accelerated the rate of increase in arterial stiffness such that it was three times higher than in those who persisted with elevated levels of blood pressure or glucose alone. Collectively these results demonstrate that insulin

resistance contributes to increased arterial stiffness independently of blood pressure both in type 2 diabetic patients and in apparently healthy individuals.

1.1g – Arterial function and cardiovascular disease

Atherosclerosis, the predominant pathology associated with CVD, is a highly complex, chronically progressive condition of the arteries. It typically involves a breakdown in the healthy functioning of the vascular endothelium as a result of, amongst other things, elevated and modified LDL; increased oxidative stress; hypertension; and hyperglycaemia and/or hyperinsulinaemia [38]. Combinations of these or other causes leads to monocyte adhesion; platelet aggregation; increased LDL peroxidation and internalisation; and smooth muscle cell migration and proliferation, which ultimately results in the formation of obstructive plaques in the vascular wall [38]. If these plaques continue to develop blood flow is occluded such that normal function of downstream tissue is reduced. They can also become unstable and rupture causing local thrombosis and further occluding blood flow as is often the cause of stroke and acute myocardial infarction [38]. The atherosclerotic process is facilitated by a pro-inflammatory and pro-thrombotic state, further inhibiting endothelial function thereby leading to a continuously progressive pathology [38, 42, 45]. As discussed previously the vascular endothelium plays a crucial role in the normal resistance to the development and progression of atherosclerosis, therefore, endothelial dysfunction is inherent in the pathogenic process of vascular disease [42, 45].

Hypertension is integrally involved in this process, with evidence showing it is causative of and caused by this atherosclerotic process [38]. Multiple mechanistic pathways have been identified around the pathogenesis of hypertension including endothelial dysfunction and/or arterial stiffening as well dysfunction of the renin angiotensin aldosterone system,

the sympathetic nervous system and baroreflex control mechanisms. [96]. Irrespective of the specific mechanisms initiating a loss in the healthy control of BP however, hypertension is consistently associated with endothelial dysfunction [42, 96, 97]. The relative contribution of endothelial dysfunction to the initiation and progression of hypertension is thought to vary between individuals, however the presence of endothelial dysfunction in hypertension presents yet another dangerous positive feedback loop towards increased CV dysfunction [42, 97]. Loss of NO dependent vasodilatation results in a sustained increase in peripheral vascular resistance, driving blood pressure up and causing increased augmentation of aortic pressures. Furthermore the loss in capacity for reactive dilatation (as demonstrated by reduced FMD) in response to changes in blood flow or biochemistry (e.g. acetylcholine), reduces the capacity to response effectively to increased physiological stress. The consequences of this are exaggerated increases in BP when cardiac output is increased (e.g. during increased physical exertion) and restricted blood flow to tissues. Increased pressure during exertion exacerbates the likelihood of rupturing an atherosclerotic plaque potentially causing myocardial infarction or cerebrovascular accident. Restricted blood flow causes reduced tissue perfusion resulting in impairing the supply of oxygen and nutrient rich blood to working tissue and therefore metabolic function.

This disruption to the homeostatic regulation of BP causes increased stress to the arterial walls further supporting pathological progression. This is partially through dysfunction of the vascular endothelium and partially as a result of other structural changes in the vessel walls [98]. With elevated blood pressure the arteries stiffen as the result of degeneration of the arterial media with fractures and fragmentation of elastic lamellae, increased collagen and calcium content, and dilation and hypertrophy of large arteries and the aorta

[98]. This process occurs to some extent as a result of aging but is accelerated through the increased mechanical stress with greater intra-arterial pressures. It is evident that vascular dysfunction is both causative of, and caused by, these deviations from normal blood vessel structure and function, creating a vicious cycle of cardiovascular disease progression.

1.1h – Arterial function as a therapeutic target

It is clear that the structural and functional integrity of the arterial tree is a likely contributor to the link between excess adiposity, insulin resistance and CV dysfunction and disease. The role of the endothelium in the development and progression of these pathologies supports the potential that endothelial dysfunction resulting from one pathological state (e.g. dyslipidaemia) may directly contribute to the development of separate pathology (e.g insulin resistance). Therefore, if the integrity of the arterial function were able to be maintained in the first instance, then not only could the progression of the initial insult be slowed, but the development of further pathologies may be avoided. The maintenance of arterial function thus becomes a logical target for early intervention in the control of metabolic or CV dysfunction. This may then slow or prevent the progression from obesity to the cluster of pathologies defined as the MetS.

This potential has been acknowledged by pharmaceutical companies and the vascular endothelium will likely become a more direct target for drug therapy in the future [99]. Endothelial mechanisms have also been elucidated for some commonly used medications including aspirin and statins and research is ongoing for effective coatings for arterial stents to support improved endothelial integrity [52, 99].

The extent to which large artery stiffness is modifiable remains a contentious subject [100]. While the structural degeneration that underlies arterial stiffness in elastic arteries is largely irreversible, stiffness in muscular arteries can be attenuated directly by vasodilator therapy including calcium antagonists [101], nitrates [102], phosphodiesterase-5 inhibitors [103], angiotensin-converting enzyme inhibitors [104, 105] and angiotensin receptor blockers [106-108]; and indirectly by improvement of endothelial function through medical [109] and lifestyle therapy [110, 111].

The potential to interrupt the progressive nature of endothelial dysfunction at a sub-clinical level has great merit when considering the increased CV and metabolic risk once MetS has developed. To implement strategies to enhance or support endothelial function in overweight/obese individuals, or those with mildly elevated BP, or reduced insulin sensitivity would appear to be a sound strategy. These individuals would be suited to lifestyle based intervention rather than pharmaceutical by present standards [112]. Some exercise and/or dietary based interventions have demonstrated the potential to improve endothelial function in these populations [112]. A significant limitation to the widespread use of lifestyle related interventions is the limited evidence for long term maintenance of significant lifestyle modification [113]. As a result the most successful lifestyle strategy is likely to be one that requires the least change from an individual‟s existing routine. This supports the potential benefit of bioactive nutrients that can be added to or substituted into an individual‟s existing routine to afford some health benefit. Some such products have demonstrated a potential to impact upon endothelial function including flavanols derived from cocoa beans [20, 114].

1.2 Exercise

The term „exercise", often used interchangeably with „physical activity" generally refers to the performance of physical work above that required at rest. It can be graded in intensity usually dependent upon the degree of exertion relative to an individual"s maximum achievable, and is commonly defined as mild, moderate or high intensity. Exercise can be further classified depending upon the type of physiological exertion into aerobic (also termed cardiovascular, cardiorespiratory or endurance) exercise, which primarily stresses the cardiovascular and respiratory systems and resistance exercise (also termed strength training) which primarily stresses localised areas of skeletal musculature.

The threshold whereby rest becomes exercise or the amount of exercise in a given timeframe that is required to define an individual as „physically active" have not been conclusively defined. Therefore these parameters will vary between studies and must be considered when interpreting data of this nature [13]. In addition to this consideration, the capacity to directly measure exercise intensity and participation is limited. Exercise intensity can be precisely measured for an individual person during exercise but the capacity to do this on a large scale is demanding of resources and expertise. Additionally to quantify the intensity and amount of exercise to determine longitudinal health effects requires continuous assessment indefinitely. The resources required to achieve this are significantly prohibitive to conducting this research. To overcome this limitation, a range of survey instruments have been developed to record long term exercise participation or regular samples of exercise participation over shorter periods. Short term samples such as 24 hour dairies are accurate records for acute exercise participation but do not indicate long term trends and are subject to bias through a hawthorn type effect [115]. Long term recall instruments are reasonable indicators of population levels of activity but lack

accuracy for determining individual activity participation compared to objective measurements [116]. This error is exaggerated when they are used in association with an exercise intervention [117]. Regardless of these limitations in the capacity to obtain an accurate indication of exercise levels in individuals, epidemiological studies have identified physical inactivity as an independent risk factor for cardiovascular and all cause mortality [118].

In addition to the benefits of exercise participation, evidence has emerged for a protective effect of aerobic exercise capacity (also termed functional capacity, aerobic fitness, or cardiorespiratory fitness) [119, 120]. This can be directly measured through exercise testing and provides a valid and reliable determinant of the functional capacity of the cardiovascular, respiratory and metabolic systems under increased stress. Exercise capacity is influenced by physical activity participation and age or disease related declines in exercise capacity are attenuated through exercise participation [121]. The extent to which habitual physical activity participation directly determines exercise capacity remains unclear [122].

The final area of evidence for the beneficial effects of exercise on health lies in the multitude of randomised controlled trials demonstrating improvements in CV and metabolic function with various exercise interventions. Whilst the exact long term benefits cannot be accurately predicted from the results seen with short term interventions, it is reasonable to suggest that if the exercise behaviours that lead to improved function are sustained in the long term that these benefits can translate to reduced premature mortality.

1.2a Exercise and Obesity

Given the underlying causative influence of energy imbalance in the development of obesity, it is obvious that the increase in energy expenditure with exercise will be beneficial in its prevention and treatment [4]. Epidemiological evidence supports this with studies demonstrating inverse relationships between exercise participation and weight gain over time [123-125]. The exact amount of exercise required to prevent weight gain is not yet known but Hill and colleagues have predicted that 15 – 20 minutes per day of (additional) exercise could prevent weight gain in most individuals based upon the present rate of weight gain seen in the American population [126] . The WHO along with most major health institutes around the world including, within Australia, the National Heart Foundation and Diabetes Australia recommend every person should participate in at least 30 minutes of moderate intensity physical activity on most if not all days of the week. Despite this, survey data from 2006 suggest that only 29% of Australian adults participate in sport or recreational exercise more than 2 times per week.

It is clear from the discrepancy between the physical activity recommendations and the self reported levels of participation that exercise is not presently being adopted to the extent required to prevent the development of obesity by most individuals. It is therefore important to consider the potential for exercise to treat existing incidents of excess adiposity. An initial review of studies comparing weight loss with diet modification and exercise to diet alone concluded that most studies found no significant differences in total weight loss but that in just about every study the absolute amount of weight lost was a little higher when diet and exercise were used together [127]. Stronger evidence for exercise was provided by a more recent review that incorporated a meta-analysis whereby an overall increased weight loss with diet and exercise intervention led to a 20% greater

weight loss than diet alone and 22% greater weight loss at 12 months after the cessation of the intervention [128]. Importantly, the mean weight loss from baseline 1 year post intervention was approximately 50% less than that at the end of the intervention indicating a weight regain of half of that lost during the intervention in both groups. When considered in isolation (i.e. without concurrent dietary restriction) exercise has not been found to be an effective strategy for weight loss [127]. It is largely agreed however that 60 – 90 minutes per day of moderate exercise can be effective for the maintenance of weight loss if adhered to [13]. When considered in the context of the typically sedentary occupational setting of the developed world to achieve this target indefinitely requires a significant contribution of leisure time physical activity. To date there is a lack evidence to suggest that the dietary and exercise changes required to effect weight loss through simple manipulation of energy balance is sustainable for the population at large [4]. Longer term follow up studies suggest that these lifestyle changes are not sustained in most individuals [129]. Other means of modifying energy balance that do not require equivalent behavioural changes may prove more successful. The potential for various compounds found within certain foods (e.g. preferential utilisation of fat for fuel with caffeine consumption), specific types of exercise (e.g. high versus low intensity) or pharmaceuticals, to influence energy balance over and above any direct contribution warrants greater investigation. Consumption of high levels of green tea have been shown to increase the energy cost of rest and exercise via an as yet unknown mechanism [130]. Additionally omega 3 fish oil supplementation has been found to increase the energy contribution from fat prior to exercise and increase the amount of fat tissue lost with participation in a 12 week exercise program [114]. The design of nutritional and exercise programs that take advantage of these type of effects may provide a more sustainable approach to the treatment and management of obesity.

1.2 b Exercise and vascular function

In addition to the potential direct effects in the prevention and treatment of obesity, exercise has the capacity to improve CV function. Exercise training of both localised muscle groups [131, 132] and whole body exercise, predominantly of the lower limbs [132-142] are associated with improvement in measures of NO dependent vasodilator function in subjects with cardiovascular risk factors and disease. Indeed, the consistency of the published data indicating that exercise training improves endothelial function in heterogeneous groups in whom it is initially depressed is remarkable, and contrasts with training studies of subjects with normal endothelial function. This strongly suggests that subjects with impaired endothelial function may be more amenable to improvement in NO function as a result of training than healthy subjects. Also the beneficial effects of exercise are believed to be systemic rather than local if the exercise involves large muscle groups (i.e. lower limb exercise improves upper limb conduit and resistance vessel function [134, 137, 141, 142].

Interestingly the effect of exercise on endothelial function in overweight and obese individuals is less well studied and is typically investigated as part of a general weight loss program incorporating diet and exercise. Three studies have reported improvements in endothelial function with exercise in obesity. Woo et al investigated the effect of diet and exercise induced weight loss and diet only weight loss over 6 weeks in otherwise healthy obese children [143]. They found that both groups improved in endothelial function and there was not an additive effect of exercise at six weeks. When followed up at 12 months however they found that only the diet plus exercise group had sustained improvement. Watts et al [144] investigated the effects of 3 x 1 hour exercise sessions per

week (combining aerobic and resistance training) for 8 weeks in otherwise healthy obese adolescents. Their results indicated improved endothelial function independent of changes in adiposity [144].The third study investigated 30 minutes of 3 days per week and dietary restriction in type 2 diabetic adults for between 10 and 16 weeks [143]. They reported that only those that lost weight showed improvement in endothelial function suggesting that the exercise, in the absence of weight loss, did not improve endothelial function. A more recent study found no effect of exercise or weight loss on endothelial function in obese diabetic subjects [145]. The relationship between aerobic fitness or physical activity participation and endothelial function has not yet been investigated. Christou et al investigated the relationship between aerobic fitness, degree of fatness, and various markers of cardiovascular risk, however they did not directly assess endothelial function [146].

The effect of regular exercise on large artery stiffness has also been documented. Subjects performing regular aerobic exercise have greater arterial compliance than sedentary subjects [110]. In addition, regular exercise for 3 months appears to restore arterial compliance in previously sedentary subjects [110]. Although the mechanism by which exercise training ameliorates arterial stiffness is unclear, a functional effect, such as improvement of endothelial function, [88] has been postulated. Conversely, some evidence suggests that resistance training may increase large arterial stiffness [147]. Acute intermittent elevation in blood pressure and sympathetic nervous activity has been proposed as possible mechanisms [147].

These results indicate the capacity for exercise to enhance arterial function in various populations including obesity. Furthermore the amount of exercise required to induce these effects appears to be significantly less than that required to induce weight loss. This

may increase the likelihood of individuals achieving the required lifestyle modification to illicit a benefit and maintaining this in the long term. Exercise may therefore be a valuable tool in resisting the deleterious effects of obesity on arterial function. This may be further enhanced by combining exercise with other therapies such as general dietary changes or dietary supplementation with bioactive nutrients.

1.3 Cocoa flavanols

1.3a Classification and bioavailability

The past decade has seen an increasing interest in potential anti-pathogenic properties of groups of polyphenolic compounds found in plant derived foods [148]. Collectively known as flavonoids, they have a typical basic structure but can be sub-divided into numerous groups depending upon deviations from this, as well as the types and positions of side groups and interaction with other compounds [148, 149]. One of these sub-classes, the flavanols, has attracted particular interest for their potential anti-atherogenic and more recently anti-obesogenic effects. Flavanols are the predominant flavonoids in cocoa, wine, tea, apples, berries and onions. Of these foods, cocoa offers potentially the richest source of flavanols per serve [150, 151]. Despite the availability and generally accepted palatability of cocoa products, when evaluated in a cross sectional evaluation of 470 European men, one third did not consume cocoa at all and the median intake was just 2.1 grams/day [152]. Additionally, variations in the manufacture of cocoa products can vastly alter the flavanol content, often deliberately in an attempt to improve taste [153].

Until recently the lack of a suitable means of testing food products for their relative concentrations of various flavonoids has limited the information obtained from trials using food products. It is now known that cocoa contains the isomeric flavanols, catechin and epicatechin, as well as oligomers/polymers of these compounds known as procyanidins.

The advancements in measuring individual flavonoid concentrations have also provided a greater understanding of their absorption and bioavailability in vivo. It was originally thought that all procyanidins were degraded by the acidic environment of the stomach,

however intact flavanols as well as procyanidins have been detected in human plasma [154-156], although they generally undergo some form of conjugation upon absorption [157]. Flavanol conjugates have been found in the plasma and urine of humans and animals and in the brain and bile of rats following cocoa administration [155, 158, 159]. Some degradation of both flavanols and procyanidins has also been reported and it is speculated that these metabolites may also be of clinical relevance [160]. Following consumption of an equal dose of epicatechin and catechin, plasma epicatechin levels were found to be ten times greater than those of catechin suggesting epicatechin is considerably more bioavailable. Part of this difference may be attributable to procyanidin breakdown [156]. In an *in vitro* study catechin and epicatechin dose-dependently accumulated to similar extents in the whole cell fraction of Jurkat cells (a cell line derived from human T-cell leukaemia), and epicatechin, but not catechin, was found in the nuclear fraction [161]. Peak plasma concentrations of flavanols in humans have been reported to occur at around 2 hours post administration [162, 163] and, contrary to previous reports [164], do not appear to be influenced by milk protein, but may be influenced by the food matrix consumed [165, 166].

1.3b Flavanols and Endothelial Function

Numerous studies have reported acute increases in endothelial function following consumption of cocoa flavanols (CF). In vivo, in patients with cardiovascular risk factors, including smoking, a cocoa drink high in flavonol content (176 to 185 mg) rapidly enhances the circulating pool of bioactive NO by more than a third and, in turn, augments flow mediated vasodilation [167, 168]. Moreover, infusion of NG-monomethyl-L-arginine, (L-NAME) an inhibitor of NO synthesis, reverses the increase in NO and the

augmentation in endothelial function associated with cocoa intake, whereas infusion of ascorbic acid has no effect. Similarly, in isolated aortic rings, concentrations of flavanols comparable to those occurring in plasma after cocoa intake induce endothelium-dependent relaxations. Chronic consumption of a high-flavanol diet is associated with a high urinary excretion of NO metabolites, consistent with an augmented NO production or diminished degradation [169].

Fisher and colleagues investigated the vascular response to forearm ischemia using peripheral arterial tonometry in subjects following 5 days of consuming a high cocoa polyphenol beverage (821mg polyphenols/day). They reported an increase in basal peripheral blood flow at day five and a non-significant trend towards an increased response to ischemia compared to day 1 values. Despite these increases the response to an acute dose of cocoa at day 5 further increased both basal and reactive hyperaemic blood flow. Again infusion of the nitric oxide synthase inhibitor L-NAME following the acute dose of cocoa attenuated the increase baseline and reactive hyperaemic blood flow, suggesting increased NO production is responsible for these differences. Acute consumption of both a high flavanol cocoa beverage or an epcatechin extract lead to similar acute increases in FMD lasting about 6 hours, suggesting this effect may be primarily mediated via epicatechin [169].

Several studies have reported sustained effects on endothelial function with ongoing daily consumption. Grassi and colleagues reported non-acute (following >12 hour fasting) increases in FMD with 15 days of dark chocolate consumption (100g/day) providing approximately 500mg of CF in healthy, insulin resistant and hypertensive subjects [20, 170]. A similarly designed study (2 week crossover) that used a high-flavanol cocoa beverage (~900mg flavanol per day) reported a sustained improvement in insulin

mediated vasodilation [22]. Heiss and colleagues provided 906mg of CF per day to smokers for 1 week and saw significant improvements in FMD compared to placebo [171]. Longer term studies have reported a sustained improvement in FMD in a T2D group [172] and increased blood flow, but not flow mediated dilatation, in hypercholeserolaemic, post-menopausal women [173]. A recent meta-analysis of the cardiovascular effects of the most studied flavonoids concluded that CF were the only group to show an overall effect on endothelial function [21]. When studies were removed from the meta-analysis based on lack of blinding an overall effect for chronic consumption was not present but the acute effect remained.

1.3c Other reported cardiovascular effects of Flavanols

Direct Antioxidant Action of Flavanols

Flavonoids in general are known for their antioxidant properties both in vitro and in vivo [174]. The structure of the flavanols, and therefore procyanidins, enables them to easily donate hydrogen ions thus facilitating a simple antioxidant action [175]. This allows them to scavenge free oxygen and nitrogen species that may otherwise play an instigatory or facilitatory role in the atherogenic process. The specific structural characteristics of these molecules also provide a metal chelating function, thus reducing the potential production of highly reactive oxygen species [176]. This has been demonstrated in vitro with cocoa and purified CF and procyanidins having been found to attenuate the copper mediated and endothelial cell-mediated oxidation of LDL cholesterol [177], reduce the production of reactive oxygen species by activated leukocytes [178], protect against erythrocyte hemolysis [179, 180] and inhibit ultraviolet C-induced DNA oxidation [181].

These results have been partially replicated in vivo with inhibition of LDL oxidation in healthy human subjects following consumption of a flavanol rich cocoa product [182] and increased resistance to induced LDL oxidation in rats with oral administration of cocoa powder. An increase in plasma antioxidant capacity has been found in humans following the consumption of both high flavanol chocolate [163] and a high flavanol cocoa beverage [183]. Daily consumption of dark chocolate and cocoa powder for 4 weeks in a placebo controlled cross over study found increased total plasma antioxidant activity and reduced susceptibility of LDL to ex vivo oxidation when tested in a fasted state [184]. Chronic feeding of cocoa powder in rats was associated with reduced DNA and glutathione oxidation [185].

Flavanols and Inflammation

Activation of proinflammatory enzymes such as lipoxygenases and myeloperoxidases (MPO) are believed to be critical steps in the progression of atherosclerosis [186]. Flavanols from cocoa have been shown in vitro to suppress the enzymatic activity of lipoyxgenases on arachidonic acid by directly binding to the active site of the enzymes [187, 188]. Lipoxygenases are responsible for the biosynthesis of leukotrienes and the catalysis of specific and non-specific lipid peroxidation [189]. Leukotrienes contribute to the atherogenic state by promoting neutrophil adhesion to endothelial cells and are a chemotactic agent for other inflammatory cells [189]. MPO are known to react with nitric oxide to produce nitrite radicals [190]. This impacts negatively on the vessel in two ways, it reduces the bioavailability of nitric oxide (NO) (a known mediator of anti-inflammatory, anti-atherosclerotic and vasodilatory actions) and precipitates the nitrite mediated peroxidation of LDL by MPO [191]. Flavanols have been found to block this process in vitro by acting as substrates for MPO and scavenging the nitrite radicals [192,

193]. The in vivo significance of this may be, at least partially, represented in the reported effects of CF on endothelium mediated vasodilatation as discussed above [194, 195].

Flavanols and procyanidins have also been reported to regulate Nuclear Factor – kappa B (NF-κB) transcription factor in vitro [161]. NF-κB is activated by multiple signals associated with the proinflammatory state and regulates the expression of numerous genes involved in inflammation, cell proliferation and survival and immune responses [196].

Flavanols and Platelet Aggregation

Platelet adhesion and activation plays a prominent role in the development and manifestation of atherosclerosis and its associated health risk [197]. Platelets can adhere to dysfunctional endothelium, exposed collagen, and macrophages and become activated. Once activated, platelets release cytokines, growth factors and thromboxane, which contributes to migration and proliferation of smooth muscle cells and monocytes and platelet aggregation [198]. Both high and moderate acute doses of cocoa were shown ex vivo to reduce both the ADP/collagen and adrenaline/collagen-activated platelet related hemostasis at 2 and 6 hours post consumption [199, 200]. These antiaggregatory effects were shown to be attributable to a reduction in the ADP and adrenaline induced expression of the GPIIa/GPIIIb surface protein [200, 201]. Following on from this, 28 days of moderate cocoa flavanol consumption resulted in reduced ADP/collagen-induced platelet aggregation, P-selectin (implicated in adhesion and rolling of leukocytes on the vascular wall) and platelet volume (a marker of platelet activation status) [202]. More research is required to elucidate the mechanism(s) of action of flavanols on platelet function, but inhibition of lipoxygenases (as described in section 1.3b above) is likely to contribute to these anti-aggregatory effects

Flavanols and Blood pressure

First evidence of a similar effect of cocoa was obtained in Kuna Indians, a native population living on islands off the coast of Panama. The Kuna belong to one of the few cultures that are protected against the age-dependent increase in blood pressure and the development of arterial hypertension. Interestingly, the Kunas consume enormous amounts of cocoa daily, sometimes even enriched with salt [203]. Clinical studies revealed that the Kunas indeed have lower blood pressure values and no age-dependent decline in kidney function [203]. Moreover, in this native population, mortality resulting from cardiovascular events is markedly lower compared with other Pan-American citizens (9.2 ±3.1 versus 83.4 ± 0.7 age-adjusted deaths per 100 000). The factors involved are clearly environmental rather than genetic because this protection is lost on migration to urban Panama City, where the home-prepared cocoa is replaced by other food with a lower flavanol content. Further epidemiological evidence has come from the Zutphen Elderly longitudinal study looking at lifestyle and cardiovascular risk in a cohort of older men [152]. This study found cocoa intake to be inversely related to blood pressure, and in a prospective analysis, higher cocoa intake was associated with a reduction of cardiovascular and all-cause mortality. Furthermore, this association has since been confirmed in another population [204].

A number of clinical trials have reported reductions in BP following short term consumption of high flavanol dark chocolate compared to low flavanol white chocolate. A recent meta-analysis [205] included five studies which compared the antihypertensive effects of CF-rich chocolate with chocolate containing few or no CF. The amount of chocolate consumed daily ranged from 46 to 105g and provided intakes of CF ranging from 246 to 500 mg/day. There was a mean BP reduction of 4.7 / 2.8 mmHg

(systolic/diastolic). In a subsequent study that was not included in the meta-analysis, Taubert et al [206] showed that consuming as little as 30 mg of CF per day in 6g of dark chocolate for 18 weeks was sufficient to lower systolic blood pressure (SBP) by 2.9 mmHg and diastolic blood pressure (DBP) by 1.9 mmHg. A more recent meta analysis by Hooper et al [21] also reported a net effect in favour in cocoa in the order of 6mmHg systolic and 3mmHg diastolic. A series of 3 studies by Grassi et al (one of which was included in each of the above mentioned Meta-analyses) repeated the same protocol in samples of healthy, non-medicated hypertensive and non-medicated insulin resistant populations [19, 20, 170]. In each of these trials participants consumed 100g of dark chocolate or 100g of white (flavanol free) chocolate in a crossover design using 24 hour ambulatory monitoring. Results were similar in all groups with significant reductions in both SBP and DBP in the hypertensive and insulin resistant groups, and SBP in the healthy group, with dark chocolate compared to white. The reported dose of CF with the 100g chocolate serve was approximately 500mg.

An anomaly within the research literature around the effects of BP is that the results reported with cocoa consumption in the form of dark chocolate have not been replicated with non-chocolate food types. Three studies have been conducted which assessed the effects of ongoing consumption of cocoa in the form of a flavanol-rich reconstituted beverage. The populations studied were postmenopausal females, adults with type 2 diabetes and adults with essential hypertension [22, 172, 173]. The cocoa beverages used delivered between 446 – 964 mg CF/day for between 14 days and 6 weeks. Despite all reporting some improvements in arterial function, none found any reduction in BP compared to placebo. The lack of BP change in these studies following chronic consumption of large doses of CF are inconsistent with the results seen in the meta-

analysis and subsequent study [205] with flavanol rich chocolate consumption. These findings suggest that the dose of flavanols alone may not determine the change in BP, but a number of differences in the design of these studies makes direct comparisons difficult. The studies that have been published to date examined different patient populations (healthy and hypertensive adults in chocolate based protocols and hypercholesterolaemic, obese and type 2 diabetic in non-chocolate protocols; medicated and unmedicated adults and have utilised different techniques for monitoring BP (seated clinic BP, supine clinic BP, or ambulatory BP monitoring).

1.3d Flavanols and Metabolic Function

The major criticism against the consumption of cocoa products (specifically chocolate) for therapeutic benefit is the high amount of sugar and triglycerides that needs to be consumed to reach what has been demonstrated to be a potentially therapeutic dose and the capacity this has to increase cardiovascular risk though the development and/or propagation of obesity. The potential risk/benefit balance is yet to be established but this remains a concern for the widespread adoption of cocoa rich chocolate products for therapeutic purposes. The development of reduced fat, high polyphenol cocoa products may go some way towards alleviating this limitation, however the efficacy of such products needs to be established.

Grassi and colleagues [19, 20, 170] found that in addition to the aforementioned increases in FMD and reductions in blood pressure, 15 days of dark chocolate consumption significantly reduced both fasting and postprandial plasma insulin and glucose levels compared to baseline and to 15 days of white chocolate consumption. Furthermore a dose

dependent reduction in blood glucose was also found in diabetic rats following 4 weeks of cocoa extract [207].

To investigate the potential influence of cocoa on body composition, an identical high fat diet plus either cocoa (12.5% of total diet) or placebo was fed to male wistar rats for 21 days [208]. Cocoa consumption led to a significant decrease in total body weight, mesenteric white adipose tissue (MES-WAT) weight, serum triglycerides but no difference in liver or gastrocnemius muscle weight. They performed DNA microarrays on liver and MES-WAT tissue samples from two rats from each group that were representative of the mean values of the above parameters. The results showed a reduction in the expression of numerous genes associated with fatty acid transport and synthesis in liver and MES-WAT and increased expression of genes associated with thermogenesis (uncoupling protein 2) in MES-WAT. The authors propose that these changes in gene expression are responsible for the observed differences in weight gain but provide no suggestion of potential mechanisms underlying such effects.

1.4 Study Aims:

In summary the key concepts underpinning the experimental work to follow in this thesis are as follows:

- Obesity appears to be causally linked with arterial dysfunction.
- Physical inactivity and/or lack of functional fitness may be causally linked with arterial dysfunction.
- Obesity related arterial dysfunction leads to insulin resistance and T2D, and hypertension and CVD.
- Exercise may be an effective therapeutic tool in attenuating obesity related arterial dysfunction and subsequent progression to cardiometabolic disease.
- Cocoa flavanols may be an effective therapeutic tool in attenuating obesity related arterial dysfunction and subsequent progression to cardiometabolic disease.
- More robust studies are required to evaluate the potential benefits of cocoa flavanols in attenuating arterial dysfunction.

Therefore the specific aims of each experiment are:

Experiment 1 (Chapter 3)

This study aimed to identify the specific relationships between adiposity and/or fitness and cardiovascular function. These results provide important mechanistic insights and supported the rationale for the main intervention study to follow.

Experiment 2 (Chapter 4)

This experiment makes up the principal intervention of this thesis whereby the effect of continuous flavanol supplementation, with and without concurrent moderate physical activity, on cardiovascular and metabolic function was evaluated.

Experiment 3 (Chapter 5)

This study builds on the results presented in experiment 2 by specifically assessing the acute BP response to exercise. This provided further insight into the functional effects of cocoa flavanol consumption in obesity.

Experiment 4 (Chapter 6)

This study specifically addressed an emerging discrepancy in the literature surrounding the efficacy of cocoa flavanols in reducing blood pressure. The study aimed to replicate the population and BP assessment technique used in previous studies, but provided a more robust design, particularly in terms of placebo control.

Chapter 2

Experimental Techniques

This chapter aims to provide an explanation of the specific data acquisition techniques utilised within the subsequent experimental chapters. Details of experimental protocols including subject inclusions, exclusions and sequencing of tests is provided within each of the individual experiment descriptions (chapters 3 through 6).

2.1 - Anthropometry

Height and weight

Height, weight and body composition were assessed in all subjects. Standing stature (height) was measured without shoes against a wall mounted telescopic stadiometer (Seca, 220, Vogel & Halke, Humberg, Germany). Body mass (weight) was measured without shoes and lightly clothed with electronic scales (Tanita „Ultimate Scale 2000", Tokyo, Japan) at each visit for all studies accurate to 0.2 kg. The classification of overweight and obesity was based upon BMI in accordance with the WHO definition of 25 – 29.9 $kg.m^{2-1}$ and ≥30 $kg. m^{2-1}$ respectively [209].

Dual Energy X-ray Absorptiometry

Body composition was determined with dual energy X-ray absorbtiometry (DEXA) assessment on a Prodigy (GE Lunar, Madison, USA) scanner with associated software. DEXA uses analysis of the scatter and reflection of a photon beam to determine tissue type. To assess body composition the DEXA machine uses what is termed a fan beam which means it runs a constant full width beam through the tissue progressively sampling from head to foot of the test subject. This process is significantly faster than the „pencil

beam“ process used for bone density assessment as less detail is required to determine tissue type compared to the assessment of bone tissue structural density. The DEXA software provides output on total mass (derived from tissue volume), and then lean mass, fat mass and an overall index of bone mineral density. It also allows the segmentation of individual regions, termed regions of interest (ROI) that can then be analysed separately. This method was used to derive a measure of abdominal adiposity by producing a quadrilateral box encasing the first four lumbar vertebrae extending laterally to the lateral iliac border. This method has been previously described and validated by Glickman and collegues [210]. Validity and reliability of DEXA body composition analysis has been reported previously including the regional determination of abdominal fat tissue against the gold standard of computer tomography [27, 211]. The relative “fatness” of an individual as determined by DEXA is expressed as the percentage of fat mass in total body.

Prior to each test the DEXA was calibrated in accordance with manufacturers specifications. This involved the measurement of a „standard“ brick containing material of known properties that represent those of human tissue, and an aluminium „phantom spine“ to represent bone mineral density. The values are checked against the internal data base to ensure the machine is reading truly.

2.2 Cardiovascular assessments

2.2a Blood Pressure Assessment

Resting Blood Pressure

Resting Blood Pressure was assessed by oscillometry in all cases in accordance with the procedures outlined by the Joint National Committee on the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (VI): US Dept of Health and Human Services [212]. Four consecutive BP readings were taken at 1 min intervals by a single observer. The first reading was discarded and an average of the remaining measurements was taken to determine eligibility for study entry. Resting posture (seated or supine) varied between studies as did the specifications of the monitoring device. The devices used were the HDI/Pulsewave CR-2000 Cardiovascular Profiler (Hypertension Diagnostics Inc, Eagan, MN) or SpaceLabs ambulatory BP monitor (Model 90217, SpaceLabs Medical, Florida, USA). However the specific device was kept consistent within a given study.

Ambulatory Blood Pressure Monitoring

For assessment of 24 hour Ambulatory Blood Pressure (ABP), volunteers wore a SpaceLabs ambulatory BP monitor (Model 90217, SpaceLabs Medical, Florida, USA) for 24 hours. An appropriately sized cuff was placed firmly around the upper non-dominant arm, centred over the brachial artery, with the monitor worn on a waist strap and ABP measurements were recorded at 15 min intervals excepting from 11 pm – 7am (30 min intervals). The cuff and monitor was only removed briefly for bathing and remained in place at all other times during the 24 hour recording period. Assessments at baseline and follow up were always carried out on the same day of the week using the same monitor, cuff size and arm. Participants were required to maintain an activity diary during each 24

hour ABP period to enable BP values to be related to activities. 24 hour ABP recordings were used to determine daytime (7am – 11pm), night-time (11pm -7am) and 24-hour averages for systolic BP (SBP), diastolic BP (DBP) and mean arterial pressure BP (MAP) and heart rate (HR).

There a number of reasons why 24-hour ambulatory blood pressure monitoring (ABPM) is preferable to in clinic measures of BP. The number of measurements with ABPM (approximately 80 in a 24 hour period) greatly exceeds that of in-clinic measurements (approx 3) this can improve accuracy and increase the sensitivity of the readings [213, 214] Single measurements of BP (or few repeats) in-clinic can be influenced by a number of factors such as diet, emotional state, physical activity and time of day these fluctuations can be absorbed in the ABPM averaging of the 80 measurements to give a better indication of the hypertensive state of a patient [215].

ABPM can help with the diagnosis of white coat hypertension where patients may present at the clinic with elevated blood pressure but have lower blood pressure when they are absent from the clinic [216]. In studies (such as the present study) in which potentially hypertensive individuals are being sought, concern of their health is often a driving factor to volunteer, thus in-clinic BP can be increased due to this anxiousness. Once at home patients become more relaxed and blood pressure can decrease and this can be detected using the ABPM [217].

Blood Pressure monitoring during exercise

Traditional assessment of BP with either oscillometry or auscultation and arterial occlusion provide isolated one-off indications of systolic and diastolic pressures. To monitor acute BP responses to stressors (such as exercise) continuous monitoring is preferable. The Finapres™ (Ohmeda Inc., Englewood, Colorado) is a device that is

designed to non-invasively monitor continuous beat-to-beat HR and BP. A photo-electric plethysmograph finger cuff measures the pulsatile changes in tissue volume at the middle phalange of the index finger. These changes in tissue volume are calibrated to represent changes in intra-arterial pressure (96). This technique has been validated against brachial pressure including real time change in intra-arterial brachial measurements during lower body exercise [218-221].

Subjects were positioned on a cycle ergometer with their left hand steadied in a support which was maintained at a standardised height for all testing. The Finapres cuff was fitted as per manufacturer‟s instructions to the left index finger and BP validated against brachial pressure measured by automated oscillometry (Model 90217, SpaceLabs Medical, Florida, USA). If the measurements on the Finapres™ were not aligned with the brachial measurements this was rectified by either adjusting the cuff position on the finger or warming the finger/hand by submersion in warm water to increase blood flow.

Data was obtained for every heart beat during the 15 minute protocol, then averaged in 30 second blocks. The final 30 seconds of the seated BP and HR assessment was taken to be the pre-exercise HR and BP. The changes in BP and HR during exercise were calculated by subtracting the average of each 30 second block during exercise from this pre-exercise average. These 30 second averages were used to calculate the area under the curve (AUC) for the change in BP from pre-exercise values to give an integrated BP response to exercise.

2.2b Endothelial Function

A means of directly assessing the functional capacity of the endothelium non-invasively was first proposed in 1992 when Celermajer [222] and colleagues published results of a novel means of measuring vasodilatation of the brachial artery. The endothelium

dependent dilatation is initiated by activation of the mechanical stress sensitive receptors of the vascular endothelium in response to ischemia induced reactive hyperaemia. The ischemia is facilitated by a period of approximately 5 minutes of suprasystolic occlusion of the arm (or leg in the case of femoral measurement) with an inflatable cuff. Ultrasound imaging of the artery is recorded prior to occlusion to establish a baseline diameter and for the 5 minutes following the release of the cuff at 30 second intervals to determine the endothelium mediated change in diameter.

The measurement of FMD was carried out in accordance with the method described above on a LOGIQ 5 GE ultrasound unit (GE Medical Systems, Wisconsin, USA). Two dimensional high frequency ultrasound images (in the form of 3 second video loops) of the brachial artery were recorded using a 12 MHz linear array transducer in the longitudinal plane. After optimising the ultrasound image the transducer was fixed in place with an adjustable clamp and three baseline images were taken. FMD was induced by 5 minutes of forearm cuff occlusion at 200mmHg and images taken at 30 second intervals following release of the cuff. Analysis of all images was done on the LOGIQ 5 1.1X software (GE Medical Systems, Wisconsin, USA) with digital callipers from the proximal to distal intima-lumen interface at an angle of 90˚ ±0.1˚ to the distal intimal surface. When the technique of FMD was first reported, ultrasound image quality only allowed measurements between the medial layers but higher frequency transducers now allow sufficient imaging of the intimal layer to use this as a measurement marker [223]. It is thought that this technique provides more accurate FMD diagnosis as it is less influenced by structural malformations in the vessel [223]. A typical ultrasound image of the brachial artery is shown in *Figure 2.1* below. Diameter measurements at each time point within the same test and subject were located within 1mm distance of each other

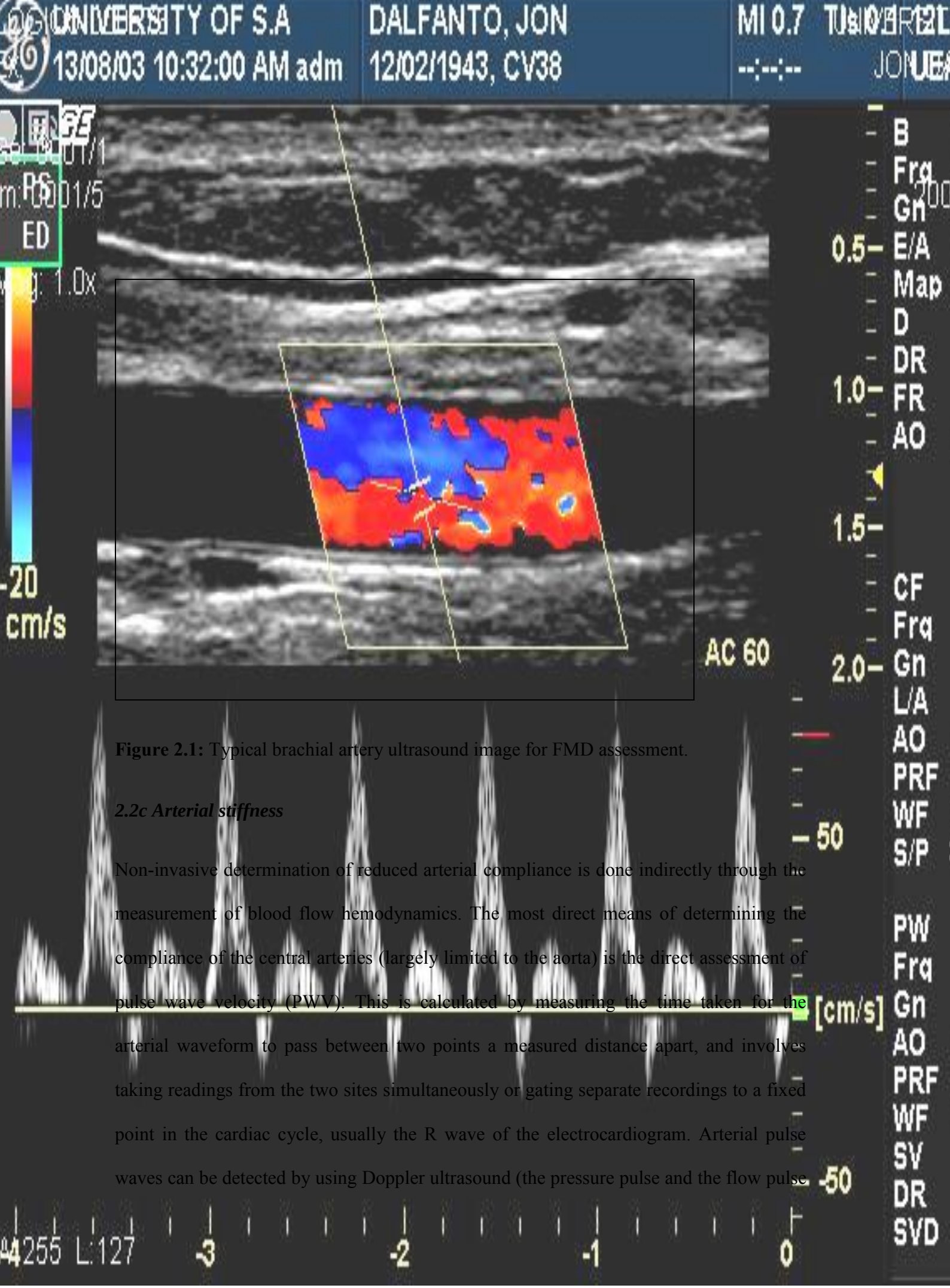

Figure 2.1: Typical brachial artery ultrasound image for FMD assessment.

2.2c Arterial stiffness

Non-invasive determination of reduced arterial compliance is done indirectly through the measurement of blood flow hemodynamics. The most direct means of determining the compliance of the central arteries (largely limited to the aorta) is the direct assessment of pulse wave velocity (PWV). This is calculated by measuring the time taken for the arterial waveform to pass between two points a measured distance apart, and involves taking readings from the two sites simultaneously or gating separate recordings to a fixed point in the cardiac cycle, usually the R wave of the electrocardiogram. Arterial pulse waves can be detected by using Doppler ultrasound (the pressure pulse and the flow pulse

propagate at the same velocity), or applanation tonometry. The less compliant the aorta the greater the velocity of pulse wave transmission.

Other techniques assessing arterial compliance rely upon the relative characteristics of the direct and reflected pulse waves measured in the periphery. These techniques can be grossly grouped in to the term pulse wave analysis (PWA). Pulse wave sampling for PWA is most commonly achieved through applanation tonomety of the radial artery. Plethysmography of the finger has also been used to derive pulse waveforms for analysis. PWA typically relies upon the timing of the reflected wave relative to the direct wave to identify compliance, whereby the earlier the reflected wave becomes superimposed upon the direct wave, the less compliant the aorta. The various methods show varying agreement to PWV measures and to each other and it is posed that each technique may provide insight into different aspects of arterial compliance at different levels of the arterial tree [224]. The two techniques employed in this thesis are PWA from radial applantion tonometry (experiment 1) and from digital volume pulse (DVP) waveform analysis derived from pulse oxymetry light absorbtion technology (experiment 2).

Pressure waveform analysis was performed using resting (supine) cardiovascular characteristics including systolic and diastolic blood pressure and large and small artery elasticity HDI/*Pulsewave* CR-2000 Cardiovascular Profiler (Hypertension Diagnostics Inc., Eagan, MN, USA). Indices of large and small vessel stiffness are derived from radial artery pressure pulse wave analysis. Simultaneous brachial artery oscillometric BP measurement is used for calibration of the pressure wave. Radial pressure waves are measured using applanation tonometry with a piezoelectric based, direct contact, acoustic sensor placed over the right radial artery. The amplified pressure wave contour is analysed with algorithmic software internal to the machine and large and small artery

elasticity indexes calculated from the diastolic decay portion of the cardiac cycle (HDI – technical notes). The equation to determine this is based upon a modified Windkessel circuit model consisting of a capacitive compliance element, interpreted as an indication of large artery compliance and an oscillatory or reflective compliance element interpreted as small artery compliance [225]. The device provides a reading of signal strength for the user to ensure that an adequate trace has been located for accurate analysis. The minimum signal strength accepted was 20% in accordance with the manufacturers specifications. Three measurements of blood pressure and compliance were taken consecutively with a five minute rest between each and the average of these used for data analysis. Rather than reporting two separate indices from the same PWA raw data only the large artery compliance was reported and is simply referred to in the experimental chapter (chapter 3) as arterial compliance (AC).

Following on from the advancements in peripheral pressure waveform analysis the concept of (DVP) waveform assessment has developed. The volume pulse waveform is a a simple and inexpensive means of assessing AC, using photoplethysmography of the finger relative to applanation tonometry of the peripheral arteries but possesses the same characteristics in terms of direct and reflected wave components. To demonstrate the extent of this relationship Millasseau [226] and colleagues developed a transfer function to derive pressure pulse waveforms from those of the volume pulse. DVP measurements were obtained from the index finger of the right hand using the commercially available, purpose designed *Pulse Trace* (MicroMedical, Gillingham, Kent, UK) analysis system. This device uses a high-fidelity photoplethysmograph finger clip transducer to obtain real-time volume pulse waveforms for processing. The transducer contains a light omitting diode (LED) on one side and photo diode receiver on the other. The amount of

light transmitted through the finger is modulated by the volume of blood present in the vascular tissue bed. Real-time waveforms are passed through a signal conditioning circuit where an average of six waveforms per measurement are smoothed and normalised for amplitude and velocity and the direct and reflected peaks are determined. A stiffness index (SI) is determined from timing of the reflected wave relative to the direct wave and the person‟s height. It derives this value by dividing subject‟s height by the time between the direct and reflected peaks of the DVP waveform (refer Figure 2.2). A typical measurement report showing the DVP waveform and direct and reflected wave peak markers in shown in *Figure 2.2* below. Three baseline measurements were taken prior to the subject commencing exercise. If a satisfactory trace was not able to be obtained the subject was asked to immerse the right hand in warm water (~35°C) for 1 minute to facilitate local vasodilatation.

NOTE:
This figure is included on page 69 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.2: Determination of Reflection and Stiffness Indexes from the digital volume pulse waveform. *From: Pulse Trace operating manual (2002).*

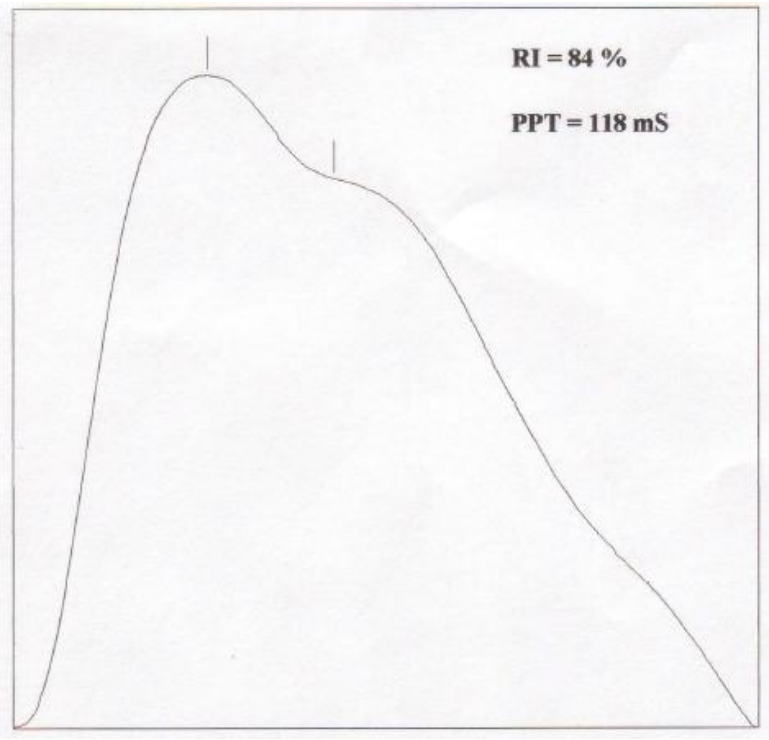

Figure 2.3: Typical Pulse Trace DVP waveform.

2.3 Exercise Testing

2.3a Prediction of maximal aerobic capacity

Maximal aerobic capacity is defined by the maximal rate of oxygen consumption that can be induced with physical exertion and is commonly expressed either in absolute units ($L.min^{-1}$) or relative to body mass ($ml.kg.min^{-2}$). Maximal aerobic capacity can be predicted from sub-maximal measurements with good accuracy due to the linearity of the relationship between HR, oxygen consumption and work load during sub-maximal exercise [227]. Maximal exercise is uncomfortable, difficult to achieve in an untrained subject and poses greater risk of adverse event. For this reason it is not considered ethically appropriate to subject research participants to maximal testing where sub-maximal predictions can suffice.

Maximal aerobic capacity was estimated using an incremental submaximal exercise test on a cycle ergometer (Egromedic 828E, Monark Exercise, Sweden). Subjects cycled for 5 mins at a cadence of 60 rpm at each of 3 incremental sub-maximal workloads. These workloads elicited a heart rate (HR) response equivalent to 50, 60 and 70 percent of age-predicted maximum respectively using the equation of Tanaka et al [228] (200-(0.7*age[yr])). HR was recorded throughout each exercise test as 5 second averages using a personal HR monitor (Acurex Plus, Polar Electro, Finland). Respiratory gases were collected for analysis with a two-way non-return breathing valve (Hans Rudolf model no. 2700, Kansas City, MO, USA). A turbine-based pneumotachometer was attached to the inspiratory port of the valve and connected to a ventilation monitor (VM 2A, PK Morgan, Gillingham, Kent, UK) for measurement of inspired gas volumes. Expired gases were sampled for relative percentages of oxygen and carbon dioxide using Ametec Applied Electrochemistry O_2 analyser S-3A/1 and CO_2 analyser CD 3A (AMETEK, Sunnyvale, CA, USA). Respiratory data was processed for constant monitoring and recording on a local PC. Baseline measurements were obtained for three minutes and then throughout the exercise protocol. Oxygen uptake was monitored in 30 sec epochs during each workload.

Maximal oxygen uptake ($\dot{V}O_2$ max) was estimated by extrapolating the linear regression plot of the HR/$\dot{V}O_2$ relationship to age-predicted maximum HR.

2.3b Fat oxidation during exercise

Indirect calorimetry refers to the estimation of energy consumption at rest or during exercise from the measurement of oxygen consumption and carbon dioxide production during normal respiration. This is done through the collection and analysis of expired gases and comparison to atmospheric concentrations (inspired gases) and comparison to normative values during metabolism. This can be further extended to determine the

relative contribution of macronutrients from the ratio of oxygen consumption and carbon dioxide production due to the different metabolic pathways of fat and carbohydrate.

This technique was used to assess fat oxidation during sub-maximal exercise at baseline and follow up visits in experiment 2. Respiratory gases were collected for analysis with a two-way non-return breathing valve (Hans Rudolf model no. 2700, Kansas City, MO, USA). Expired gas volume and relative concentrations were analysed using a proprietary metabolic cart (True One 2400; Parvo Medics, Sandy, UT). Subjects walked on a treadmill (Quinton Instruments, Model Q65, Washington, USA) at a comfortable speed, and at an incline that elicited a heart rate equivalent to 75% of their age predicted maximum (220 – age [yr] x 0.75) for 10 minutes [228]. The same treadmill speed and incline was used at each follow up assessment. Fat oxidation was estimated from steady-state measurements of oxygen uptake and carbon dioxide output during the final minute of exercise using the stochiometric equation of Frayn [229].

2.3c Blood pressure response to submaximal exercise challenge

Subjects undertook an exercise test on a cycle ergometer (Monark, Model 828, Vansbro, Sweden) to determine the required workload for subsequent exercise tests. They were required to ride at a constant cadence of 60 rpm for 10 minutes and resistance was adjusted to elicit a HR equivalent to 75% of their age-predicted maximum (208–(0.7 x age [yr]))*0.75) [228]. Electrocardiographic monitoring was performed by a medical practitioner to confirm their suitability to continue exercising. This workload was recorded and reproduced for each of the 10 minute bouts of exercise during testing.

2.4. Blood Chemistry

A fasted venous blood sample was obtained by venipuncture from all subjects by an experienced practitioner and collected into EDTA containing tubes (6ml) (Venoject needle and Vaccuette tube, Grenier Bio-one, Austria). Plasma was separated by 10 minutes of spinning at 4000 rpm and 4˚C in a centrifuge (Model 32, Hettich Universal, Tuttlingen, Germany) and stored at -80°C for subsequent analysis.

For experiment 1 (chapter 3) analysis of plasma samples was performed at the CSIRO department of human nutrition, Kintore Ave, Adelaide, South Australia. Lipid levels and glucose concentrations were assessed by enzymatic colorimetric kits (Hoffman-LA Roche Diagnostica, Basel, Switzerland) on an automated Cobas-Bio centrifugal analyser (Roche Diagnostica, Basel, Switzerland). Glucose was determined using the method described by Bondar [230] total cholesterol by Allain [231], and triglycerides by Fossati [232].

For experiment 2 (chapter 4) plasma samples were performed at the UC Davis Department for Human Nutrition (One Shields Avenue, Davis, CA) on behalf of the industry sponsor. Glucose and lipid concentrations were determined using a commercial assay kit with a SYNCHRON LX20 autoanalyzer (Beckman Coulter Inc., Fullerton, CA, USA). Fasting plasma insulin concentrations were determined by radioimmunoassay (Human Insulin Specific RIA Kit, Millipore, Billerica, MA, USA) using a Packard Cobra Auto-Gamma counter (PerkinElmer, Meriden, CT, USA). Insulin resistance was estimated from fasting glucose and insulin concentrations using the modified homeostasis model assessment of insulin resistance (HOMA2) as described by Wallace et al [233]. This model provides an index of insulin resistance (HOMA2 – IR), beta cell function (%B) and insulin sensitivity (%S) [233].

2.5 Physical activity and dietary assessment

Background physical activity was assessed by sampling 3 days of normal activity with a 3-day diary which included two week days and one weekend day [115]. Subjects were advised not to modify their normal activity patterns while completing the diary. Background dietary behaviour was sampled by completion of 3 day food diaries and total energy and macro/micro nutrient analysis was performed using Foodworks Professional Edition (version 3.02; Xyris Software, Highgate Hill, Australia). The timing and frequency of dietary records varied between studies and is detailed within each experimental chapter.

2.6 Cocoa supplementation

All cocoa supplementation was provided by Mars incorporated following development of a patented technique for the production of cocoa powder with a high retention of flavanol content. Validation of samples of the flavanol contents was performed by Mars before and following the trials by patented High Performance Liquid Chromatography technique as described by Kwik-Uribe et al [234]. Powdered cocoa drinks matched for nutrient content, colour and flavour were provide in the specified flavanol doses and controls with negligible flavaonl content. The drink powders were reconstituted in water using hand held battery operated mixing devices provided to each participant. All participants were instructed in how to reconstitute the beverages to ensure consistency of food matrix in all studies. The cocoa was provided to volunteers in sachets labelled with a three-digit

numerical code, blinding both volunteers and investigators to their identity throughout the study. Empty sachets were collected to monitor compliance.

2.7 Statistical analysis

Specific statistical analyses are described within each experimental chapter. All statistics were performed on current versions of either SPSS (SPSS Inc, Chicago, IL, USA) or STATISTICA for windows software (StatSoft Inc, Tulsa, OK).

Chapter 3

Differential effects of body fat and cardiorespiratory fitness on cardiovascular function.

3.1 Introduction

Obesity and cardiorespiratory fitness (CRF) are predictors of cardiovascular (CV) and all cause mortality [120, 235-238]. The mechanisms which mediate the relationships between obesity, CRF and CV mortality risk are not entirely understood [238, 239]. However, given that the protective effects of CRF and the detrimental effects of obesity appear to influence CV mortality independently of other CV risk factors, it is of interest to investigate their influences on established markers of CV health. This will allow for a better understanding of the potential mechanisms by which obesity and CRF may influence the risk of CV mortality.

Increased adiposity, in particular visceral adiposity, is associated with reduced vascular endothelial function [240, 241]. Endothelial function refers to the general functional capacity of vascular endothelial cells, primarily mediated by their capacity to synthesize and release nitric oxide [45]. Reduced synthesis and/or availability of NO is associated with increased vascular permeability, inflammation, adhesion and thrombosis and a reduced vasodilatory capacity and abnormalities of endothelial function have been associated with a number of CV risk factors [242].

The non-invasive technique of measuring flow-mediated dilatation (FMD) in the brachial artery provides a marker of endothelium-mediated dilatory function. Previous studies have reported strong associations between FMD and risk of CVD [222, 243, 244]. Furthermore, a relatively low FMD has recently been identified as an independent risk factor for future CV events [245]. The influence of CRF on endothelial function has not been clearly established. However, exercise training has been shown to improve FMD in various populations [246] [247-249].

Decreased arterial compliance (elasticity) is an early indicator of increased CVD risk which may precede elevated BP and/or arterial stenosis [250]. Arterial compliance (AC) is reduced in subjects with, or at high risk of developing, arterial disease [251] and can be abnormal well before overt CVD develops [252]. Previous studies have found both decreased CRF [253] and increased adiposity [146] to be associated with reduced AC. Intervention studies have indicated that exercise training can improve compliance of the large capacitance arteries, either independently of or in association with improvements in CRF [254] [255]. It is not clear whether the protective effects of CRF in terms of reduced CV mortality risk might be in some way mediated by improvements in arterial compliance.

The aim of this study was to investigate the influence of obesity and CRF on arterial stiffness and function in a sedentary population in order to provide some insight into potential mechanisms by which obesity and CRF may mediate CV mortality risk. A sedentary population was chosen for this study to avoid the potentially confounding effect of exercise training and/or regular physical activity.

3.2 Method

Subjects

Twenty seven obese (BMI >30kg/m^2) and 26 lean (BMI 18 – 24.9 kg/m^2) sedentary male and female volunteers (40 - 65 years) were recruited from the general community in Adelaide, South Australia using flyers and radio advertising. Initial screening for inclusion was conducted via written questionnaire and telephone interview. Applicants were excluded from participating if their BMI or age fell outside of the prescribed ranges, if they had a history of cardiovascular, metabolic, hepatic or renal disease, were not sedentary (i.e. exercised >1 time per week for the purpose of improving health), were taking blood pressure or cholesterol lowering medication, were smokers, or were pregnant or lactating. Written informed consent was obtained from all subjects prior to participation. The research was approved by the University of South Australia Human Research Ethics Committee.

Study design

Subjects were required to attend the research clinic on two occasions separated by not more than 1 week. Both visits were scheduled for the same time of day and subjects were required to attend each visit following an overnight fast (minimum 12 hours fasted). During the first visit blood samples were collected for assessment of blood glucose, triglycerides and cholesterol after which blood pressure (BP), AC and FMD were assessed. During the second visit anthropometric, body composition and CRF assessments were conducted.

Blood analyses

Fasting blood (10 ml) was obtained by venepuncture. Plasma concentrations of glucose, triacylglycerols, and total cholesterol were measured on an automated centrifugal analyzer (Cobas-Bio, Rotkreuz, Switzerland) using standard commercial kits (Roche Diagnostica,Indianapolis, USA).

Cardiovascular assessments

After participants had been lying supine for 10-min, BP and AC were measured using the HDI/Pulsewave CR-2000 Cardiovascular Profiler (Hypertension Diagnostics Inc, Eagan, MN). Recordings were made in triplicate at 5 min intervals. Endothelial function was assessed whilst supine by FMD as previously described by Raitakari and Celermajer [256] [222]. For FMD assessments the diameter of the brachial artery was measured by a single operator using 2-dimensional B-mode ultrasound (LOGIQ 5; GE Medical Systems, Waukesha, WI). To induce reactive hyperemia, a sphygmomanometer cuff was placed around the proximal region of the forearm (ie, distal to the imaged brachial artery) and inflated to a supra-systolic pressure (200mm Hg) for 5 min. Images of the artery were taken before cuff inflation, 10 s before cuff release, 10 s after cuff release, and then every 30 s for an additional 3 min. Arterial diameter was measured as the maximum perpendicular distance between the intima with the use of digital calipers (Logiq software, version 5 1.1X; GE Medical Systems).

Anthropometry and body composition

Each subject‟s height (Seca 220 stadiometer, Vogel & Halke, Germany) and weight (Ultimate Scale 2000, Tanita, Japan) were recorded to calculate BMI. Body composition was assessed by dual energy x-ray absorptiometry (DEXA) (Lunar Prodigy; General Electric, Madison, WI). During DEXA scans subjects wore a hospital gown and scans were performed in accordance with the manufacturer‟s instructions. Abdominal fat content was estimated from regional analysis of the DEXA scan by drawing a quadrilateral box with the base of the box touching the top of the iliac crest, the lateral borders extending to the edge of the abdominal soft tissue, and the upper margin touching the most inferior aspect of the ribs.

Cardiorespiratory fitness

CRF was estimated using an incremental submaximal exercise test on a cycle ergometer (Egromedic 828E, Monark Exercise, Sweden). Subjects cycled for 5 mins at a cadence of 60 rpm at each of 3 incremental sub-maximal workloads. These workloads elicited a heart rate (HR) response equivalent to 50, 60 and 70 percent of age-predicted maximum respectively using the equation of Tanaka et al [228] (200- (0.7*age[yr])). HR was recorded throughout each exercise test as 5 second averages using a personal HR monitor (Acurex Plus, Polar Electro, Finland). Oxygen uptake was monitored in 30 sec epochs during each workload by indirect calorimetry. Maximal oxygen uptake ($\dot{V}O_2max$) was estimated by extrapolating the linear regression plot of the HR/$\dot{V}O2$ relationship to age-predicted maximum HR.

Physical activity

All subjects completed a 3-day physical activity diary which included two week days and one weekend day in order to assess habitual activity levels [115].

Statistical analysis

Statistical analysis was performed using SPSS (version 12, SPSS Inc, Chicago, IL, USA). Unpaired t-tests were used to compare means between the lean and obese groups. Analysis of variance was used to compare FMD measurements between groups, using the baseline arterial diameter as a covariate. Pearson‟s correlation coefficient was used to identify relationships between variables. Statistical significance was set at an alpha level of 0.05. All data are shown as mean $\pm$ standard error of the mean (SEM).

3.3 Results

	Obese	Lean	P
Number (M/F)	9/18	8/18	-
Age (years)	49.6 ± 1.6	50.1 ± 1.3	0.8
BMI ($kg.m^{-2}$)	35.3 ± 0.9	22.5 ± 0.3	<0.001
Body fat (%)	43.5 ± 1.2	27.2 ± 1.6	< 0.001
Abdominal body fat (%)	48.6 ± 0.9	28.9 ± 1.8	< 0.001
Total cholesterol ($mmol.L^{-1}$)	6.7 ± 0.4	5.7 ± 0.3	0.08
Triacylglycerols ($mmol.L^{-1}$)	1.8 ± 0.7	0.8 ± 0.1	< 0.01
Glucose ($mmol.L^{-1}$)	5.2 ± 0.2	5.3 ± 0.2	0.31
Systolic BP (mmHg)	127.0 ± 2.6	118.6 ± 3.1	< 0.05
Diastolic BP (mmHg)	72.4 ± 2.0	67.0 ± 1.8	0.06
Flow Mediated Dilatation (%)	3.2 ± 0.4	5.7 ± 0.7	<0.01
Arterial compliance ($ml \cdot mmHg^{-1} \cdot 10$)	16.5 ± 0.9	15.5 ± 0.6	0.36
$\dot{V}O_2max$ ($L \cdot min^{-1}$)	2.3 ± 0.2	1.8 ± 0.1	< 0.01
$\dot{V}O_2max$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	23.0 ± 1.4	27.9 ± 1.1	< 0.01
$\dot{V}O_2max$ ($ml \cdot kgFFM^{-1} \cdot min^{-1}$)	44.4 ± 1.7	42.0 ± 1.4	0.28

Table 3.1: Comparative characteristics of lean and obese groups. Note: FFM - fat free mass; values represent mean ± SEM

Subject characteristics are shown in Table 3.1. Age and gender was well matched between groups and no difference was seen in physical activity diary results between groups (Obese = 16 086 ± 650 kJ vs Lean 16 321 ± 875 kJ). Body composition analysis confirmed the higher adiposity of the obese group in total (43.5 ± 1.2 vs. 27.2 ± 1.6%) percentage body fat (%BF) and abdominal (48.6 ± 0.9 vs. 28.9 ± 1.8%) percentage body fat (%ABF) compared to the lean group. Systolic BP was 8.4 mmHg greater and plasma triglyceride concentrations 1.0 $mmol.L^{-1}$ greater in the obese group compared to lean. Mean FMD in the obese group was approximately half (56%) of that observed in the lean group. There were no differences in Diastolic BP, HR, AC, plasma cholesterol or glucose between groups. Absolute $\dot{V}O_2max$ ($L.min^{-1}$) was significantly higher in the obese group compared with the lean, but when expressed relative to body mass ($ml.kg^{-1}.min^{-1}$) it was

lower in the obese compared with lean. When expressed relative to fat-free mass there was no difference in $\dot{V}O_2$max between groups (refer Table 3.1).

FMD was not related to $\dot{V}O_2$max when expressed in either absolute or relative terms. AC was positively related to $\dot{V}O_2$max (CRF) expressed in either absolute terms or relative to lean mass (see Table 3.2 and Figure 3.1).

	Age	BMI	%BF	%AF	SBP	DBP	TAG	TChol	FG	FMD	AC	VO2max(A)	VO2max(R-TM)
BMI	-0.17	.											
%BF	-0.01	**0.74****	.										
%AF	-0.1	**0.81****	**0.89****	.									
SBP	0.16	0.25	0.06	**0.29***	.								
DBP	0.14	0.17	0.09	0.19	**0.81****	.							
TAG	**-0.34***	**0.62****	**0.37****	**0.5****	0.28	0.26	.						
TChol	0.01	0.22	**0.35***	**0.28***	0.02	0.02	**0.30***	.					
FG	0.06	0.002	0.12	0.03	0.21	-0.01	0.03	-0.11	.				
%FMD	-0.14	**-0.40****	**-0.28***	**-0.34***	**-0.41****	**-0.46****	**-0.3***	-0.18	-0.19	.			
AC	-0.28	0.18	0.05	0.06	**-0.29***	-0.05	0.1	-0.19	0.08	0.03	.		
VO2max(A)	-0.18	**0.46****	0.03	0.24	**0.31***	**0.38****	**0.35***	-0.25	0.15	-0.2	**0.64****	.	
VO2max(R-TM)	-0.12	**-0.33***	**-0.67****	**-0.47****	0.06	**0.30***	-0.19	**-0.41****	0.05	0.11	**0.4****	**0.6****	.
VO2max(R-FFM)	-0.14	0.21	0.01	0.15	0.06	0.25	0.1	**-0.29***	-0.01	-0.04	**0.5****	**0.74****	**0.73****

Table 3.2 : Correlations between parameters of fitness, fatness and cardiovascular function. Abbreviations are as follows: BMI, Body mass index; %BF, percentage body fat; %AF, percentage abdominal fat; SBP, systolic blood pressure; DBP, diastolic blood pressure; TAG, triacylglycerol; TChol, total cholesterol; FG, fasting glucose; %FMD, percentage flow mediated dilatation; AC, large artery compliance; $\dot{V}O_2$max (A), predicted maximal oxygen uptake (absolute); $\dot{V}O_2$max (R-TM), predicted maximal oxygen uptake (relative to total mass); $\dot{V}O_2$max (R-FFM), predicted maximal oxygen uptake (relative to fat-free mass). * $p < 0.05$; ** $p < 0.01$.

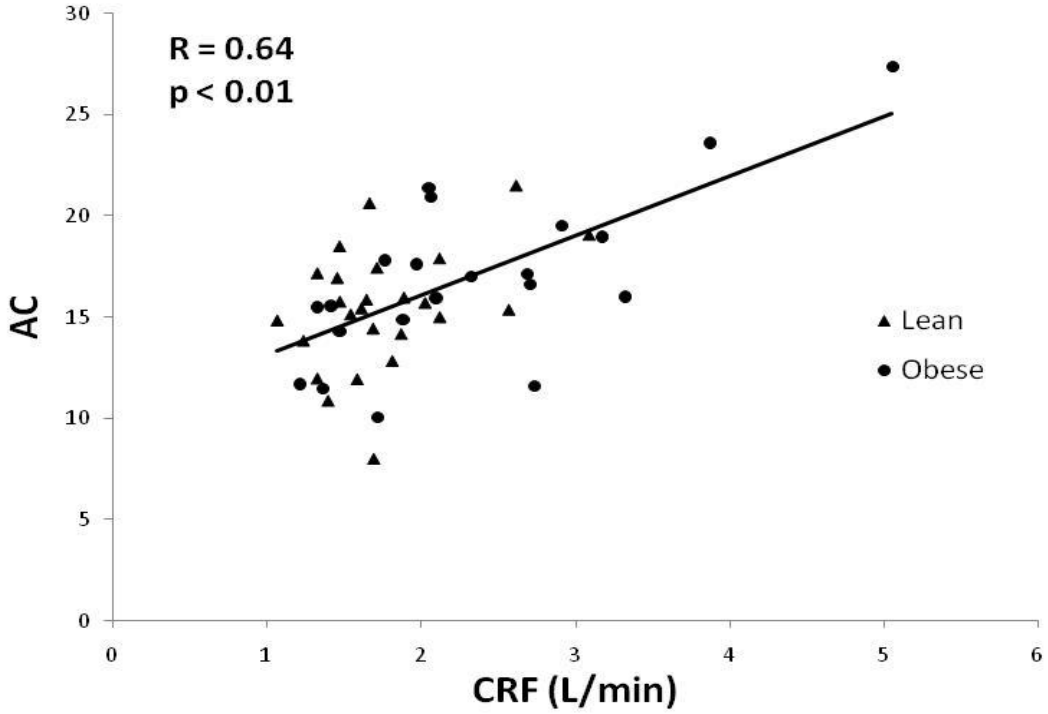

Figure 3.1: Relationship between large artery compliance (AC) and absolute cardiorespiratory fitness ($\dot{V}O_2max$).

3.4 Discussion

Long term prospective studies have found both CRF and obesity to be strong predictors of CV mortality [235-238]. (2, 3). CRF may be a more influential moderator of CV risk than obesity because the influence of obesity is reduced or negated with high CRF [120, 237]. The mechanisms underlying the influence of CRF on CV disease outcomes has yet to be clearly identified. Identification of these mechanisms may help to better inform therapeutic approaches to reduce CVD risk. A previous cross sectional study by Christou and associates [146] of the relationships between CRF, obesity and CV risk factors in healthy adults found obesity to be associated with numerous CV risk factors including AC, assessed by aortic pulse wave velocity, but CRF was only associated with metabolic markers of risk (triacylglycerols, fasting insulin and insulin sensitivity). It is however difficult to determine any potential causal mechanism from this study due to the inclusion of subjects across a broad range of activity levels, thereby introducing potential confounding effects of physical activity habits. This is particularly so due to the systematic inclusion of a highly endurance exercise trained group. Therefore the present study is the first to directly compare markers of CV function between obese and lean individuals whilst controlling for the influence of habitual physical activity.

The results presented in this paper demonstrate a clear differentiation between the influences of obesity and CRF on CV function. FMD in the obese group was almost half that seen in the lean group, indicating an impairment in endothelial function in the obese group. While the mechanism underlying the relationship between endothelial dysfunction and obesity is yet to be fully elucidated, evidence suggests multiple potential pathways [257]. Obesity is associated with alterations in production and secretion of lipids and lipoproteins, and adipokines (increased leptin and resistin and decreased adiponectin)

[258]. These changes can impact negatively on vascular endothelial function through increased proinflammatory pathways including increased endothelin-1, monocyte chemoattractant protein 1, pentraxin, tumor necrosis factor α and interleukin 6 & 12 and through direct action on endothelial cells and vascular smooth muscle [258]. Impaired endothelial function may feedback on metabolic function by impairing muscle tissue perfusion response to insulin and during exercise [259]. This could result in reduced substrate mobilisation, delivery and oxidation and possibly contribute to higher levels of obesity and dyslipidaemia.

While FMD was reduced in obesity, it was not associated with CRF. This suggests that the cardio-protective effects of CRF may not be mediated via improved endothelial function. When considered in the context of previous findings of improvements in FMD with exercise training [260], this may suggest that these benefits are mediated through different mechanisms to those responsible for improvements in CRF. However, because the population in the present study were sedentary the variance in CRF is due to underlying basal cardio-respiratory function not due to a training effect. Therefore, the influence of higher CRF or increases in CRF due to exercise training on FMD cannot be predicted from these results.

The present study found no relationship between markers of obesity and AC, indicating that within a non-diseased, age-matched and sedentary population, obesity does not negatively impact on AC. This is in contrast to the previously mentioned result of Christou and associates [255] who found that BMI and waist circumference were inversely associated with AC. This discrepancy may be due to the different methods of AC assessment (pulse wave velocity vs the Windkessel model of the CR-2000 in the present study), although good correlations have been previously reported between these

two techniques [224]. Alternatively it may be due to methodological differences between the two studies including the inclusion by Christou et al [146] of a range of BMI levels and physical activity/exercise training status as discussed previously. This is in contrast to the present study involving a direct comparison between an inactive obese and inactive lean sample. Irrespective of any underlying reasons, this apparent discrepancy warrants further investigation in follow up studies.

CRF when expressed as an absolute value ($L.min^{-1}$ $\dot{V}O_2max$) was significantly higher in the obese group compared to lean. This is not entirely surprising as both groups were sedentary (untrained) and the obese group had higher fat free mass as well as fat mass, indicating greater total muscle tissue and so greater maximal aerobic capacity [261]. When expressed in the more traditional method relative to total mass (ml.kg-$1.min^{-1}$ $\dot{V}O_2$ max), CRF was significantly lower in the obese group compared to lean. This again is not unexpected due to the large (and deliberate) discrepancy in total mass between groups. When expressed relative to lean mass ($ml.kg^{-1}$fat free $mass.min^{-1}$ $\dot{V}O_2max$), there was no significance difference in fitness between groups. Because the latter is a better indication of the relative oxidative capacity of the more metabolically active muscle tissue, it can be argued that this may be a more appropriate way to account for fitness when comparing a lean and obese population. It does not however account for the true functional capacity as the obese group have a larger mass to move for a given aerobic capacity. The strongest predictor of AC was absolute fitness, suggesting that regardless of body composition a higher absolute oxidative capacity represents the greatest protection.

Whether expressed in absolute or relative terms CRF was significantly related to AC, whereas AC was not related markers of obesity and hence did not differ significantly

between lean and obese subjects (i.e. no group effect). This implies that regardless of the level of adiposity, AC is positively associated with CRF. Previous studies have shown that regular participation in endurance type exercise can result in improvements in AC [114] although it is unclear whether this is due to increased fitness or the chronic exposure to exercise, or a combination of both factors. Further work is, therefore, required to determine the most appropriate exercise intervention for improving AC. As discussed above, this study sought to control for the influence of regular exercise exposure and, therefore recruited only sedentary subjects. This means that the results cannot be extended to the effects of regular exercise participation on CV function. Likewise, they cannot be interpreted to predict any modification in CV function as a result of interventions designed to modify either fatness or fitness.

In summary, this study demonstrated that obesity is associated with substantial impairment in FMD and therefore, potentially a reduction in general arterial endothelial function. There was no impact of obesity on AC, indicating that obesity may not contribute to CV mortality through a loss of AC. CRF, however, was significantly associated with AC, indicating that the cardio-protective effects of a higher CRF may be related to maintenance of elasticity in central conduit arteries. The implication of these findings in terms of their possible contribution to CV related diseases and mortality is depicted in Figure 3.2. Increased obesity through reduced endothelial function may augment the progression of arterial disease, thrombosis and hypertension as well as, through decreased NO and muscle blood flow, metabolic dysfunction leading to increased CV mortality risk. Reduced CRF with the associated decrease in AC may lead to increased cardiac afterload and, in turn, left ventricular hypertrophy, reduced coronary perfusion and increased CV mortality risk. Strategies aimed at reducing CV mortality

must therefore, aim to reduce adiposity in the obese and increase or maintain CRF in all people.

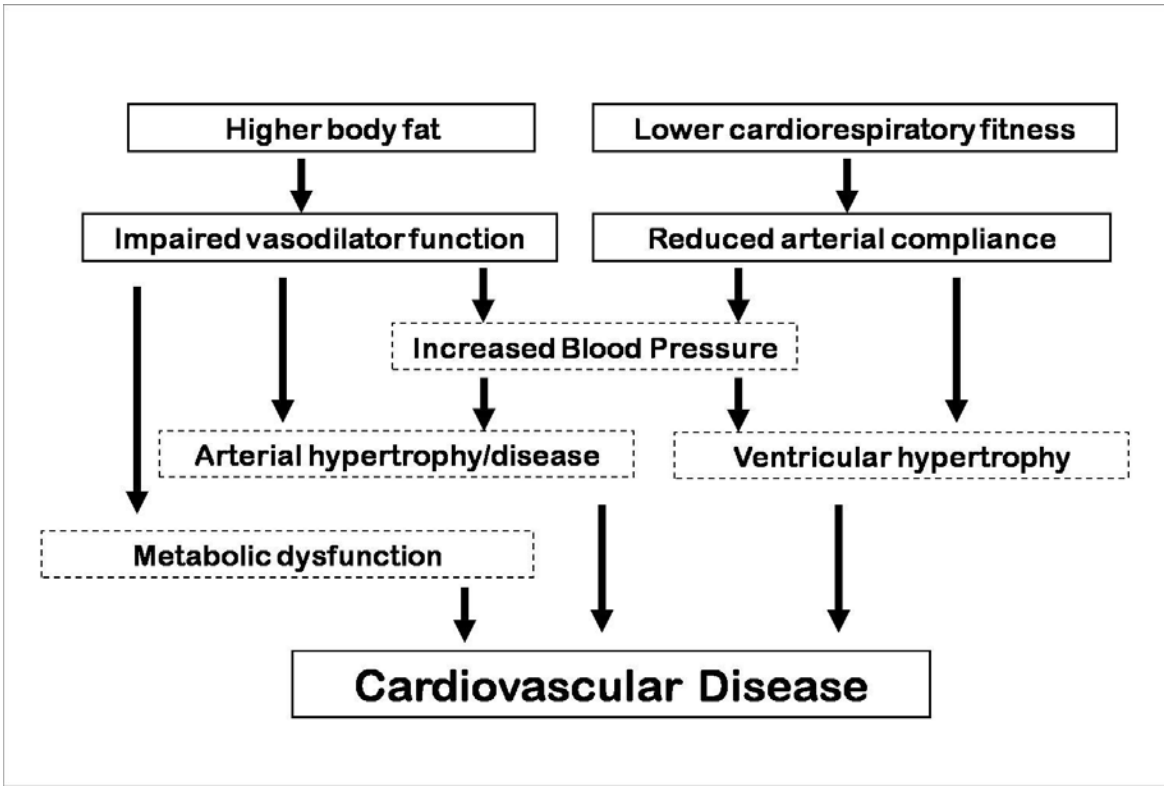

Figure 3.2: Proposed model of impact of obesity and lower cardiorespiratory fitness on cardiovascular risk. Solid steps indicate those supported by this study and other evidence, dashed steps represent those proposed from other evidence as referenced in this discussion.

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Chapter 4

Effect of cocoa flavanols and exercise on cardio-metabolic risk factors in overweight and obese subjects

Kade Davison[a,b], Alison M Coates[a], Jonathan D Buckley[a] and Peter RC Howe[ab]

[a] Nutritional Physiology Research Centre and ATN Centre for Metabolic Fitness, School of Health Sciences, University of South Australia, SA 5001 Australia and [b] School of Molecular & Biomedical Sciences, University of Adelaide, SA 5005, Australia.

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Statement of Authorship

Effect of cocoa flavanols and exercise on cardio-metabolic risk factors in overweight and obese subjects.

Kade Davison (Candidate)

Contributed to conceptualisation and design of protocol, Recruited and Screened and tested all volunteers; analysed and interpreted data, prepared manuscript.

I hereby certify that the statement of contribution is accurate

Signed

…… *……Date…18/12/09……*

Alison Coates (co-author)

Contributed to conceptualisation and design of protocol, interpretation of data and development of manuscript.

I hereby certify that the statement of contribution is accurate

Signed …… *……Date…18/12/09…*

Jon Buckley (co-author)

Contributed to conceptualisation and design of protocol, interpretation of data and development of manuscript.

I hereby certify that the statement of contribution is accurate

Signed …… *……Date…18/12/09……*

Peter Howe (co-author)

Contributed to conceptualisation and design of protocol, interpretation of data and development of manuscript.

I hereby certify that the statement of contribution is accurate

Signed……. *……Date…18/12/09……*

4.1 Introduction

Endothelial dysfunction is associated with reduced production of nitric oxide (NO) by the endothelium. There is evidence that, in addition to the well documented adverse cardiovascular effects, reduced availability of NO can impair metabolic function [259]. Among the proposed mechanisms for this is a reduction in blood flow to areas of high metabolic demand such as skeletal muscle [259].

Flow-mediated dilatation (FMD) of the brachial artery is used to assess the NO dependent endothelium-mediated dilatory response of an artery and, as such, provides a marker of endothelial function. In recent years, the consumption of cocoa products containing flavanols and procyanidins (oligomeric flavanols), has been shown to elicit dose-dependent acute improvements in FMD, and regular consumption for up to 14 days provides a sustained improvement in FMD [19, 171, 194, 262]. Improvements in FMD are associated with increased skeletal muscle blood flow during exercise [263], suggesting that improvements in FMD might facilitate increased blood flow and nutrient delivery to sites of metabolism, particularly skeletal muscle during exercise, with resultant improvements in metabolic control and body composition. Indirect support for this hypothesis comes from studies which have shown that the consumption of flavanol - containing cocoa products, and L-arginine, (the primary substrate for NO), not only improve FMD [264], but also reduce body fat [208, 265].

Aerobic exercise is strongly advocated for increasing energy expenditure and reducing fat stores, but the amount of exercise required to achieve weight loss may be difficult to

achieve for obese individuals who are unaccustomed to exercise [13]. However, if cocoa flavanols can improve circulatory function and fat metabolism, it may result in an augmentation of substrate utilisation and/or energy expenditure during exercise. Thus a less demanding and more achievable exercise regimen may be effective for the treatment of obesity. Such an approach has been used successfully in another recent study where the combination of dietary omega-3 fatty acid supplementation with modest exercise provided cardiovascular and metabolic benefits and improved body composition in an obese population [114].

The primary aim of this study was to investigate whether cocoa flavanol consumption can improve cardio-metabolic function and ultimately body composition in an overweight/obese population. A secondary aim was to determine whether the addition of a moderate exercise program may enhance the impact of such effects on adiposity.

4.2 Materials and Methods

This study used a randomised, double blind, placebo-controlled, parallel, 2 x 2 factorial design incorporating consumption of cocoa beverages and regular exercise. The study was conducted at the Nutritional Physiology Research Centre at the University of South Australia (Adelaide, South Australia) between Sept 2005 and Dec 2006. Ethical approval for the study was obtained from the Human Research Ethics Committees of both the University of South Australia and the University of Adelaide and written informed consent was obtained from all subjects prior to their participation.

Subjects

Sedentary (not exercising > 1 per week for the purpose of improving health) male and female volunteers aged 18 – 65 years with a body mass index (BMI) > 25 $kg \cdot m^{-2}$ were recruited from the general public. People with habitually high cocoa consumption (daily consumption of dark chocolate or >26g milk chocolate or powdered cocoa), stage 2 hypertension (resting systolic blood pressure (BP) >160 mmHg or diastolic BP> 100 mmHg), existing cardiovascular disease or who were taking any cardiovascular medications (including aspirin) or fish oil supplements were excluded. Initial screening for suitability to participate was based on diet and lifestyle questionnaires, measurement of height, weight and BP and a medical clearance for exercise based on electrocardiogram monitoring during a graded sub-maximal treadmill test.

Intervention

Volunteers were block-matched into two groups according to BMI, gender, age and BP. The groups were then randomised to the daily consumption of either high cocoa flavanol (HF) or low cocoa flavanol (LF; placebo) cocoa drink for 12 weeks. Volunteers in each of the two groups were then further randomised to either undertake a program of regular modest exercise for 12 weeks, or to remain sedentary. At baseline (Week 0), subjects attended the clinic following an overnight fast (≥12 hours) for collection of blood samples and measurement of BP, FMD, body composition (Dual Energy X-Ray Absorptiometry [DEXA]) and fat oxidation during submaximal exercise. Each subject then consumed a single packet of the appropriate beverage product (or cocoa drink) (acute intervention) and FMD was re-assessed 2 hours later. On the day following the baseline testing subjects commenced the chronic (12 week) intervention and the baseline tests were repeated after 6 and 12 weeks. Body composition was assessed at baseline and Week 12 only. On test days at Week 6, cocoa drinks were not consumed until all assessments had been completed. Subjects were instructed not to change their dietary habits during the study aside from consuming the appropriate drink, restricting caffeine intake to no more than 2 caffeinated beverages per day and avoiding red wine and dark chocolate throughout the intervention.

Dietary Intervention

The cocoa containing product used in this study was a dairy based powder mix. Table 4.1 shows the macro/micro-nutrient content of the HF and LF drink mixes. They were closely matched for all ingredients, other than the cocoa flavanol content. The cocoa flavanol

content refers the total amount of epicatechin, catechin, and procyanidins up to decamers in the drinks. The drink mixes were mixed with 150ml of cold water immediately prior to consumption. Subjects were instructed to consume the beverages twice daily throughout the intervention period, which provided 902 mg and 36mg of cocoa flavanols per day in the HF and LF groups, respectively. To monitor compliance with the intervention, subjects recorded their drink consumption in a diary each day. Empty sachets were returned at Weeks 6 and 12 and counted as a further check of compliance.

	High Flavanol cocoa	**Low Flavanol cocoa**
Flavanols (mg)	451	18
Energy (kJ)	497	490
Total fat (g)	1.4	1.5
Sat fat (g)	0.8	0.8
Total CHO (g)	17.1	16.5
Sugars (g)	9.4	9.2
Protein (g)	9.4	9.2
Fibre (g)	3	3.9
Caffeine (mg)	18	21
Theobromine (mg)	337	327

Table 4.1: Nutritional contents of high and low flavanol cocoa drinks.

Three day physical activity [115] and food diaries (analysed with FOODWORKS PROFESSIONAL EDITION [version 3.02; Xyris Software, Highgate Hill, Australia]) were recorded by all subjects during the week prior to commencing the intervention and the final week of the intervention study to identify any changes to background energy intake/expenditure that may have influenced study outcomes. Subjects were instructed to substitute the cocoa beverages for other foods rather than simply adding the cocoa drink on top of their usual energy intake.

Exercise Protocol

Volunteers randomised to undertake regular exercise walked or jogged 3 times per week for 45 minutes at an exercise intensity that elicited 75% of their age-predicted maximum heart rate (220 – age in years) for 12 weeks. The non-exercising volunteers were asked to maintaining their normal levels of physical activity. To assist with maintaining compliance, subjects in the exercise group were required to attend at least one supervised exercise session per week and to maintain a training diary.

Assessment of Flow-mediated Dilatation and Blood Pressure

Endothelial function was assessed by FMD. The diameter of the brachial artery was measured by a single operator with the use of 2-dimensional B-mode ultrasound (LOGIQ 5; GE Medical Systems, Waukesha, WI). Optimal imaging of the artery has been described by Raitakari and Celermajer [256, 266] For the production of reactive hyperemia, a sphygmomanometer cuff was placed around the midpoint of the forearm (ie, distal to the scanned part of the artery) and inflated to a pressure of

200 mm Hg for 5 min. Images of the artery were taken before cuff inflation, 10 s before cuff release, 10 s after cuff release, and then every 30 s for an additional 3 min. Based on repeat assessments of FMD on two consecutive days in 12 subjects the standard error of the estimate for this technique in our laboratory, expressed as a coefficient of variation, is 5%. Resting BP and heart rate were measured after 10 min lying supine by automated oscillometry using a Spacelabs ambulatory BP monitor (Spacelabs Healthcare, WI, USA).

Four readings were taken over a 15 minute period, the first value was discarded and BP was calculated as the mean of the remaining three measurements.

Anthropometric assessment

Body mass and waist circumference were assessed at Weeks 0, 6 and 12. Body composition was assessed at Weeks 0 and 12 only using dual energy X-ray absorptiometry (DEXA) (Lunar Prodigy; General Electric, Madison WI, USA). Abdominal fat content was determined using regional analysis of the body segment bordered superiorly and inferiorly by the lowest point of the rib cage and the uppermost aspect of the iliac crests respectively, and extended laterally to the outer

edge of the rib cage.

Fat Oxidation

Fat oxidation during exercise was assessed at Weeks 0, 6 and 12 by indirect calorimetry (True One 2400; Parvo Medics, Sandy, UT). Subjects walked on a treadmill (Quinton Instruments, Model Q65, Washington, USA) at a comfortable speed, and at an incline that elicited a heart rate equivalent to 75% of their age predicted maximum (220 – age [yr] x 0.75) for 10 minutes. The same treadmill speed and incline was used at Weeks 0, 6 and 12. Fat oxidation was estimated from steady-state measurements of oxygen uptake and carbon dioxide output during the final minute of exercise using the stochiometric equation of Frayn [267]. Both oxygen consumption and the respiratory exchange ratio reached a plateau by the 7th minute of the exercise test in all cases.

Blood Sampling and Analysis of Biomarkers

Fasting blood samples were obtained by venepuncture and plasma was stored at -80°C until analysis. Fasting plasma glucose and lipid concentrations were determined using a commercial assay kit with a SYNCHRON LX20 autoanalyzer (Beckman Coulter Inc., Fullerton, CA, USA). Fasting plasma insulin concentrations were determined by radioimmunoassay (Human Insulin Specific RIA Kit, Millipore, Billerica, MA, USA) using a Packard Cobra Auto-Gamma counter (PerkinElmer, Meriden, CT, USA). Insulin resistance was estimated from fasting glucose and insulin concentrations using the modified homeostasis model assessment of insulin resistance (HOMA2) as described by Wallace et al [233]. As previously reported this model provides an index of insulin resistance (HOMA2 – IR), beta cell function (%B) and insulin sensitivity (%S) [233].

Statistical Analysis

Statistical analysis was performed using STATISTICA for WINDOWS software (version 5.1; StatSoft Inc, Tulsa, OK). Analysis of variance with repeated measures was used to determine the effect of the treatments, time of measurement and their interactions on the dependent variables. Where ANOVA showed no interaction effect between exercise and cocoa, factorial analysis was performed for each of these separate treatments to detect independent effects. Where ANOVA showed a statistically significant main effect, pair wise comparisons were performed using Tukeys HSD to determine differences between means. To optimise the analysis of differences between treatments, where appropriate, a

nested ANOVA design was used to examine changes in dependent variables from baseline with the treatment factor (i.e. cocoa or exercise) nested in time. Statistical significance was set at an *a* level of 0.05. Data are expressed as mean +/- SEM in tables and figures. Power calculation indicated a minimum of 10 participants in each of the four arms would be required to detect a change of 2.0% in total body fat with the combined treatment at an alpha level of 0.05 and probability of 0.8. Standard deviation of the change in % body fat was estimated from our previous studies to be 1.46%.

4.3 Results

General

A total of 98 subjects were screened for inclusion in the study; of these 65 overweight/obese volunteers were enrolled and 49 completed the full 12 weeks of the intervention. Of those that completed, there was an average compliance of 98% for cocoa drink consumption and 86% for the exercise component (i.e. of a required 3 sessions / wk of exercise the average achieved was 2.6). 14 subjects withdrew during the study due to time restrictions or changes in personal circumstance unrelated to the requirements of the intervention and 2 subjects were excluded due to non-compliance with study requirements (1 failed to comply with exercise protocol and 1 went on a calorie restricted diet). Baseline characteristics are shown in Table 2.

		LF + Ex	HF + Ex	LF - Ex	HF - Ex
Number	(M/F)	4/9	6/7	3/8	4/8
Height	(cm)	168.7 ± 2.7	168.7 ± 2.9	165.3 ± 4.1	171.5 ± 3.6
Weight	(kg)	94.7 ± 2.2	94.7 ± 5.2	94.6 ± 6.1	97.2 ± 5.8
BMI	($kg \cdot m^{-2}$)	33.5 ± 1.1	33.2 ± 1.6	34.5 ± 1.8	32.8 ± 1.1
Age	(years)	45.2 ± 3.0	45.5 ± 4.0	44.4 ± 4.4	45.3 ± 4.4
Waist	(cm)	107 ± 2.0	105 ± 6.0	109 ± 4.0	107 ± 4.0
FMD	(%)	5.37 ± 0.68	4.05 ± 0.51	3.65 ± 1.40	4.12 ± 0.75
SBP	(mmHg)	121 ± 3.6	126 ± 2.7	124 ± 1.8	124 ± 3.0
DBP	(mmHg)	74 ± 1.6	78 ± 2.4	77 ± 1.5	76 ± 1.8
HR	(bpm)	60 ± 1.4	66 ± 2.1	61 ± 1.9	66 ± 3.1
Total	($mmol \cdot L^{-1}$)	5.39 ± 0.25	5.94 ± 0.24	6.1 ± 0.22	5.43 ± 0.37
LDL Chol	($mmol \cdot L^{-1}$)	3.25 ± 0.18	3.74 ± 0.2	3.75 ± 0.22	3.26 ± 0.33
HDL Chol	($mmol \cdot L^{-1}$)	1.52 ± 0.09	1.53 ± 0.18	1.48 ± 0.13	1.54 ± 0.15
Trigs	($mmol \cdot L^{-1}$)	1.28 ± 0.16	1.56 ± 0.14	1.89 ± 0.18	1.37 ± 0.2
Glucose	($mmol \cdot L^{-1}$)	5.2 ± 0.1	5.5 ± 0.2	5.1 ± 0.2	5.6 ± 0.1
Insulin	($\mu U \cdot mL^{-1}$)	15.2 ± 1.1	21 ± 2.7	19.2 ± 2.3	19.3 ± 2.4
HOMA2 %Beta		140.7 ± 5.2	165.7 ± 24.7	171.6 ± 16.7	165.7 ± 14.8
HOMA2 %Sensitivity		51.7 ± 3.5	44.6 ± 5.7	48.7 ± 6.9	48.2 ± 6.6
HOMA2 – IR		2.03 ± 0.15	2.70 ± 0.35	2.45 ± 0.29	2.46 ± 0.30
Fat Oxid	($g \cdot min^{-1}$)	0.28 ± 0.02	0.30 ± 0.13	0.35 ± 0.12	0.42 ± 0.04
%BF	(%)	43.8 ± 1.7	43.2 ± 2.4	44.9 ± 2.5	44.1 ± 2.6
%ABF	(%)	45.5 ± 1.4	45.0 ± 1.4	48.2 ± 1.8	44.4 ± 1.0

Table 4.2: Baseline characteristics of all 4 treatment groups and of the combined cocoa and exercise treatment groups. LF = low flavanol drink; HF = high flavanol drink; Ex = exercise program; BMI = Body Mass Index; FMD = % change in FMD; SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate; HOMA2 %Beta = Percentage Beta cell function; HOMA2 % Sensitivity = Percentage insulin sensitivity; HOMA2 IR = Insulin Resistance; Fat Oxid = Rate of Fat Oxidation During Exercise; %BF = Total Percentage Body Fat; %ABF = Total Percentage Body Fat in Abdominal Region.

Cardiovascular Parameters

There were no significant three-way interactions (cocoa x exercise x time) for any of the cardiovascular outcomes (FMD p = 0.957; SBP p = 0.856; DBP p = 0.92; LDL p = 0.93; HDL p = 0.17; Tg p = 0.18).

FMD increased significantly in the HF group compared with LF two hours after consuming a single 450 mg dose of the cocoa drink (p=0.02; Figure 4.1; Table 4.3). Resting (pre-occlusion) vessel diameter did not differ between groups pre- or post-cocoa consumption (p = 0.26) nor did it change as a result of regular consumption of cocoa for 12 weeks (p =0.52). FMD increased significantly at weeks 6 and 12 relative to baseline in the HF group compared with LF (p=0.002, cocoa x time interaction; Figure 4.1). Thus chronic HF consumption had a sustained effect on FMD which was still evident at least 12 hours after consumption of the previous dose.

Percentage FMD	*n*	Pre-supplementation	Post-supplementation	Δ Post – Pre
LF	28	4.3 ± 0.59	4.9 ± 0.54	0.6 ± 0.49
HF	27	4.4 ± 0.41	6.9 ± 0.61	2.5 ± 0.51*

Table 4.3: Percentage FMD before and 2 hours following consumption of 902mg cocoa flavanols at baseline visit. LF = low flavanol group; HF = high flavanol group
* significantly different from LF.

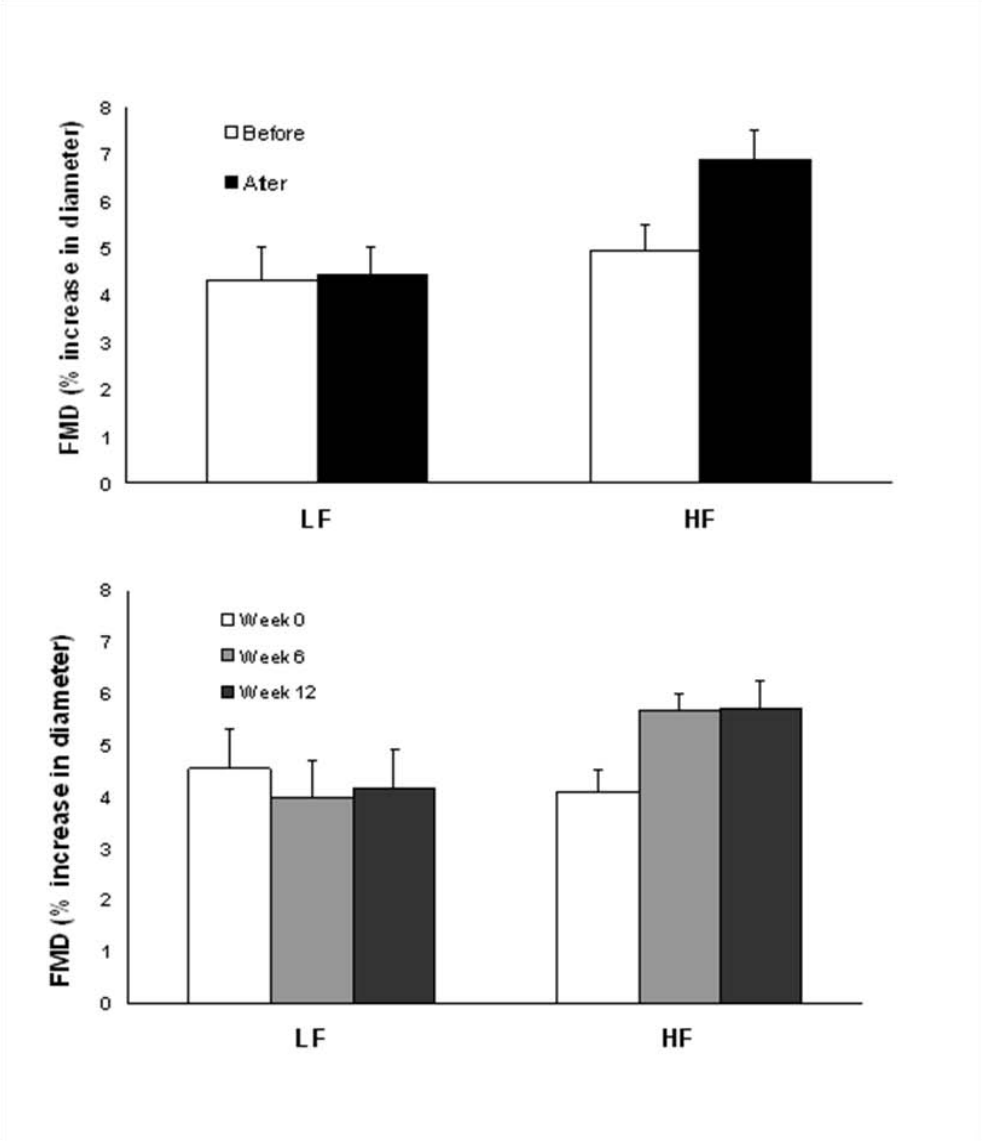

Figure 4.1: Flow Mediated Dilatation, expressed as % increase in brachial artery diameter, A) before and 2 hours after initial cocoa administration at baseline and B) following a 12 hour fast at baseline, 6 and 12 weeks. LF = low flavanol; HF = high flavanol. * represents significantly different change from baseline between treatments ($p<0.01$).

BP and HR measurements tended to decrease over time with HF compared to LF treatment (Figure 4.2), although the differences between treatments were not significant at either 6 or 12 weeks. However, the effects at both time points were taken into consideration by nesting the effects of cocoa on BP in time. This combined analysis

revealed a significant reduction in both diastolic (p=0.04) and mean arterial pressure (p=0.05) with HF versus LF but there were no differences in systolic BP or HR.

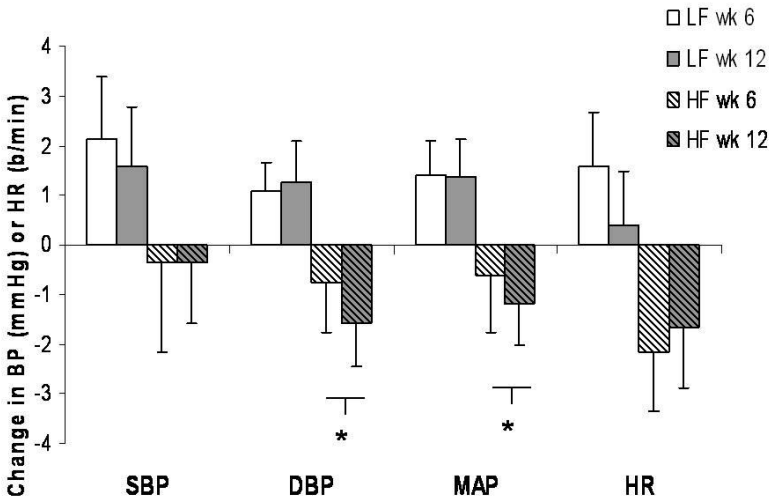

Figure 4.2: Changes in blood pressure and heart rate from baseline to week 6 and to week 12. SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure; HR = heart rate; LF = low flavanol; HF = high flavanol. * HF significantly different from LF by nested analysis of week 6 and 12 data ($p<0.05$).

ANOVA revealed a significant main effect of time on total cholesterol (p=0.002) and a significant interaction between exercise and LP supplementation (p=0.03). The change between groups from weeks 0 to 12 were not significantly different from one another. Table 4.4 displays the change data for all cardiovascular assessments in each intervention group.

Metabolic and Anthropometric Parameters

There was no significant effect of cocoa on the rate of fat oxidation at the same absolute workload at week 6 or 12. There was a significant interaction between exercise and time ($p=0.006$) and post hoc analysis revealed significant differences between week 0 and weeks 6 ($p=0.001$) and 12 ($p=0.015$) in the combined exercise groups compared to non-exercise. ANOVA showed no significant main effects of exercise or cocoa supplementation on total body mass, lean mass or fat mass. ANOVA showed a significant interaction between time and exercise treatment on percentage abdominal fat ($p=0.005$). Post hoc revealed a significant difference between week 0 and 12 associated with exercise compared to those not exercising ($p=0.004$).

There were was no significant effect of cocoa or exercise on fasting glucose, insulin or triglycerides. There was a significant interaction between time and cocoa supplement on HOMA – IR ($p=0.01$). Nesting the effects of cocoa in time revealed a significant improvement in all HOMA2 parameters with HF compared with LF. This difference in HOMA2 parameters remained when differences at baseline were controlled for using ANCOVA ($p<0.05$).

The addition of gender as a factor in the ANOVA revealed no interaction with the effects of exercise or cocoa on any parameter. Table 4.4 displays the change data for metabolic and anthropometric assessments in each intervention group.

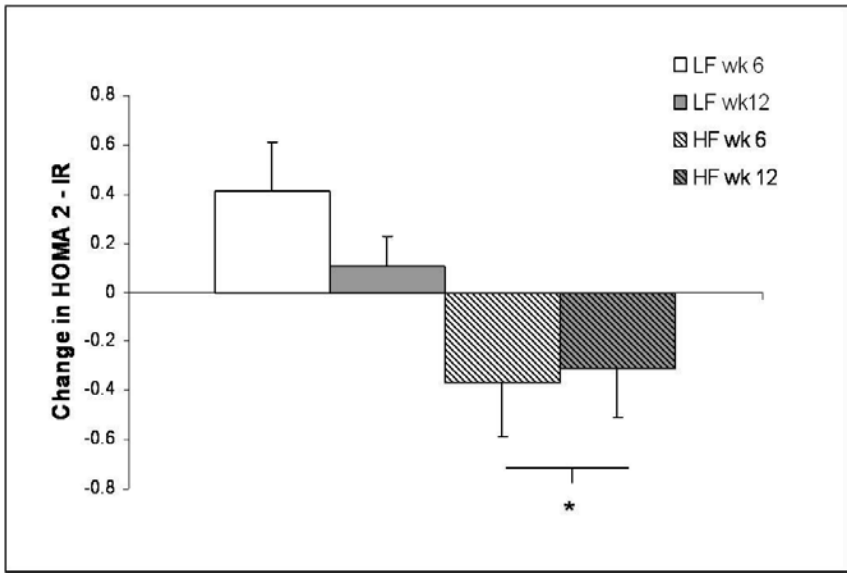

Figure 4.3: Change in HOMA2 – IR from baseline to week 6 and 12. LF = low flavanol; HF = high flavanol. * HF significantly different from LF by nested analysis of week 6 and 12 data ($p<0.05$).

Total Mass (kg)	*n*	Week 0	Week 6	Week 12	Δ Week 12 – 0
LF + Ex	13	94.7 ± 2.1	95.2 ± 2.2	94.9 ± 2.4	0.20 ± 0.55
HF + Ex	13	94.7 ± 5.2	95.4 ± 5.2	95.1 ± 5.3	0.48 ± 0.77
LF - Ex	11	94.6 ± 6.1	95.2 ± 6.1	95.5 ± 6.1	0.89 ± 0.41
HF - Ex	12	97.2 ± 5.8	97.5 ± 5.6	96.7 ± 5.6	-0.44 ± 0.61
LF (combined)	24	94.65 ± 1.1	95.20 ± 1.1	5.2 ± 1.2	0.55 ± 0.28
HF (combined)	25	95.95 ± 2.6	96.45 ± 2.6	95.9 ± 2.6	0.02 ± 0.39
+ Ex (combined)	26	94.70 ± 3.5	95.30 ± 3.5	95.0 ± 3.5	0.34 ± 0.21
- Ex (combined)	23	95.90 ± 2.9	96.35 ± 2.8	96.1 ± 2.8	0.23 ± 0.31
Percentage Flow Mediated Dilatation					
LF + Ex	13	5.4 ± 0.68	4.8 ± 0.73	4.9 ± 0.81	-0.4 ± 0.77
HF + Ex	13	4.1 ± 0.51	5.5 ± 0.44	5.5 ± 0.76	1.5 ± 0.68
LF - Ex	11	3.7 ± 0.35	3.0 ± 0.47	3.4 ± 1.31	-0.3 ± 0.53
HF - Ex	12	4.1 ± 0.75	5.8 ± 0.55	5.9 ± 0.75	1.8 ± 0.89
LF (combined)	24	4.6 ± 0.76	4.0 ± 0.72	4.2 ± 0.76	-0.4 ± 0.47
HF (combined)	25	4.1 ± 0.44	5.7 ± 0.34	5.7 ± 0.52	1.6 ± 0.54 ^{a}
+ Ex (combined)	26	4.8 ± 0.62	5.2 ± 0.53	5.2 ± 0.55	0.4 ± 0.45
- Ex (combined)	23	4.3 ± 0.54	4.4 ± 0.47	4.7 ± 0.55	0.4 ± 0.47
Systolic Blood Pressure (mmHg)					
LF + Ex	13	121 ± 3.6	125 ± 3.2	120 ± 2.5	-0.5 ± 2.3
HF + Ex	13	126 ± 2.7	125 ± 3.4	127 ± 2.8	1.1 ± 1.6
LF - Ex	11	124 ± 1.8	125 ± 2.5	129 ± 3.7	4.2 ± 2.7
HF - Ex	12	124 ± 3.0	124 ± 3.2	122 ± 3.1	-1.9 ± 1.9
LF (combined)	24	123 ± 2.1	125 ± 2.0	125 ± 2.3	1.6 ± 1.8
HF (combined)	25	125 ± 2.0	125 ± 2.3	124 ± 2.1	-0.4 ± 1.2 ^{a}
+ Ex (combined)	26	123 ± 2.4	125 ± 2.5	123 ± 2.1	0.6 ± 1.5
- Ex (combined)	23	124 ± 1.8	125 ± 2.0	125 ± 2.4	0.9 ± 1.6
Diastolic Blood Pressure (mmHg)					
LF + Ex	13	74 ± 1.6	74 ± 1.8	74 ± 2.0	-0.1 ± 1.4
HF + Ex	13	78 ± 2.4	78 ± 3.0	77 ± 2.5	1.3 ± 1.2
LF - Ex	11	77 ± 1.5	79 ± 2.2	80 ± 2.4	2.1 ± 1.2
HF - Ex	12	76 ± 1.8	76 ± 2.2	75 ± 3.1	-1.9 ± 1.5
LF (combined)	24	75 ± 1.2	76 ± 1.5	76 ± 1.7	1.3 ± 1.0
HF (combined)	25	78 ± 1.5	77 ± 1.8	76 ± 1.9	-1.6 ± 0.9 ^{a}
+ Ex (combined)	26	77 ± 1.5	77 ± 1.7	76 ± 1.7	-1.0 ± 0.9
- Ex (combined)	23	76 ± 1.3	76 ± 1.6	77 ± 2.0	0.4 ± 1.1
Heart Rate (bpm)					
LF + Ex	13	60 ± 1.4	63 ± 1.5	61 ± 2.2	1.1 ± 2.1
HF + Ex	13	66 ± 2.1	65 ± 2.3	62 ± 2.7	-3.2 ± 1.2
LF - Ex	11	61 ± 1.9	62 ± 2.1	61 ± 2.9	0.1 ± 1.3
HF - Ex	12	66 ± 3.1	63 ± 2.4	66 ± 2.5	-0.1 ± 2.1
LF (combined)	24	62 ± 1.4	64 ± 1.6	62 ± 1.7	0.4 ± 1.2
HF (combined)	25	66 ± 1.8	65 ± 1.7	65 ± 1.6	-1.7 ± 1.2
+ Ex (combined)	26	63 ± 1.6	64 ± 1.5	62 ± 1.6	-1.2 ± 1.3
- Ex (combined)	23	65 ± 2.0	64 ± 1.9	65 ± 1.8	-0.2 ± 1.2

Total Cholesterol (mmol.L^{-1})					
LF + Ex	13	5.39 ± 0.25	5.09 ± 0.15	4.82 ± 0.15	-0.56 ± 0.20
HF + Ex	13	5.94 ± 0.24	5.61 ± 0.24	5.52 ± 0.21	-0.42 ± 0.18
LF - Ex	11	6.10 ± 0.22	5.86 ± 0.20	5.83 ± 0.27	-0.28 ± 0.23
HF - Ex	12	5.43 ± 0.37	5.43 ± 0.42	5.27 ± 0.44	-0.16 ± 0.25
LF (combined)	24	5.73 ± 0.18	5.46 ± 0.15	5.30 ± 0.15	-0.43 ± 0.15
HF (combined)	25	5.70 ± 0.22	5.52 ± 0.23	5.40 ± 0.23	-0.30 ± 0.16
+ Ex (combined)	26	5.67 ± 0.18	5.35 ± 0.15	5.17 ± 0.15	-0.46 ± 0.13
- Ex (combined)	23	5.73 ± 0.18	5.46 ± 0.15	5.30 ± 0.18	-0.43 ± 0.15
HDL Cholesterol (mmol.L^{-1})					
LF + Ex	13	1.52 ± 0.09	1.51 ± 0.08	1.41 ± 0.08	-0.11 ± 0.08
HF + Ex	13	1.53 ± 0.18	1.42 ± 0.17	1.37 ± 0.14	-0.15 ± 0.09
LF - Ex	11	1.48 ± 0.13	1.49 ± 0.13	1.48 ± 0.12	0.00 ± 0.09
HF - Ex	12	1.54 ± 0.15	1.54 ± 0.14	1.46 ± 0.13	-0.08 ± 0.05
LF (combined)	24	1.50 ± 0.07	1.50 ± 0.07	1.44 ± 0.07	-0.06 ± 0.06
HF (combined)	25	1.53 ± 0.11	1.49 ± 0.11	1.41 ± 0.09	-0.12 ± 0.05
+ Ex (combined)	26	1.53 ± 0.10	1.46 ± 0.09	1.39 ± 0.08	-0.12 ± 0.05
- Ex (combined)	23	1.50 ± 0.07	1.50 ± 0.07	1.44 ± 0.07	-0.06 ± 0.06
LDL Cholesterol (mmol.L^{-1}					
LF + Ex	13	3.25 ± 0.18	2.96 ± 0.11	2.94 ± 0.12	-0.31 ± 0.12
HF + Ex	13	3.74 ± 0.20	3.54 ± 0.24	3.47 ± 0.20	-0.27 ± 0.22
LF - Ex	11	3.75 ± 0.22	3.54 ± 0.15	3.45 ± 0.20	-0.31 ± 0.15
HF - Ex	12	3.26 ± 0.33	3.24 ± 0.33	3.11 ± 0.32	-0.15 ± 0.24
LF (combined)	24	3.50 ± 0.15	3.24 ± 0.11	3.18 ± 0.13	-0.31 ± 0.09
HF (combined)	25	5.70 ± 0.22	5.52 ± 0.23	5.40 ± 0.23	-0.21 ± 0.16
+ Ex (combined)	26	3.50 ± 0.14	3.25 ± 0.14	3.21 ± 0.13	-0.27 ± 0.11
- Ex (combined)	23	3.51 ± 0.20	3.39 ± 0.18	3.28 ± 0.18	-0.22 ± 0.13
Triglycerides (mmol.L^{-1})					
LF + Ex	13	1.28 ± 0.16	1.36 ± 0.22	1.11 ± 0.14	-0.17 ± 0.14
HF + Ex	13	1.56 ± 0.14	1.49 ± 0.15	1.54 ± 0.13	-0.02 ± 0.07
LF - Ex	11	1.89 ± 0.18	1.87 ± 0.21	1.97 ± 0.20	0.09 ± 0.13
HF - Ex	12	1.37 ± 0.20	1.51 ± 0.30	1.47 ± 0.26	0.10 ± 0.10
LF (combined)	24	1.57 ± 0.13	1.61 ± 0.16	1.52 ± 0.15	-0.04 ± 0.10
HF (combined)	25	1.47 ± 0.12	1.50 ± 0.17	1.50 ± 0.14	0.04 ± 0.06
+ Ex (combined)	26	1.42 ± 0.11	1.43 ± 0.13	1.33 ± 0.10	-0.09 ± 0.08
- Ex (combined)	23	1.62 ± 0.14	1.69 ± 0.19	1.71 ± 0.17	0.09 ± 0.08
Glucose (mmol.L^{-1})					
LF + Ex	13	5.24 ± 0.14	5.20 ± 0.17	5.42 ± 0.14	0.18 ± 0.10
HF + Ex	13	5.46 ± 0.16	5.34 ± 0.15	5.42 ± 0.13	-0.04 ± 0.11
LF - Ex	11	5.09 ± 0.16	5.12 ± 0.18	5.01 ± 0.21	-0.08 ± 0.08
HF - Ex	12	5.16 ± 0.15	5.08 ± 0.14	4.94 ± 0.16	-0.21 ± 0.12
LF (combined)	24	5.16 ± 0.11	5.16 ± 0.12	5.21 ± 0.13	0.05 ± 0.07
HF (combined)	25	5.31 ± 0.11	5.21 ± 0.10	5.18 ± 0.11	-0.13 ± 0.08
+ Ex (combined)	26	5.35 ± 0.11	5.27 ± 0.11	5.42 ± 0.09	0.07 ± 0.08
- Ex (combined)	23	5.12 ± 0.11	5.10 ± 0.11	4.97 ± 0.12	-0.15 ± 0.07

Insulin ($\mu U \cdot mL^{-1}$)					
LF + Ex	13	15.2 ± 1.1	18.83 ± 2.62	16.00 ± 2.07	0.82 ± 1.3
HF + Ex	13	21 ± 2.7	18.71 ± 2.58	17.54 ± 2.61	-3.5 ± 1.7
LF - Ex	11	19.2 ± 2.3	22.22 ± 3.62	19.97 ± 2.53	0.79 ± 1.34
HF - Ex	12	19.3 ± 2.4	22.04 ± 5.69	17.87 ± 3.66	-1.4 ± 2.7
LF (combined)	24	17.20 ± 0.61	20.52 ± 1.31	17.98 ± 1.04	0.78 ± 0.8
HF (combined)	25	20.15 ± 1.3	20.37 ± 1.29	17.70 ± 1.31	-2.45 ± 0.9
+ Ex (combined)	26	18.10 ± 1.27	18.77 ± 1.81	16.77 ± 1.26	-1.33 ± 0.83
- Ex (combined)	23	19.25 ± 1.12	22.13 ± 2.85	18.92 ± 1.83	-0.33 ± 0.67
HOMA2 – IR					
LF + Ex	13	2.02 ± 0.2	2.31 ±0.33	2.16 ± 0.28	0.14 ± 0.17
HF + Ex	13	2.71 ± 0.4	2.45 ± 0.33	2.29 ± 0.36	-0.41 ± 0.22
LF - Ex	11	2.45 ± 0.3	2.80 ± 0.44	2.53 ± 0.33	0.08 ± 0.17
HF - Ex	12	2.46 ± 0.3	2.15 ± 0.32	2.25 ± 0.45	-0.21 ± 0.34
LF (combined)	24	2.24 ± 0.17	2.65 ± 0.28	2.35 ± 0.21	0.11 ± 0.12
HF (combined)	25	2.58 ± 0.23	2.31 ± 0.23	2.27 ± 0.28	-0.31 ± 0.20 *a*
+ Ex (combined)	26	2.38 ± 0.20	2.39 ± 0.23	2.23 ± 0.22	-0.15 ± 0.15
- Ex (combined)	23	2.45 ± 0.20	2.48 ± 0.28	2.38 ± 0.28	-0.07 ± 0.19
Fat Oxidation ($g.min^{-1}$)					
LF + Ex	13	0.28 ± 0.02	0.33 ± 0.03	0.33 ± 0.03	0.06 ± 0.03
HF + Ex	13	0.30 ± 0.13	0.35 ± 0.03	0.31 ± 0.03	0.01 ± 0.04
LF - Ex	11	0.35 ± 0.12	0.35 ± 0.03	0.33 ± 0.04	-0.02 ± 0.02
HF - Ex	12	0.42 ± 0.04	0.45 ± 0.04	0.35 ± 0.05	-0.04 ± 0.03
LF (combined)	24	0.31 ± 0.02	0.34 ± 0.02	0.33 ± 0.02	0.02 ± 0.02
HF (combined)	25	0.36 ± 0.03	0.43 ± 0.03	0.39 ± 0.02	0.03 ± 0.03
+ Ex (combined)	26	0.29 ± 0.02	0.34 ± 0.02	0.33 ± 0.02	0.04 ± 0.02 *b*
- Ex (combined)	23	0.39 ± 0.03	0.41 ± 0.03	0.036 ± 0.03	-0.03 ± 0.02
Percentage total body fat					
LF + Ex	13	43.83 ± 1.68	-	43.37 ± 1.53	-0.46 ± 0.38
HF + Ex	13	43.19 ± 2.35	-	42.77 ± 2.31	-0.42 ± 0.34
LF - Ex	11	44.87 ± 2.47	-	44.92 ± 2.42	0.05 ± 0.26
HF - Ex	12	44.07 ± 2.62	-	44.18 ± 2.68	0.11 ± 0.29
LF (combined)	24	44.31 ± 1.42	-	44.08 ± 1.36	-0.23 ± 0.24
HF (combined)	25	43.63 ± 1.71	-	43.43 ± 1.73	-0.20 ± 0.22
+ Ex (combined)	26	43.51 ± 1.41	-	43.07 ± 1.36	-0.44 ± 0.25
- Ex (combined)	23	44.47 ± 1.76	-	44.51 ± 1.78	0.04 ± 0.19
Percentage abdominal fat					
LF + Ex	13	45.53 ± 1.36	-	45.01 ± 1.74	-0.52 ± 0.28
HF + Ex	13	44.99 ± 1.41	-	43.68 ± 1.61	-1.31 ± 0.49
LF - Ex	11	48.16 ± 1.84	-	48.10 ± 1.86	-0.06 ± 0.34
HF - Ex	12	44.37 ± 1.01	-	44.79 ± 1.02	0.43 ± 0.32
LF (combined)	24	46.74 ± 1.1	-	46.43 ± 1.16	-0.31 ± 0.22
HF (combined)	25	44.72 ± 0.86	-	44.19 ± 0.96	-0.52 ± 0.34
+ Ex (combined)	26	45.26 ± 0.96	-	44.35 ± 1.05	-0.92 ± 0.29 *b*
- Ex (combined)	23	46.21 ± 1.08	-	46.35 ± 1.08	0.14 ± 0.24

Table 4.4: Baseline and week 6 & 12 values with absolute change from baseline to week 12 for key variables. LP = low polyphenol supplement; HP = high polyphenol supplement; Ex = exercise program; HOMA2 IR = Insulin Resistance;. [a] significantly different change from baseline between cocoa treatments ($p<0.05$); [b] significantly different change from baseline between exercise treatments ($p<0.05$).

Diet and physical activity

Diet and background physical activity did not change in either group during the study period (Table 4.5). The apparent discrepancy between energy intake and expenditure is likely due to under reporting of total food intakes and over reporting of physical activity in this subject population.

		Week 0			Week 12		
Energy (kJ)	**LF + Ex**	16 320	±	575	15534	±	1006
	HF + Ex	15855	±	814	15976	±	1058
	LF - Ex	16086	±	1650	16321	±	1675
	HF - Ex	16700	±	1203	16258	±	1201
Energy intake (kJ)	**LF + Ex**	9587	±	545	9139	±	637
	HF + Ex	8388	±	420	8502	±	611
	LF - Ex	9243	±	1136	8971	±	1210
	HF - Ex	8107	±	463	7701	±	501
Protein [a]	**LF + Ex**	18.7	±	1.24	18.5	±	1.29
	HF + Ex	18.9	±	1.48	17.8	±	1.56
	LF - Ex	16.1	±	1.73	17.5	±	1.68
	HF - Ex	19.6	±	1.41	19.1	±	1.28
Fat [a]	**LF + Ex**	34.4	±	2.21	34.4	±	3.39
	HF + Ex	33.4	±	2.30	32.0	±	3.16
	LF - Ex	32.9	±	3.13	37.2	±	6.79
	HF - Ex	32.4	±	3.13	30.8	±	2.98
Sat. Fat [a]	**LF + Ex**	14.1	±	1.22	12.9	±	1.52
	HF + Ex	14.4	±	1.50	13.1	±	1.83
	LF - Ex	11.7	±	1.04	14.2	±	2.79
	HF - Ex	13.0	±	1.61	11.9	±	1.61
Poly. Fat [a]	**LF + Ex**	4.6	±	0.53	5.0	±	0.87
	HF + Ex	4.1	±	0.34	3.8	±	0.47
	LF - Ex	5.1	±	0.67	6.0	±	1.03
	HF - Ex	4.8	±	0.80	4.77	±	0.48
Mon. Fat [a]	**LF + Ex**	11.7	±	0.83	11.7	±	1.21
	HF + Ex	11.9	±	1.08	10.5	±	1.62
	LF - Ex	13.1	±	1.70	13.7	±	2.62
	HF - Ex	11.2	±	1.04	11.1	±	1.19
CHO [a]	**LF + Ex**	44.9	±	3.29	44.4	±	2.73
	HF + Ex	44.7	±	3.15	48.5	±	3.38
	LF - Ex	46.2	±	10.4	38.9	±	5.98
	HF - Ex	41.7	±	3.13	45.8	±	5.80

Table 4.5: Energy expenditure and intake of energy and macronutrients at weeks 0 and 12 derived from 3 day physical activity diary and weighed food diary. [a] Expressed as percentage of total energy intake.

4.4 Discussion

These results confirm previous findings of enhanced endothelial vasodilator function following acute ingestion of HF cocoa and maintenance of this effect with ongoing consumption. Furthermore they demonstrate that this effect on FMD remains during 12 weeks of ongoing beverage consumption, which is 10 weeks longer than previously reported. They also confirm the previous finding of increased insulin sensitivity with chronic HF cocoa consumption. However, this is the first study to examine these effects in an overweight/obese population and to do so using a double blind placebo controlled study design. However, despite the fact that the regular consumption of a HF cocoa drink improved FMD and insulin resistance, these improvements were not associated with any changes in body composition. Only exercise resulted in an independent effect on body composition by reducing abdominal fat.

An increase in arterial dilatation, determined from changes in digital pulse wave amplitude following forearm occlusion, has been demonstrated previously in healthy adults after consuming 851mg cocoa flavanols/day for 5 days [262]. Recently Heiss et al also reported an increase in arterial dilatation, assessed by FMD, in a group of smokers following 7 days of consuming 918mg/day of cocoa flavanols [171]. Whilst these studies provide clear evidence of an effect of short-term consumption of cocoa flavanols on endothelial function, the lack of placebo control prevents a definitive interpretation of the results as being directly caused by the cocoa flavanols. A previous placebo controlled study conducted in post-menopausal hypercholesterolaemic females was able to demonstrate an increase in brachial artery blood flow following 6 weeks of HF cocoa consumption (446mg flavanols/day) but fell marginally short of showing a significant

improvement in FMD [173], potentially due to a lack of statistical power. Prior to the present study, Grassi et al had provided the strongest evidence for benefits of chronic consumption of cocoa containing flavanols on FMD when it was demonstrated, using a cross-over design, that consuming 100g of dark chocolate each day (containing around 500mg of cocoa „polyphenols") for 15 days resulted in significant improvements in FMD, BP and insulin sensitivity compared with consuming white chocolate containing no polyphenols [20]. A potential limitation of this study was the lack of blinding of the treatment due to the use of dark chocolate and white chocolate. The present study design overcame this limitation by using reconstituted cocoa beverages that were well matched for taste and appearance. This also allowed a consistent dose of methylxanthine between cocoa treatments, whereas other studies have used methylxanthine free white chocolate as a control. A further advantage of the powders used in the current study was that we were able to deliver a high flavanol load with considerably lower total energy than chocolate. The energy per mg of flavanol in the HF cocoa drink was approximately 1.1 kJ compared to 4.02 kJ in the chocolate product used by Grassi and colleagues. This is an important consideration when long term dietary intervention is being investigated, particularly in overweight subjects and when body composition is a primary outcome measure.

Improved endothelial function has been identified as a likely mechanism for the antihypertensive effects of cocoa flavanols and the concurrent improvements seen in FMD and BP here, as well as the effects previously reported by Grassi et al [20], provide further evidence to support this hypothesis. The magnitude of change in the present study was relatively small when compared to that reported by Grassi et al, despite the higher intake and longer duration of cocoa consumption in the present study [20]. Grassi et al

reported a reduction of 11.9/8.5 mmHg (systolic/diastolic) in hypertensives after 15 days [20] and, even in a normotensive population, they saw a reduction of 7/3 mmHg (diastolic not significant) with the same protocol [19]. The present intervention saw a 1.6mmHg drop in diastolic and 1.2 mmHg in mean BP over 12 weeks. The magnitude of difference between treatments was greater than this, however, as BP tended to rise in the placebo group. The net difference from placebo treatment was still considerably lower than reported by Grassi et al but is comparable to the change seen over 18 weeks in essential hypertensives by Taubert et al [206] with a surprisingly low dose of polyphenols/flavanols (30mg/day). Although Taubert et al controlled background dietary polyphenol consumption more tightly than in the present study, there remains an apparent discrepancy in the magnitude of response to a given level of polyphenol/flavanol consumption between these studies. Therefore, additional studies are warranted to better understand the relationship between flavanol consumption and changes in BP.

A recent review described multiple mechanisms by which reduced availability of NO, as seen in endothelial dysfunction, can impair metabolic function [259]. Among these are potential decreases in mitochondrial biogenesis and oxidative phosphorylation; activation of specific transcription factors and signaling pathways; and reductions in blood flow to areas of high metabolic demand such as skeletal muscle. It is plausible therefore, that improvements in muscle blood flow, and insulin sensitivity, which occur secondarily to improvements in endothelial function may be useful for the prevention or management of obesity. Supplementation with L-arginine, the primary substrate for NO, in Zucker diabetic fatty rats increased NO production by 71-85% and attenuated increases in fat mass over 10 weeks compared to those supplemented with alanine [265] indicating a role

of improved endothelial function in obesity management. More direct evidence of a potential role of cocoa in this was provided by a recent Japanese study [208]. Inclusion of cocoa powder in a high-fat diet significantly blunted the increase in fat mass compared to placebo in male Wistar rats, potentially as a result of reduced gene expression for fat synthesis. Although not evaluated in this study, a potential obesity resisting effect of HF cocoa consumption would be beneficial in itself if it could be replicated in humans and warrants further investigation as energy imbalance is the underlying cause of obesity [4]. This was the first study to investigate the effects of cocoa flavanol consumption on body composition in humans but, while regular HF cocoa consumption was able to independently improve FMD and insulin sensitivity, presumably through increased NO availability, there was no effect on body composition over a 12 week period. Only regular exercise resulted in an independent, although modest, reduction in abdominal body fat, and this reduction was associated with an increase in fat oxidation during sub-maximal exercise.

In conclusion this was the first study to evaluate the cardio-metabolic effects of HF cocoa consumption with and without regular moderate exercise in an obese population. Regular exercise for 12 weeks lead to an increased rate of fat oxidation during exercise and reduced abdominal adiposity. Although HF cocoa consumption did not directly affect body composition, nor augment the effect of exercise on body composition, it was associated with enhanced endothelial function; reductions in diastolic and mean arterial BP and reduced insulin resistance. These observations indicate that HF cocoa consumption may be useful, particularly when combined with a program of regular

modest physical activity, for reducing cardio-metabolic risk factors in an obese population.

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Additional data

The following results were not included in published work on reviewer‟s request. Arterial stiffness was assessed by Pulsetrace stiffness index (SI) supine in association with the described blood pressure assessments at baseline and weeks six and 12.

No significant change was seen in the SI data in any main or pooled group analysis.

	n	Week 0	Week 6	Week 12	Δ Week 12 - 0
LP + Ex	13	8.0 ± 0.5	7.5 ± 0.4	7.9 ± 0.5	-0.1 ± 0.3
HP + Ex	13	7.9 ± 0.6	7.5 ± 0.5	7.8 ± 0.6	-0.1 ± 0.2
LP - Ex	11	8.5 ± 0.6	8.4 ± 0.5	8.9 ± 0.7	0.3 ± 0.6
HP - Ex	12	7.4 ± 0.5	7.4 ± 0.5	7.3 ± 0.6	-0.1 ± 0.5
LP (combined)	24	8.2 ± 0.5	7.9 ± 0.4	8.3 ± 0.5	-0.0 ± 0.4
HP (combined)	25	7.7 ± 0.4	7.5 ± 0.4	7.6 ± 0.4	-0.1 ± 0.3
+ Ex (combined)	26	7.5 ± 0.5	7.1 ± 0.4	7.5 ± 2.0	-0.1 ± 0.2
- Ex (combined)	23	7.9 ± 0.4	7.9 ± 0.4	8.0 ± 0.5	0.2 ±0.4

Table 4.6: Stiffness Index ($s.m^{-1}$) at baseline, weeks 6 and 12, and change from baseline at week 12 in all groups. Values represent Mean ± SEM

Chapter 5

Impact of cocoa flavanol consumption on blood pressure responsiveness to exercise

Narelle M Berry[a], Kade Davison[a,b], Alison M Coates[a], Jonathan D Buckley[a] and Peter RC Howe[ab]

[a] Nutritional Physiology Research Centre and ATN Centre for Metabolic Fitness, School of Health Sciences, University of South Australia, SA 5001 Australia and [b] School of Molecular & Biomedical Sciences, University of Adelaide, SA 5005, Australia.

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Statement of Authorship

Impact of cocoa flavanol consumption on blood pressure responsiveness to exercise

Kade Davison (Candidate)

Contributed to conceptualisation and design of protocol. Recruited, Screened and tested all volunteers and; analysed and interpreted data in association with co author Berry N. Contributed to manuscript development.

I hereby certify that the statement of contribution is accurate

Signed …… *……Date…18/12/09…*

Narelle Berry (co-author)

Contributed to conceptualisation and design of protocol. Recruited, Screened and tested all volunteers and; analysed and interpreted data in association with candidate. Prepared manuscript.

I hereby certify that the statement of contribution is accurate

Signed …….. *……..Date……18/12/09*

Alison Coates (co-author)

Contributed to conceptualisation and design of protocol, interpretation of data and development of manuscript.

I hereby certify that the statement of contribution is accurate

Signed …….. *……Date…18/12/09…*

Jon Buckley (co-author)

Contributed to conceptualisation and design of protocol, interpretation of data and development of manuscript.

I hereby certify that the statement of contribution is accurate

Signed …….. *……Date…18/12/09*

Peter Howe (co-author)

Contributed to conceptualisation and design of protocol, interpretation of data and development of manuscript.

I hereby certify that the statement of contribution is accurate

Signed *……Date…18/12/09*

5.1 Introduction

Impaired endothelial vasodilator function is an important contributor to the development of cardiovascular disease [268], but it is not yet clear how it impacts on other biomarkers of cardiovascular health.

It has been well-established that obesity and hypertension are associated with impaired nitric oxide (NO) dependent vasodilatation [29, 269]. Reduced availability of NO significantly impairs the degree of blood vessel dilatation in response to cardiovascular stressors, such as exercise [270].

The typical cardiovascular response to aerobic exercise is an increase in heart rate (HR) and cardiac output which elicits an increase in systolic BP (SBP). Diastolic BP (DBP) either remains unchanged or decreases slightly due to vasodilatation in the exercising muscles, resulting in increased pulse pressure. Impaired endothelial function (as measured by flow mediated dilatation [FMD]) has been associated with exaggerated BP responses to exercise [271] which have been linked to an increased risk of developing future hypertension [272-275]. This therefore suggests that individuals with impaired vasodilatation such as those who are obese or have elevated BP may have exaggerated BP responses to exercise, thus making them predisposed to acute risk during exercise [276].

Previous attempts to evaluate this emerging risk factor have been limited by the techniques available to measure BP responsiveness to exercise. Most studies have used graded exercise tests with single BP measurements taken at the end of each workload. The introduction of non-invasive techniques for continuous beat-to-beat monitoring of BP enables cardiovascular responses to be measured *during* rather than *after* exercise, thus offering a more physiological representation of the effects of impaired dilatation on

cardiovascular function, compared with the commonly used but somewhat artificial FMD response to passive hyperaemia.

Recent studies have shown that the short-term intake of cocoa polyphenols can lower blood pressure (BP) and improve endothelium-dependent vasodilatation [19, 20, 206, 277]. The mechanism by which cocoa exerts its antihypertensive effect is yet to be determined but the effect may be mediated through enhanced endothelial function [278], with the cocoa polyphenols increasing the activity of nitric oxide synthase in endothelial cells [167, 279], which can lead to enhanced endothelium-dependent vasodilatation [168, 280] and improved blood pressure. . Thus, there may also be potential for cocoa flavanols to attenuate the BP increases in response to physiological stressors such as exercise.

The aim of this study was to see whether improvements in FMD seen in overweight individuals following consumption of flavanol-rich cocoa [277] can also improve their exaggerated BP responses to aerobic exercise (measured by decreased area under the curve for SBP, DBP and mean arterial pressure (MAP) during submaximal exercise).

5.2 Method

A randomised, double-blind, cross-over trial to test acute effects of cocoa flavanols on BP responsiveness to exercise was conducted. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the University of South Australia Human Research Ethics Committee and conducted at the Nutritional Physiology Research Centre. Written informed consent was obtained from all subjects.

Men and post-menopausal women who were overweight or obese (BMI> 25 kg/m^2) but otherwise healthy were recruited. Volunteers had no history of cardiovascular disease, diabetes or renal disease, were not taking diabetic or BP lowering medication and had seated clinic SBP ≤160 mmHg and DBP ≤100 mmHg. Participants were not intolerant to alkaloids (caffeine, theobromine) or dairy, currently smoking or using nicotine replacement therapy.

Volunteers visited the Centre at the same time of day on 5 occasions. At the first (screening) visit, they undertook an exercise test on a cycle ergometer (Monark, Model 828, Vansbro, Sweden) to determine the required workload for subsequent exercise tests. They were required to ride the cycle ergometer for 10 minutes at a workload eliciting a HR equivalent to 75% of their age-predicted maximum (208–(0.7 x age [yr]))*0.75) [228] and their ECG was monitored by a medical practitioner to confirm their suitability to continue exercising.

Volunteers were then provided with a dairy-based cocoa beverage powder which was either high (HF) or low (LF) in cocoa flavanols (refers herein to epicatechin and catechin as well as their procyanidin oligomers up to and including decamers). Reconstituted in 200 ml water, the cocoa beverages provided a total of either 701 mg of cocoa flavanols (HF: 139 mg epicatechin, 39 mg catechin and 523 mg procyanidins) or 22 mg of cocoa flavanols (LF: 0 mg epicatechin, 9 mg catechin and 13 mg procyanidins). The LF and HF cocoa drinks were matched in appearance, macronutrient, micronutrient, and alkaloid (caffeine and theobromine) content, with the only exception being the cocoa flavanol content, see Table 5.1 for nutrient composition of products. The sachets were labelled with a three-digit numerical code, blinding both volunteers and investigators to their identity throughout the study. Empty sachets were collected to monitor compliance.

The sequence of events on each of the four visits was as follows:

1. Volunteers fasted from food and drink (except water) for ≥ 4 hours

2. Supplement was consumed.

3. FMD was conducted 2 hours after supplementation. Previous research has demonstrated a peak effect of cocoa flavanols on FMD at 2 hours after consumption [167] which returns to baseline 6 hours after consumption [171, 172]. The assessment of FMD in the brachial artery was performed using 2-dimensional B-mode ultrasound (LOGIQ 5; GE Medical Systems, Waukesha, WI). Optimal imaging of the artery was achieved using the method of Raitakari and Celermajer [256]. A sphygmomanometer cuff was placed around upper forearm in line with the cubital fossa (ie, distal to the scanned part of the artery) and inflated to supra-systolic pressure (200 mmHg) for 5 min. Images of the artery were taken before cuff inflation, 10 s before cuff release, 10 s after cuff release, and then every 30 s for an additional 3 min to assess the EVF response to reactive hyperaemia.

4. Clinic BP was measured by oscillometry (SpaceLabs Model 90217, SpaceLabs Medical, Florida, USA) while subjects were seated on the cycle ergometer prior to the commencement of exercise.

During the subsequent exercise test BP and HR were measured continuously using a Finapres™ BP monitor (Ohmeda Inc., Englewood, Colorado) with the hand steadied in a support which was maintained at a constant height for all occasions. The test commenced with a 5 minute pre-exercise period of sitting on the cycle ergometer before a 10 min bout of exercise at a workload eliciting 75% of the subject‟s age predicted maximum HR [228].

Diet and lifestyle requirements during the study

This protocol was repeated twice with each cocoa drink (LF or HF) in random order at with a 3-7 day washout between visits and the repeat measures for each supplement were averaged.

Volunteers were asked to consume a low-flavanol diet during the study period, specifically participants were asked to limit their intake of fruit or fruit containing juices, apples, tea (green, black, herbal, chai, brewed or bottled), coffee or caffeinated beverages, cocoa/chocolate or cocoa/chocolate containing products, honey, soybeans, and soy containing products, nuts/nut products containing nut skins, red wine. Participants were provided with written and verbal reminders to ensure compliance with this request.

Flavanols (mg)	22	701
Weight (g)	39	39
Energy (kJ)	603	610
Total fat (g)	1.7	1.7
Sat fat (g)	0.9	0.9
Total CHO (g)	20.6	20.6
Sugars (g)	11.5	11.8
Protein (g)	11.4	11.4
Caffeine (mg)	31.1	27.2
Theobromine (mg)	268.1	307.0

Table 5.1: Nutritional profile for each cocoa dose (2 sachets) of cocoa product.

Data analysis

Using the Finapres™ BP monitor, data was obtained for every heart beat during the 15 minute protocol, then averaged in 30 second blocks. The final 30 seconds of the seated BP and HR assessment was taken to be the pre-exercise HR and BP.

The changes in BP and HR during exercise were calculated by subtracting the average of each 30 second block during exercise from this pre-exercise average. These 30 second averages were used to calculate the area under the curve (AUC) for the change in BP from pre-exercise values to give an integrated BP response to exercise.

Brachial artery diameter was assessed manually at each time point using the integrated digital callipers by a single observer who was blinded to the treatment group. FMD was reported as the maximum % change from baseline in blood vessel diameter following the cuff occlusion, as described previously [277].

FMD and BP data from both visits under each condition (HF or LF) were averaged prior to analysis. Two-way analysis of covariance (ANCOVA) was used to compare the effects of cocoa supplementation on both FMD and the BP response to exercise, with baseline arterial diameter and pre-exercise BP used as covariates using STATISTICA v5.1 (StatSoft Inc, Tulsa, OK, USA). Relationships between FMD and the BP responses to exercise were determined by linear regression analysis. $P < 0.05$ was taken to indicate statistical significance. All data are presented as mean ± standard error unless otherwise stated.

A power calculation was performed on the data obtained on the first 9 participants to determine the number required to achieve a statistically significant difference in the change in Diastolic BP (AUC) between treatments. This analysis indicated that 21 participants were required to detect a change of 3091 units with a standard deviation of 4691 at a power of 81 percent and significance level of 0.05.

5.3 Results

A total of 21 volunteers (13 men and 8 women, age 55 ± 2.2 years, height 1.7 ± 0.02 m, weight 94.1 ± 3.5 kg, BMI 31.6 ± 0.8 kg/m2, SBP 134 ± 2 mmHg, DBP 87 ± 2 mmHg) completed the trial. Using an existing database of potential volunteers, individuals identified as having a BMI> 25 kg/m^2 were invited to return to perform this trial. Of the 25 people who were contacted, 21 volunteers agreed to participate. There were no withdrawals from this study.

Flow Mediated Dilatation

Figure 5.1 demonstrates the FMD response to HF and LF cocoa. Two hours after consumption, the HF cocoa beverage resulted in a significantly greater FMD response than the LF cocoa beverage, $p<0.001$.

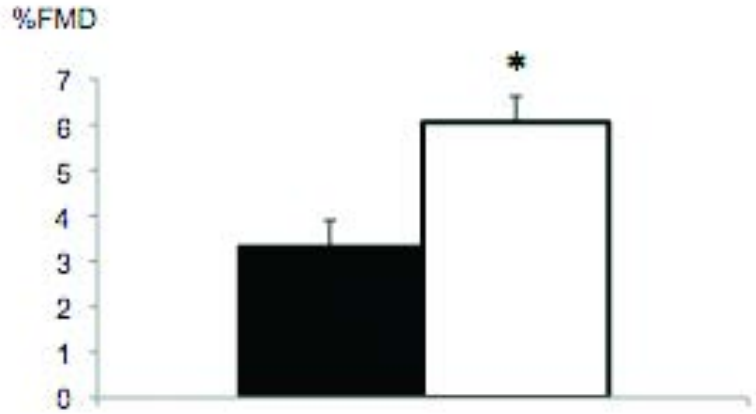

Figure 5.1: %FMD following high and low flavanol cocoa consumption. * = significant difference ($p<0.05$) between HF (white bars) and LF (black bars).

Pre-exercise BP

Pre-exercise BP and HR were measured by Finapres finger plethysmography whilst on cycle ergometer. Data were averaged from the final 30 seconds of the pre-exercise period prior to the commencement of exercise. These readings are likely to differ from the clinic BP readings due to hydrostatic differences between the relative heights of the finger cuff and the brachial artery. There were no significant differences between HF and LF cocoa beverage consumption on the pre-exercise BP (HF: SBP/DBP 153 ± 3/88 ± 3 mmHg, HR 79 ± 2 bpm; LF: SBP/DBP 153 ± 4/88 ± 2 mmHg, HR 79 ± 2 bpm).

Responses to exercise

Both HR and BP increased in response to the cycling exercise. However, there were no significant differences in the HR response following consumption of the HF or LF cocoa

beverages. On the other hand, the increases in BP were attenuated by HF cocoa consumption compared with LF cocoa consumption. Fig 5.2 demonstrates the changes from pre-exercise values for the BP responses to exercise for each 30 second block over the entire 10 minute exercise bout. Table 5.2 shows the integrated responses for BP increases during exercise. After adjusting for pre-exercise BP, the AUC was reduced 68% for DBP (P=0.03) and 14% for MAP (P=0.05) following HF cocoa consumption compared to LF cocoa consumption.

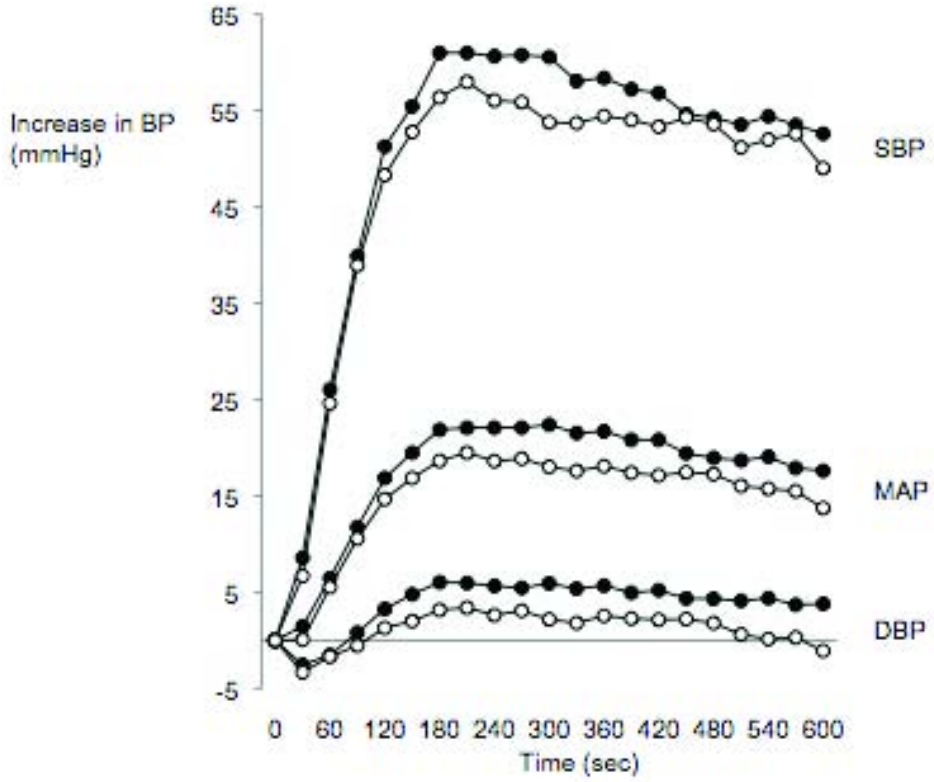

Figure 5.2: BP responses to exercise represented high (white circles) and low flavanol (black circles) cocoa consumption as change from pre-exercise values.

	SBP (mmHg x sec)		DBP (mmHg x sec)		MAP (mmHg x sec)		PP (mmHg x sec)	
	mean	SE	mean	SE	mean	SE	mean	SE
LF (22 mg)	30155	± 2021	2359	± 822	10570	± 1090	27796	± 1913
HF (712 mg)	28850	± 2234	743	± 1098	9067	± 1281	28106	± 1971
Δ	-1305	± 1904	-1616	± 902*	-1503	± 962*	310	± 59

Table 5.2 Area under the curve for the blood pressure responses to exercise between high flavanol and low flavanol cocoa (mean and standard error). *= significant decrease in the BP response to exercise when analysed with pre-exercise BP as the covariate ($p<0.05$). SBP, systolic blood pressure, DBP, diastolic blood pressure, MAP, mean arterial pressure, PP, pulse pressure LF, low flavanol, HF, high flavanol, Δ, difference between high flavanol and low flavanol response.

Relationship between changes in FMD and changes in BP response to exercise

Comparison of differences between HF and LF in the FMD and BP responses to exercise revealed no significant relationships between the differences in FMD and the differences in SBP (r=0.06, P=0.78), DBP (r= 0.42, P=0.06) or MAP (r=0.28, P=0.22), although the relationship between FMD and DBP approached statistical significance.

5.4 Discussion

Results of the present study confirm that consuming a single dose of HF cocoa results in a significant improvement in FMD after two hours. Moreover, they demonstrate that acute ingestion of HF cocoa can also attenuate the BP response to exercise.

Conditions such as obesity, diabetes and hypertension are known to impair vasodilatation [29, 269] and may potentially cause an increase in DBP during exercise. In the present study, there was indeed an exercise-induced increase in DBP (Figure 5.2), but the increase was attenuated by supplementation with HF cocoa.

This reduction in DBP response tended to correlate with the increase in FMD following HF consumption (P = 0.06), suggesting that this benefit may be due to improved endothelium dependant dilatation. While some published studies have also found a relationship between exercise BP and FMD [271, 281], others have not [282]. Green et al [260] found no relationship between improvements in conduit vessel function (as measured by FMD) or resistance vessel function (strain gauge plethysmography of total forearm blood flow) and improvements in exercise-induced vasodilatation following exercise training. The role of NO as a mediator of exercise-induced vasodilatation is controversial. A recent review by Tzemos et al. [270] concluded that there may be a role for NO in mediating exercise-induced vasodilatation but, of the studies that have examined the role of NO in exercise-induced vasodilatation [271, 281, 282], most have reported that, while NO does contribute [271, 281], there are also many other factors which mediate the vasodilatory response to exercise. Although the relationship between improvement in FMD and attenuation of the blood pressure response to exercise was not significant, it is likely that this study was not sufficiently powered to confirm this relationship.

The finding of an improvement in FMD following consumption of HF in the current study is consistent with a growing body of evidence indicating beneficial effects of cocoa flavanols for endothelial function [20, 168, 171, 206, 277, 280, 283]. The mechanism by which cocoa flavanols influence vasodilatation is yet to be clearly identified although it appears to be via an increase in the bioavailability of NO due to increased NO production [206, 262, 284]. Previous research has shown that after consumption of a similar cocoa product, plasma levels of flavanols peak at approximately 2 hours post-consumption. In addition pure epicatechin consumption closely mimicked the effect of the cocoa beverage,

suggesting that epicatechin may be the flavanol responsible for the improvements in vascular function [169], however this study was a proof of concept study with N=3. Therefore further research is required to fully elucidate which flavanols in cocoa can provide the observed benefits in vascular function.

It is important to note that the flavanol rich cocoa beverages used in this study may not deliver the same benefits as dark chocolate consumption. In a study by Hammerstone et al 2000, it was demonstrated that dark chocolate contains approximately 4.3 mg of flavanols per gram. To achieve the amount of flavanols seen in this study (701mg) would require the consumption of 163g of dark chocolate or approximately double that amount of milk chocolate [150]. Given that 163g of dark chocolate provides approximately 3526 kJ and 28g of saturated fat [285] it would be preferable to deliver cocoa flavanols for health benefits in a beverage form such as that used in this study which delivered 610 kJ and 0.9g of saturated fat.

In conclusion, the results of the present study provide further support for acute consumption of cocoa flavanols to improve FMD, and they provide new evidence that cocoa flavanols can also attenuate the BP responses to exercise.

These findings suggest that the consumption of cocoa flavanols may be able to enhance muscle blood flow to allow for improved nutrient delivery and removal to exercising muscles and attenuate the blood pressure responses to exercise, which could allow for safer and more efficient exercise performance in an at risk population such as that include in this study, thus placing less stress on the cardiovascular system during exercise. Furthermore these improvements in FMD and BP response to exercise add to growing evidence that HF cocoa consumption may benefit individuals with cardiovascular risk factors.

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All authors contributed to study design, interpretation of outcomes and preparation of the manuscript; NB and KD collected and analysed the data.

The authors do not have any financial or personal conflicts of interest.

Chapter 6

Dose-related effects of flavanol-rich cocoa on blood pressure

Kade Davison[a,b], Narelle M Berry[a], Alison M Coates[a], Jonathan D Buckley[a] and Peter RC Howe[ab]

[a] Nutritional Physiology Research Centre and ATN Centre for Metabolic Fitness, School of Health Sciences, University of South Australia, SA 5001 Australia and [b] School of Molecular & Biomedical Sciences, University of Adelaide, SA 5005, Australia.

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Statement of Authorship

Dose-related effects of flavanol-rich cocoa on blood pressure

Kade Davison (Candidate)

Contributed to conceptualisation and design of protocol. Recruited, Screened and tested all volunteers and; analysed and interpreted data in association with co author Berry N. Prepared manuscript.

I hereby certify that the statement of contribution is accurate

Signed …… *……Date…18/12/09…*

Narelle Berry (co-author)

Contributed to conceptualisation and design of protocol. Recruited, Screened and tested volunteers and; analysed and interpreted data in association with candidate. Contributed to manuscript development.

I hereby certify that the statement of contribution is accurate

Signed …… ……..Date……18/12/09

Gary Misan (co-author)

Contributed to conceptualisation and design of protocol. Recruited, Screened and tested some volunteers in association with candidate and co-author Berry N. Contributed to manuscript development.

I hereby certify that the statement of contribution is accurate

Signed …… ……Date…18/12/09…

Alison Coates (co-author)

Contributed to conceptualisation and design of protocol, interpretation of data and development of manuscript.

I hereby certify that the statement of contribution is accurate

Signed … ……Date…18/12/09

Jon Buckley (co-author)

Contributed to conceptualisation and design of protocol, interpretation of data and development of manuscript.

I hereby certify that the statement of contribution is accurate

Signed …… ……Date…18/12/09……

Peter Howe (co-author)

Contributed to conceptualisation and design of protocol, interpretation of data and development of manuscript.

I hereby certify that the statement of contribution is accurate

Signed…. *……Date…18/12/09…*

6.1 Introduction

Several intervention studies have reported beneficial effects of consuming cocoa containing products on blood pressure (BP) [19, 20, 195, 286, 287] and a recent meta-analysis confirmed this effect by demonstrating that the consumption of cocoa-rich foods for seven or more days has the capacity to lower resting BP in normotensive and mildly hypertensive adults [205]. This effect of cocoa products has been widely attributed to its content of flavanols and procyanidins (oligomeric and polymeric flavanols), which for the purpose of this paper will be included in the term "cocoa flavanols" (CF) [167, 278].

The recent meta-analysis [205] included five studies which compared the antihypertensive effects of CF-rich chocolate with chocolate containing few or no CF. The amount of chocolate consumed daily ranged from 46 to 105g and provided intakes of CF ranging from 246 to 500 mg/day. There was a mean BP reduction of 4.7 / 2.8 mmHg (systolic/diastolic). In a subsequent study that was not included in the meta-analysis, Taubert et al [206] showed that consuming as little as 30 mg of CF per day in 6g of dark chocolate for 18 weeks was sufficient to lower systolic blood pressure (SBP) by 2.9 mmHg and diastolic blood pressure (DBP) by 1.9 mmHg. A more recent meta analysis by Hooper et al [21] also reported a net effect in favour in cocoa in the order of 6mmHg systolic and 3mmHg diastolic. Although the potential antihypertensive effect of chocolate consumption is interesting, the high sugar and fat content of chocolate undermines its potential as a health enhancing food [288, 289].

Concern about delivering CF in a high sugar and high fat chocolate food matrix has led to investigations of the potential antihypertensive and other cardiometabolic health benefits of relatively low fat drinks enriched with CF. In a study which examined the acute effect of consuming a flavanol-rich cocoa beverage, Schroeter et al [169] demonstrated an

increase in flow mediated dilatation of the brachial artery (FMD). Four studies have been conducted examining the longer-term effects of consuming flavanol-rich cocoa beverages on cardiovascular health in various populations. In the first of these daily consumption of 446mg of cocoa flavanols by post-menopausal women for 6 weeks produced a significant improvement in arterial function but no change in BP compared to placebo [173]. In another study individuals with type 2 diabetes took 963mg /day for a period of 30 days and also experienced improvements in endothelial function but not BP [172]. In a third study in which overweight or obese individuals consumed 902mg/day of flavanols for 12 weeks, there were improvements in endothelial function with modest yet significant reductions in BP (MAP reduced by 1.2 mmHg) [277]. The fourth study provided approximately 900mg/day of CF to individuals with essential hypertension for 2 weeks [22]. Despite the similarity of this protocol to that by which Grassi et al [20] showed BP reductions with chocolate, and despite providing almost double the daily dose of CF, there was no change in BP although there was some improvement in endothelial function. The lack of BP change in three of these studies and the relatively small change in the other following chronic consumption of large doses of CF are inconsistent with the results seen in the meta-analysis [205] and subsequent study [206] with flavanol rich chocolate consumption. These findings suggest that the dose of CF alone does not determine the change in BP, however a number of differences in the design of these studies makes direct comparisons difficult. The studies that have been published to date examined different patient populations (healthy and hypertensive adults in chocolate based protocols and hypercholesterolaemic, overweight/obese and type 2 diabetic in non-chocolate protocols), and the type 2 diabetes study was confounded by participant medications. A further complication with comparison is a lack of consistency in the technique of BP assessment. Most studies have used seated clinic BP assessments with

only three studies to date utilising the preferred technique of ambulatory blood pressure (ABP) monitoring [19, 20, 170]. These three studies used the same protocol of a 15 day crossover study with dark chocolate (delivering approximately 500mg of CF) or white chocolate (CF free) in three separate subject groups including essential hypertensives and have reported the largest reductions in BP to date . With these factors in mind, a single study evaluating the potential BP lowering effects of CF in a homogenous patient population using the preferred method of ABP assessment and delivering the CF in a consistent food matrix was warranted. Therefore, the primary aim of the present study was to determine the dose-response effect of CF delivered using a flavanol-rich cocoa beverage on 24-hour ambulatory mean arterial pressure (MAP) in an untreated borderline/mild hypertensive population. Secondary outcomes include clinic BP assessment and additional measures of 24-hour ABP.

6.2 Method

Participants

Male and post-menopausal female adults with high-normal BP or untreated mild hypertension (SBP 130-160 mmHg or DBP 85-100 mmHg) were recruited by public advertisement. Participants were excluded if they had a known diagnosis of cardiovascular disease (or a history of cardiovascular or cerebrovascular incidents); Diabetes (Type 1 or Type 2) or were taking prescribed anti-diabetic medication; renal failure; were taking BP lowering medication or supplements that may influence BP (i.e. fish oil, liquorice, polyphenols) in the preceding 3 months, or were likely to do so during the study period; an intolerance to alkaloids (caffeine, theobromine) or dairy; were currently smoking or using nicotine replacement therapy; or had any other medical condition which may have influenced the outcome of the study. The study was approved by the Human Research Ethics Committee of the University of South Australia. Each participant provided written and informed consent prior to participation. Recruitment was carried out between February and August of 2007 and the intervention was progressively conducted from April to October of 2007.

Eligibility screening

Potential participants were initially screened for eligibility by completing a health and lifestyle questionnaire and undertaking a seated clinic BP assessment. Participants with a seated BP between SBP 130-160 mmHg or DBP 85-100 mmHg were enrolled in the study. Enrolled participants returned to the clinic approximately one week later to have their BP measured under the same conditions as the initial screening to confirm the presence of high-normal BP or mild hypertension. If they requalified (i.e. SBP 130-160

mmHg or DBP 85-100 mmHg), they commenced the study protocol. If their BP fell outside these limits they were invited to return for an additional qualifying BP assessment. Participants with screening BP consistently above thresholds for BP (i.e. diastolic > 100 mmHg; systolic > 160 mmHg) were referred to their GP and not entered into the study.

Protocol outline

Participants were block-matched by the minimisation convention [290] on BP, gender, age and BMI into four treatment groups which were randomised to consume reconstituted cocoa beverages containing 33, 372, 712 or 1052 mg/d of CF for 6 weeks in a double-blind, parallel comparison. Randomisation of groups was undertaken independently of group minimisation procedure by separate staff members of the research centre not otherwise involved with the trial. Trial investigators remained blinded to treatment allocation until after the completion of data analysis. Participants then had body weight assessed and underwent seated clinic BP and 24 hour ABP assessments at baseline and were then required to consume a cocoa drink daily for the next 6 weeks. Seated clinic BP and 24 hour ABP assessments were repeated after 3 and 6 weeks (Table 2).

Cocoa supplements

The cocoa drinks were prepared by participants mixing the contents of three cocoa beverage sachets with 300 mL of water each morning and drinking 30 minutes prior to breakfast. Nutritional information and cocoa flavanol composition of the cocoa beverages are provided in Table 6.1.

Group No.	**1**	**2**	**3**	**4**
Total flavanol dose	**33**	**372**	**712**	**1052**
Epicatechin	0	69	138	208
Catechin	12	28	43	58
Other flavanols (polymeric/oligomeric)	20	275	530	785
Macronutrient				
Weight (g)	58	58	58	58
Energy (kJ)	904.5	907.9	911.2	914.6
Total fat (g)	2.6	2.6	2.6	2.6
Sat fat (g)	1.4	1.4	1.4	1.4
Total CHO (g)	31.0	31.2	31.5	31.8
Sugars (g)	17.3	17.4	17.6	17.7
Protein (g)	17.1	17.1	17.1	17.1
Caffeine (mg)	46.6	44.7	42.7	40.8
Theobromine (mg)	402.2	421.6	441.1	460.5

Table 6.1: Flavanol composition (monomers and polymers/oligomers) of daily beverage for each dose group.

On all testing days (at 3 and six weeks), volunteers were instructed not to consume their cocoa beverage until after testing was completed to eliminate any acute effects of cocoa consumption. All empty sachets were returned at 3 and 6 weeks to monitor compliance. Participants were instructed to report any signs or symptoms of ill health immediately to the researchers and any action that was taken (e.g. medication) to identify any potential adverse effect of the intervention. In addition to this participants were specifically asked at each visit whether any signs or symptoms of ill health had been experienced. All reported events were documented by the researchers in each participant‟s case report form.

Blood pressure monitoring

Seated clinic BP was assessed after sitting quietly for at least 5 min using an automated oscillometric BP monitor (HDI/Pulsewave CR-2000 Cardiovascular Profiler, Hypertension Diagnostics Inc, Eagan, MN) in accordance with the procedures outlined by the Joint National Committee on the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (VII): US Dept of Health and Human Services [291]. Four consecutive BP readings were taken at 1 min intervals by a single observer. The first reading was discarded and an average of the remaining measurements was taken to determine eligibility for study entry.

For assessment of 24 hour Ambulatory Blood Pressure (ABP), volunteers were fitted with a SpaceLabs ambulatory BP monitor (Model 90217, SpaceLabs Medical, Florida, USA) for 24 hours. An appropriately sized cuff was placed firmly around the upper non-dominant arm, centred over the brachial artery, with the monitor worn on a waist strap; ABP measurements were recorded at 15 minute intervals excepting from 11 pm – 7am (30 minute intervals). The cuff and monitor was only removed briefly for bathing and remained in place at all other times during the 24 hour recording period. Assessments at baseline, 3 and 6 weeks were carried out on the same day of the week using the same monitor, cuff size and arm. Participants were required to maintain an activity diary during each 24 hour ABP period to enable BP values to be related to activities. 24 hour ABP recordings were used to determine daytime (7am – 11pm), night-time (11pm -7am) and 24-hour averages for MAP, SBP, DBP and heart rate (HR).

Diet and lifestyle requirements

Volunteers were asked to maintain their normal physical activity patterns and were provided with a list of dietary exclusions to ensure a low-flavanol diet for 1 week prior to and during the study period. Given the energy content of the beverages (~900 kJ), volunteers were advised on appropriate diet substitutions to avoid increasing total energy intake. To monitor compliance with this request body weight was measured at each clinic visit.

Statistical analysis

It was estimated that a sample size of 12 subjects per arm would have at least 80% power to detect a statistically significant difference in reduction in MAP between the four treatment groups (allowing for multiple comparisons) using an ANOVA at the 0.05 significance level. The estimates were based upon the assumption of a linear dose response effect achieving a 5mmHg change in 24 hour MAP with the highest dose and a standard deviation of 4mmHg as indicated by unpublished pilot data. These estimates are supported by the previously reported ABP results with CF consumption (3, 4, 18).

Baseline characteristics were compared between groups by one-way analysis of variance (ANOVA). Comparison of changes in ABP between treatment groups (i.e. CF dose) across time (i.e. 3 and 6 week assessments) was made using repeated measures analysis of covariance (ANCOVA) using baseline ABP as the covariate. Where ANCOVA showed a statistically significant main effect, post-hoc comparisons (Tukey‟s HSD) were conducted to identify differences between means. To optimise the analysis of dose effects on ABP, a nested ANOVA design was used to examine changes in ABP from baseline by 3 and 6 weeks with time (i.e. weeks 3 and 6) nested in dose. Comparison of changes in HR

between treatment groups across time was made using repeated measures ANOVA. Statistical significance was set at $p<0.05$. All data are presented as mean ± SEM.

6.3 Results

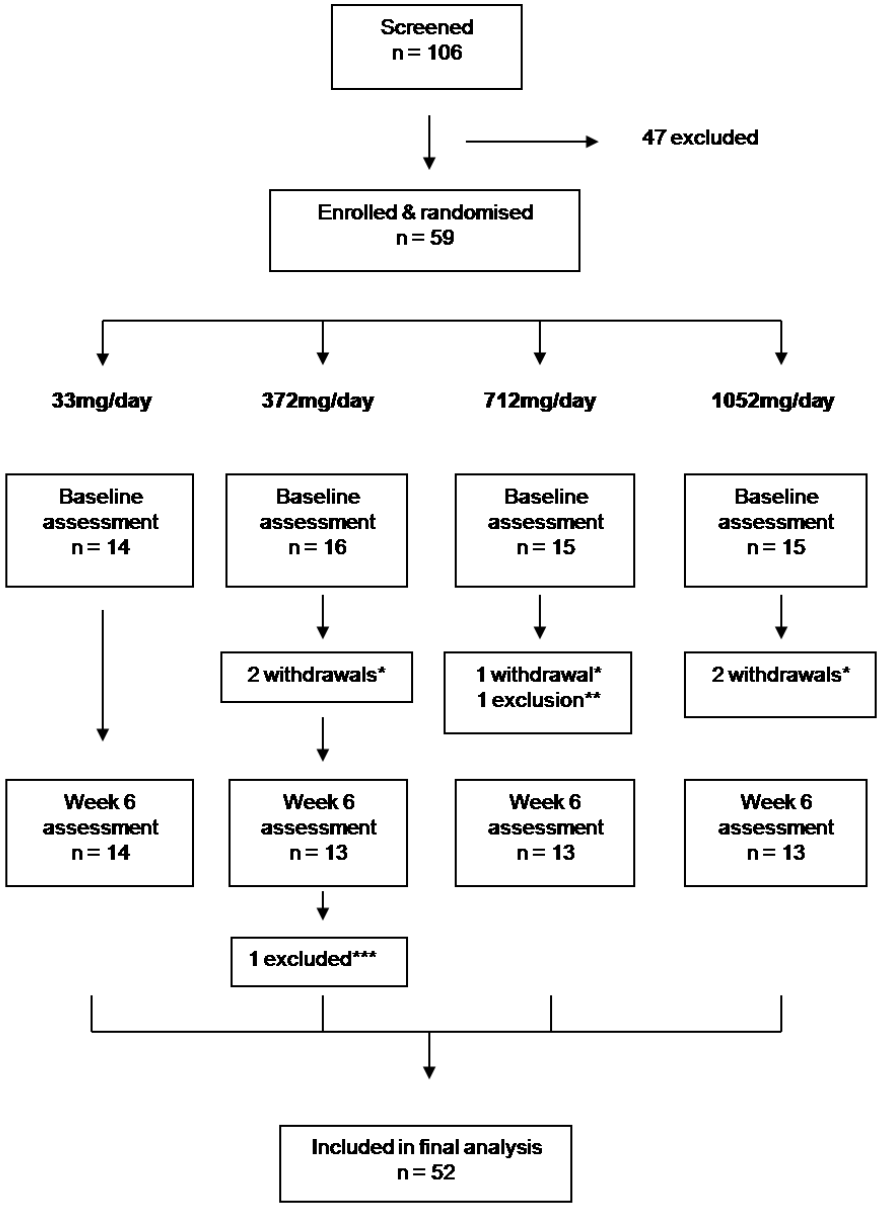

Figure 6.1: Consort diagram * withdrawn for personal or health reasons not related to study protocol; ** excluded due to gastric symptoms possibly related to cocoa beverage; ***excluded due to non-compliance to study protocol (deliberate weight loss during intervention period).

Participant flow is provided in Figure 6.1 above. In total, 53 volunteers completed the study. One volunteer was excluded due to mild persistent (>3 day) gastric symptoms that may have been related to the test beverage. No other adverse events related to the study

were reported. Five volunteers withdrew during the study due to personal circumstances unrelated to the study. One volunteer was excluded from the analysis due to non-compliance to the study protocol, leaving 52 volunteers, or n=12-14 volunteers per arm. Trial withdrawals and exclusions did slightly impact upon the success of the minimisation process on the matching of groups for the stated baseline parameters as can be seen in Table 6.2. However, no significant between group differences were seen for the blocking parameters including age (p=0.45), BMI (p=0.28), and BP (Systolic p=0.86; Diastolic p=0.45). Compliance with consumption of cocoa supplements was greater than 98% in all groups. Body mass did not change during the intervention (p = 0.48 for treatment x time).

Group (mg flavanol/day)	**33 mg**	**372 mg**	**712 mg**	**1052 mg**
N	14	12	13	13
Age (years)	53.0 ± 6.7	56.2 ± 14.2	60.2 ± 13.7	56.8 ± 9.7
M/F	10/4	7/5	8/5	7/6
BMI (kg/m^2)	33.7 ± 16.8	28.7 ± 5.5	26.9 ± 16.2	27.9 ± 5.0

Table 6.2: Baseline characteristics. **(**mean ± SEM). M/F = ratio male to female; BMI = body mass index.

SBP, DBP, MAP or HR for 24-hour, night, day, and seated clinic measurements for each treatment group are provided in Tables 6.3 – 6.6. There were no dose x time interactions for seated clinic BP, but a significant time effect for SBP (p=0.02) and MAP p=0.01) was evident. However, there were significant dose x time effects for 24 hour MAP (p=0.047) and overnight HR (p = 0.041) assessed by ABP monitoring. ANCOVA of the change from baseline (week 0) to weeks 3 and 6 (with baseline values as a covariate) revealed a significant effect of dose on 24-hour ambulatory SBP (p=0.019), DBP (p=0.017) and MAP (p=0.008) and night ambulatory SBP (p=0.0006), DBP (p=0.005) and HR (p=0.043).

Dose (mg)	BP (mmHg)	Week 0			Week 3			Week 6			Δ week 3			Δ week 6		
33	24hr	133.3	±	11.6	132.5	±	14.6	133.9	±	13.8	-0.8	±	-5.6	0.6	±	7.1
	Night	117.1	±	14.2	116.9	±	15.3	118.6	±	10.5	-0.2	±	6.4	1.5	±	8.6
	Day	140.1	±	11.2	140.0	±	15.0	141.2	±	15.7	-0.1	±	-7.1	1.1	±	10.1
N=14	Clinic	145.4	±	9.7	142.1	±	10.5	143.3	±	13.1	-3.3	±	-6.0	-2.1	±	-9.0
372	24hr	133.1	±	11.2	132.9	±	13.3	133.4	±	13.0	-0.2	±	-5.0	0.3	±	6.5
	Night	115.5	±	12.3	115.6	±	10.5	115.4	±	13.7	0.1	±	6.5	-0.1	±	-8.7
	Day	141.1	±	12.3	140.8	±	15.1	142.5	±	14.8	-0.3	±	-6.5	1.4	±	6.9
N=12	Clinic	142.9	±	10.5	142.0	±	11.9	139.6	±	9.0	-0.9	±	-9.0	-3.3	±	-7.9
712	24hr	127.4	±	7.6	127.9	±	9.0	128.9	±	8.3	0.5	±	6.2	1.5	±	5.9
	Night	111.0	±	9.7	113.1	±	10.0	114.7	±	10.7	2.1	±	8.7	3.7	±	5.5
	Day	134.6	±	7.6	134.4	±	9.4	135.6	±	8.0	-0.2	±	-5.5	1.0	±	6.6
N=13	Clinic	143.0	±	9.0	140.2	±	10.4	140.0	±	9.4	-2.8	±	-6.6	-3.0	±	-9.7
1052	24hr	127.8	±	9.4	121.0	±	10.7	124.0	±	10.7	-6.8	±	-5.9	-3.8	±	-5.5
	Night	113.5	±	11.8	106.8	±	9.7	110.1	±	7.3	-6.7	±	-8.0	-3.4	±	-8.0
	Day	133.2	±	9.4	127.4	±	12.1	129.8	±	13.5	-5.8	±	-5.2	-3.4	±	-7.3
N=13	Clinic	143.0	±	8.0	139.8	±	11.1	138.9	±	13.5	-3.2	±	-3.1	-4.1	±	-12.1

Table 6.3: Systolic BP (mmHg) for 24 hour, night, day and seated clinic measurement in each treatment group (mg flavanol/day) at each assessment point (mean ± SD).

Dose (mg)	BP (mmHg)	Week 0			Week 3			Week 6			Δ week 3			Δ week 6		
33	24hr	82.2	±	7.1	81.6	±	7.9	82.8	±	7.9	-0.6	±	-3.4	0.6	±	4.1
	Night	70.0	±	8.6	69.7	±	9.0	71.6	±	6.4	-0.3	±	-5.6	1.6	±	4.5
	Day	87.2	±	6.4	86.7	±	8.2	87.6	±	9.0	-0.5	±	-4.5	0.4	±	6.4
N=14	Clinic	88.0	±	6.0	87.7	±	7.5	88.1	±	7.9	-0.3	±	-3.7	0.1	±	4.9
372	24hr	81.3	±	8.7	80.6	±	10.0	81.4	±	10.0	-0.7	±	-2.8	0.1	±	3.6
	Night	69.3	±	7.6	69.1	±	9.0	69.3	±	8.7	-0.2	±	-4.5	0.0	±	5.8
	Day	87.0	±	10.0	86.1	±	11.4	87.2	±	11.1	-0.9	±	-3.5	0.2	±	3.6
N=12	Clinic	86.4	±	7.6	85.9	±	8.0	83.9	±	9.7	-0.5	±	-5.2	-2.5	±	-5.8
712	24hr	78.3	±	9.4	78.2	±	9.4	79.1	±	10.5	-0.1	±	-3.2	0.8	±	2.5
	Night	66.9	±	8.7	67.8	±	10.5	69.6	±	11.5	0.9	±	5.4	2.7	±	5.4
	Day	83.2	±	10.1	82.6	±	9.7	83.6	±	10.5	-0.6	±	-3.2	0.4	±	3.2
N=13	Clinic	85.9	±	9.4	84.4	±	11.5	84.2	±	10.1	-1.5	±	-5.8	-1.7	±	-5.0
1052	24hr	76.3	±	9.0	72.5	±	9.4	74.2	±	8.7	-3.8	±	-3.6	-2.1	±	-3.2
	Night	66.0	±	8.3	63.3	±	8.3	65.0	±	6.9	-2.7	±	-4.7	-1.0	±	-4.3
	Day	80.0	±	9.4	76.5	±	10.1	78.3	±	9.4	-3.5	±	-3.2	-1.7	±	-4.7
N=13	Clinic	83.2	±	9.4	81.2	±	10.8	81.2	±	8.7	-2.0	±	-6.1	-2.0	±	-3.2

Table 6.4: Diastolic BP (mmHg) for 24 hour, night, day and seated clinic measurement in each treatment group (mg flavanol/day) at each assessment point (mean ± SD).

	BP (mmHg)	Week 0			Week 3			Week 6			Δ week 3			Δ week 6		
33	24hr	98.5	±	8.2	97.8	±	9.4	98.9	±	9.4	-0.7	±	-3.4	0.4	±	4.9
	Night	85.8	±	9.4	85.3	±	10.5	87.4	±	7.1	-0.5	±	-5.2	1.6	±	4.9
	Day	104.1	±	7.5	103.5	±	9.7	104.3	±	10.5	-0.6	±	-4.5	0.2	±	6.7
N=14	Clinic	107.1	±	6.7	105.9	±	8.2	106.5	±	9.4	-1.2	±	-3.4	-0.6	±	-4.9
372	24hr	98.3	±	8.0	98.4	±	9.7	98.9	±	9.4	0.1	±	3.5	0.6	±	4.5
	Night	85.8	±	8.3	85.8	±	8.0	85.9	±	8.7	0.0	±	5.2	0.1	±	6.0
	Day	104.0	±	8.7	104.0	±	11.4	105.1	±	10.7	0.0	±	4.8	1.1	±	5.2
N=12	Clinic	105.3	±	6.9	104.6	±	8.7	102.5	±	8.7	-0.7	±	-5.5	-2.8	±	-4.1
712	24hr	95.4	±	6.9	95.2	±	7.2	96.0	±	8.3	-0.2	±	-4.0	0.6	±	3.6
	Night	82.6	±	7.6	84.1	±	8.3	85.8	±	9.7	1.5	±	6.9	3.2	±	4.7
	Day	101.0	±	6.5	99.9	±	7.6	100.9	±	7.9	-1.1	±	-4.0	-0.1	±	-4.0
N=13	Clinic	105.0	±	8.3	103.0	±	9.7	102.8	±	8.7	-2.0	±	-5.4	-2.2	±	-5.8
1052	24hr*	93.8	±	6.9	89.2	±	8.7	90.8	±	7.2	-4.6	±	-4.0	-3.0	±	-3.2
	Night	82.8	±	7.9	79.2	±	7.6	80.8	±	5.8	-3.6	±	-5.4	-2.0	±	-6.1
	Day	97.9	±	7.2	93.8	±	9.7	95.2	±	8.7	-4.1	±	-4.0	-2.7	±	-4.7
N=13	Clinic	103.1	±	7.6	100.7	±	9.7	100.4	±	9.4	-2.4	±	-7.2	-2.7	±	-7.9

Table 6.5: Mean arterial pressure (mmHg) for 24 hour, night, day and seated clinic measurement in each treatment group (mg flavanol/day) at each assessment point (mean ± SD). ***** Significant dose x time interaction (p=0.047)

Dose (mg)	BP (mmHg)	Week 0			Week 3			Week 6			Δ week 3			Δ week 6		
33	24hr	68.1	±	9.7	70.8	±	10.5	70.6	±	10.9	2.7	±	5.2	2.5	±	6.0
	Night	60.4	±	8.6	62.4	±	8.2	61.6	±	10.1	2.0	±	3.4	1.2	±	4.9
	Day	71.5	±	10.9	74.2	±	12.0	74.4	±	11.2	2.7	±	6.4	2.9	±	6.7
N=14	Clinic	64.6	±	13.1	65.0	±	13.1	63.9	±	12.3	0.4	±	4.9	-0.7	±	-6.4
372	24hr	72.7	±	10.4	72.5	±	9.7	73.5	±	10.4	-0.2	±	-2.4	0.8	±	5.9
	Night	64.4	±	10.7	64.3	±	11.4	64.3	±	9.7	-0.1	±	-3.8	-0.1	±	-5.9
	Day	76.3	±	10.0	76.3	±	9.4	77.9	±	10.7	0.0	±	-3.5	1.6	±	5.2
N=12	Clinic	72.4	±	14.2	70.0	±	10.7	69.2	±	11.1	-2.4	±	-6.6	-3.2	±	-6.6
712	24hr	68.6	±	8.7	70.4	±	10.5	69.7	±	8.3	1.8	±	4.0	1.1	±	3.2
	Night	61.2	±	7.9	63.1	±	10.8	61.9	±	7.2	1.9	±	4.7	0.7	±	2.5
	Day	71.9	±	9.7	73.4	±	10.8	73.2	±	9.0	1.5	±	4.3	1.3	±	4.7
N=13	Clinic	64.8	±	8.7	65.9	±	9.0	64.6	±	7.6	1.1	±	6.1	-0.2	±	-4.3
1052	24hr	74.1	±	5.4	74.3	±	9.0	76.9	±	9.4	0.2	±	7.2	2.8	±	7.2
	Night	67.3	±	6.5	66.3	±	7.6	70.8	±	7.2	-1.0	±	-5.0	3.5	±	4.3
	Day	77.0	±	6.1	78.1	±	10.5	79.8	±	10.5	1.1	±	9.4	2.8	±	9.0
N=13	Clinic	74.3	±	7.2	74.1	±	11.9	71.8	±	10.8	-0.2	±	-9.7	-2.5	±	-9.4

Table 6.6: Heart Rate (beats/min) for 24 hour, night, day and seated clinic measurement in each treatment group (mg flavanol/day) at each assessment point (mean ± SD).

Nested analysis (time nested in dose) revealed significant dose effects for 24 hour MAP ($p=0.0004$), SBP (0.001) and DBP ($p=0.002$); night time MAP ($p=0.01$; Figure 6. 3) and SBP ($p=0.003$) and day time MAP ($p=0.02$; Figure 6.3) and SBP ($p=0.02$). Post hoc analysis showed that for the 1052 mg flavanol dose, the reductions in 24 hour MAP ($p<0.001$), SBP ($p<0.02$) and DBP ($p<0.04$) were significantly different from all other doses (refer Figure 6.2). There were no significant effects on HR. Figure 6.3 displays a consistent reduction in day and night ABP to that seen over the full 24 hours, with significant effects observed at a CF level of 1052 mg/d.

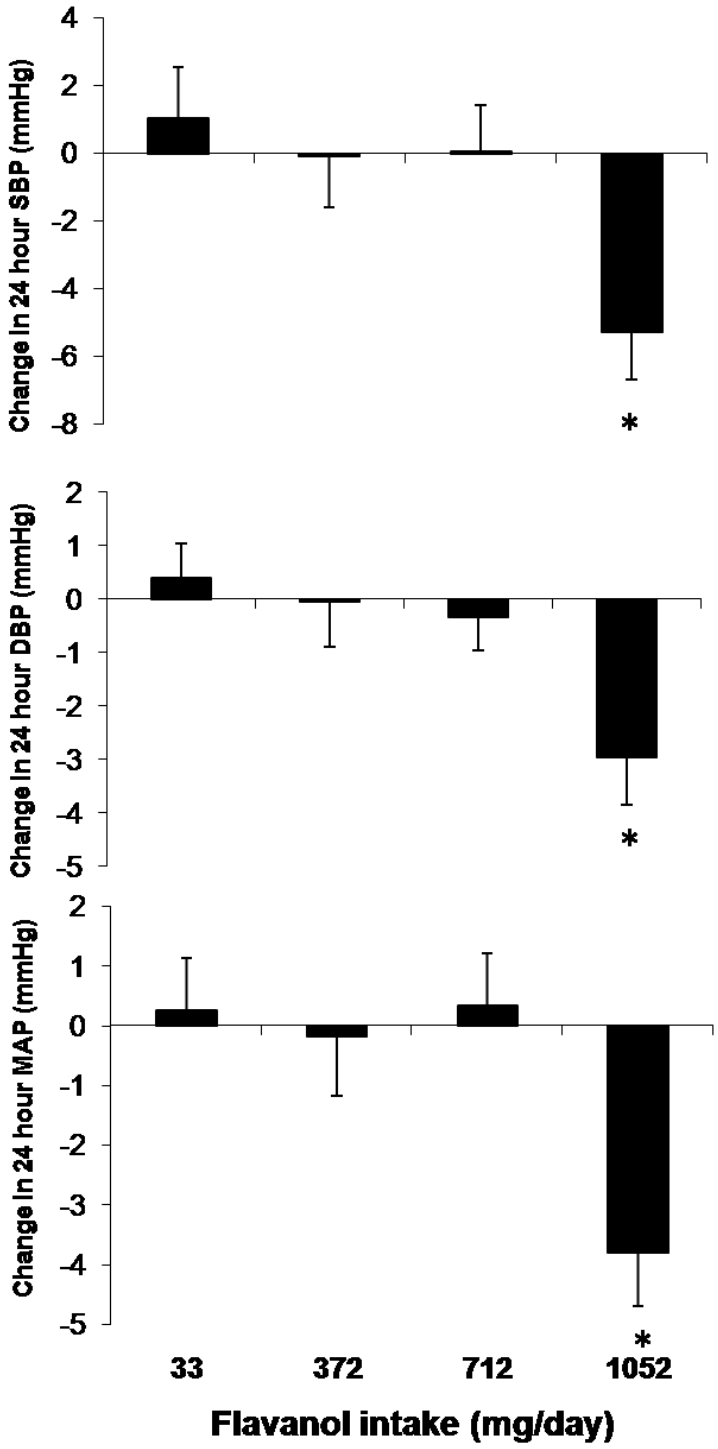

Figure 6.2: Dose-related effects of cocoa flavanols on 24 hour ambulatory blood pressure. Values represent means ± SEM of changes from baseline (average of changes to 3 and 6 weeks). *Significantly different from all other doses by nested analysis ($p<0.001$).

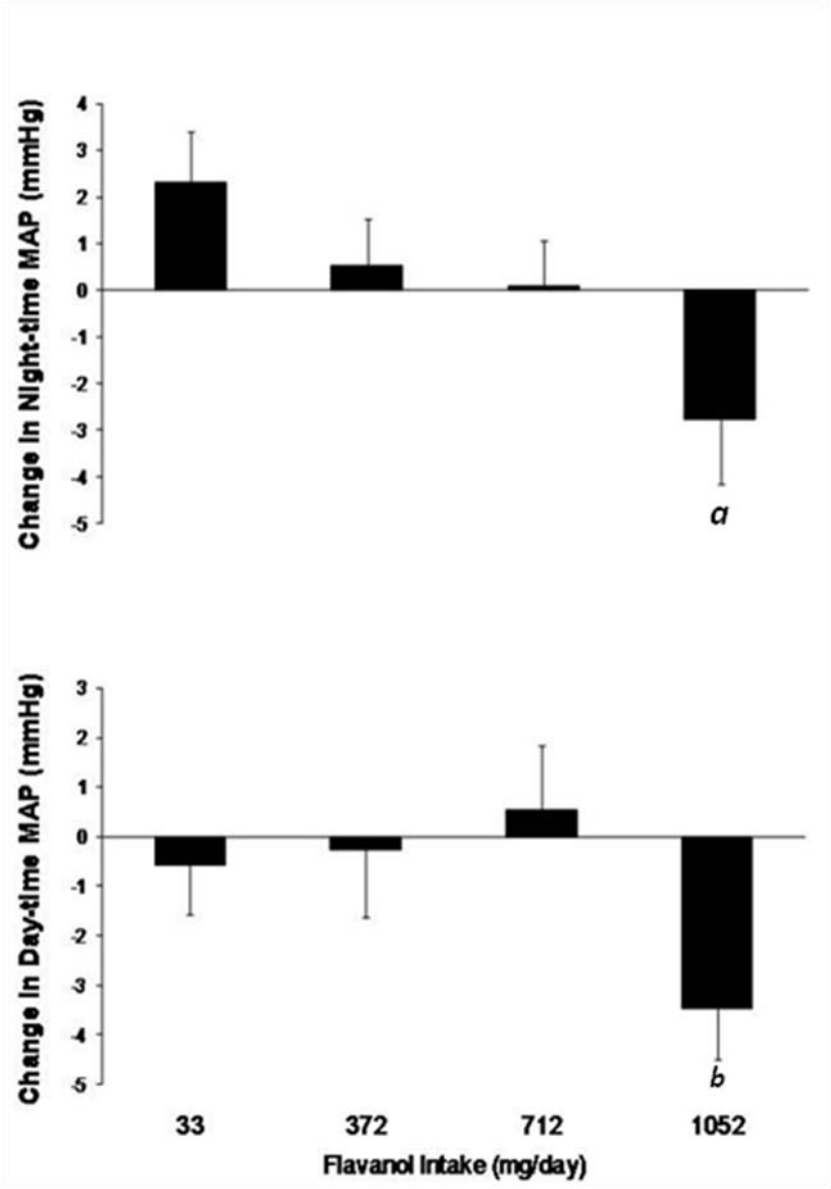

Figure 6.3: Dose-related effects of cocoa flavanols on daytime and overnight ambulatory blood pressures. Values represent means ± SEM of changes from baseline (average of changes to 3 and 6 weeks). ^{a} = significantly different from 33mg (p=0.02). ^{b} = significantly different from 712mg (p=0.01).

6.4 Discussion

The principal finding of the current study was that the regular consumption of flavanol-rich reconstituted cocoa beverages reduced BP in untreated patients with borderline/mild hypertension. This antihypertensive effect was only evident at the highest dose of 1052 mg flavanols/day, with doses up to and including 712 mg/day failing to provide any significant reduction in BP in this study. This is the first study to directly compare the efficacy of differing dose levels of isolated CF. The doses used were based upon the range of CF levels that have been previously reported to lower BP. These findings suggest that BP can be lowered through the regular consumption of flavanol-rich cocoa beverages, though higher levels of CF may be required than has been observed with consumption of chocolate based CF rich products. Although this study provided the highest dose of CF to date the lack of effect on BP with lower doses is supported by previous studies using a non-chocolate food matrix. Whilst there was no evidence of a dose response effect over the doses tested in this trial, it may be the case that the highest dose in this study is on the lower end of a dose response curve with this type of CF product. Additionally, the maximum potential effect on BP is yet to be determined.

Four previous studies have investigated the longer-term effects of consuming flavanol-rich cocoa beverages on BP [172, 173, 277] using beverages providing daily flavanol doses ranging from 446 – 964 mg. Of these studies only one demonstrated a reduction in BP and this was a study which provided one of the highest doses of flavanols (902 mg/day). The finding of this study is in agreement with the outcome of the current study which demonstrated that a high daily flavanol dose is required in order to achieve a reduction in BP. However, these findings are in contrast with those which have examined

the BP lowering effects of cocoa flavanols delivered in a chocolate food matrix where antihypertensive effects have been demonstrated with flavanol doses which were reported to be significantly lower than the levels used in the studies with cocoa beverages. A recent meta-analysis [206] which examined the antihypertensive effects of flavanol-rich chocolate found that daily consumption of chocolate providing relatively modest doses of cocoa flavanols and procyanidins ranging from 246 mg to 500 mg provided significant reductions in BP. Subsequently, Taubert et al [206] showed that consuming as little as 30 mg of cocoa polyphenols per day in dark chocolate for 18 weeks was sufficient to lower systolic blood pressure (SBP) by 2.9 mmHg and diastolic blood pressure (DBP) by 1.9 mmHg. This latter flavanol dose is similar to the lowest flavanol dose provided in the current study where no effect on BP was observed. Thus, it appears there may be a discrepancy in the effective dose of flavanols when delivered in a chocolate matrix compared to a beverage mix. A previous bioavailability study showed that there was no difference in the acute increases in plasma or urinary concentrations of flavanols following consumption of chocolate or dry cocoa containing the same quantities of flavanols [292], and similar bioavailability of cocoa flavanols from chocolate and other sources is also supported by comparisons from other studies [169, 195, 292]. There has been some debate over the potential for the addition of milk protein to cocoa products to reduce flavanol bioavailability [293] and this would potentially impact on efficacy when consumed in milk drinks. However, other recent studies have not supported any effect of milk on reducing flavanol bioavailability [164, 166, 293].

A noteworthy protocol consideration when comparing the studies using flavanol-rich chocolate and flavanol-rich beverages are the degree of placebo control and blinding.

Studies using chocolate have typically used a flavanol-poor white chocolate placebo which limits subject blinding and is not matched for all potentially bioactive nutrients including methylxanthines [20, 205, 206]. The use of flavanol-rich cocoa beverages allows close matching of placebo beverage nutrient composition as well as close matching for colour and flavour to facilitate a double blinded protocol [169, 172, 173, 277]. Muniyuppa et al [22] propose that the discrepancy between the results seen with dark chocolate and flavanol-rich cocoa beverages may be entirely due to these factors. The robust placebo control and blinding used in the present study combined with ABP monitoring provides a superior assessment of the anti-hypertensive potential of CF to those conducted previously.

The mechanism by which CF reduce BP has been largely attributed to their capacity to improve endothelial dilatory function [278, 294]. A number of studies have demonstrated that the regular intake of cocoa flavanols can improve endothelium-mediated vascular dilatory function, with these improvements being associated with an increased bioavailability of nitric oxide (NO) [2, 4, 25, 26, 28]. Recently Taubert et al [206] established a direct relationship which suggested that the BP lowering effects of cocoa flavanols were mediated by improved NO availability when they found a statistically significant correlation between the magnitude of reduction in BP and the magnitude of increase in S-nitrosoglutathione (a marker of NO availability) following 18weeks of consuming chocolate containing cocoa flavanols. However, some studies have demonstrated improvements in vascular dilatory function independently of changes in BP, suggesting that other mechanisms might also contribute to the antihypertensive effect. Two recent studies [172, 173] reported a sustained (non-acute) improvement in markers of endothelium-mediated dilatory function without concurrent changes to BP,

while a third study [277] conducted in our laboratory showed improvement in both parameters but only a modest reduction was seen in BP while endothelium-mediated dilatory function improved by approximately 40%.

The lack of a demonstrable effect of cocoa consumption on seated clinic BP in the present study may also reflect the relative lack of sensitivity of this technique compared with ABP monitoring. Seated clinic BP reduced with time independently of flavanol dose, reflecting a habituation effect (regression to the mean) which most likely masked any treatment effect on BP. An habituation effect was not evident with ABP monitoring. In a previous study, we were able to demonstrate a statistically significant reduction in BP with clinic assessments [277], but the BP assessment protocol in that study was different from that used in the current study with clinic BP measured while participants were supine after a longer pre-assessment rest period. Additionally for the previous study patients with elevated BP were not specifically recruited which may have reduced the likelihood of a „white coat" hypertensive effect masking any effect of the cocoa supplement.

A key concern with the consumption of bioactive nutrients for health benefits is the question of potential adverse effects of the other ingredients. In the case of cocoa flavanols this concern is particularly associated with the high energy and saturated fat content of chocolate. According to HPLC analysis of flavanol content of various food types, to achieve the effective dose of flavanol seen in this study (1052mg) would require the daily consumption of 210g of standard dark chocolate or approximately double this amount of milk chocolate [150]. Given that 210 grams of dark chocolate provides in the

order of 4700Kj and 35g of saturated fat a net health effect may be questionable [285]. This is in contrast to the 914kJ and 1.4g of saturated fat provided by the beverage used in this trial. However, depending upon the underlying causes of the previously discussed discrepancy between the effect of chocolate and flavanal rich cocoa beverages on BP, this comparison may not prove valid. It should be noted that the intention of this trial was not to evaluate the use of a flavanol rich beverage as a potential food supplement, but rather to determine the effect of various doses of cocoa flavanols on BP.

Ideally the lowest dose would deliver 0mg of flavanol; however, this would be not possible with our cocoa based product without significantly altering the flavour and or consistency of the drink, thereby compromising the treatment blinding. Although Taubert et al [206] reported an antihypertensive effect of 30mg CF/day, this has only been seen with a white vs. dark chocolate protocol and not with a well matched control so interpretation of these data are subject to the limitations listed above. Moreover others have failed to show effects on BP of considerably higher doses of isolated CF [172, 173]. Other concerns regarding the 33mg dose results in the present study relate to the lower age and BMI and greater proportion of males in this group. While care was taken to avoid this in the blocking process, with the relatively small subject numbers per treatment arm small numbers of participant attrition can disrupt this balance. It could be argued that this may reduce the potential responsiveness of the control group, however the lack of BP reduction seen at the two intermediate doses suggests this has not influenced the overall results. Additionally, the differences seen in BMI and age were not statistically significant and the most important variable (BP) remained well matched across the four groups. The final limitation was the lack of explicit assessment of the effectiveness of the

blinding protocol. The close matching of the cocoa products implies successful blinding, however these results could have been strengthened by assessing this.

In summary, this study provides the most robust assessment of the effects of cocoa flavanols on blood pressure to date. A significant antihypertensive effect of daily cocoa flavanol intake was observed at the highest dose of 1052mg flavanols/day with no BP lowering effect seen at the lower doses. This study supports the potential for cocoa flavanols to lower BP, but further research is required to determine the extent of antihypertensive benefit that can be achieved with different dietary sources and doses of flavanols in various cardiovascular pathologies.

Acknowlegements

The authors would like to acknowledge the contribution of all the study participants and MARS Inc. for the supply of the test product and financial support (by way of an Australian Government Food Innovation Grant) to conduct study.

What is known about this topic?	*What this study adds*
• **Consumption of cocoa rich chocolate has been shown to lower blood pressure (BP)** A meta-analysis of several intervention trials has demonstrated a BP lowering effect of cocoa rich dark chocolate compared to cocoa free white chocolate. • **Cocoa flavanols (CF)improve endothelial function** Isolated CF and cocoa solids have been shown to enhance endothelial vasodilatation in the same manner as cocoa rich chocolate. • **Lack evidence for effect of CF on BP** Chronic consumption of isolated cocoa solids delivering CF doses in excess of those provided in chocolate have not lowered BP.	• **Robust evaluation of effects of isolated cocoa solids on BP** Dose response evaluation of CF on ambulatory BP in borderline/mild hypertensives. • **The study identifies a threshold dose for cocoa flavanols** Only the highest dose of CF (1052mg/day) lowered BP. This would account for lack of effect on BP with sub-threshold doses of CF in previous studies.

Table 6.7: Summary of study (note - this table is a requirement of the Journal of Human Hypertension for publication).

Chapter 7

Discussion and Conclusion

7.1 Discussion

Collectively the results contained within this thesis provide significant insight into the potential for cocoa flavanols to interrupt the progression from obesity to cardiometabolic dysfunction by counteracting impairment of endothelial vasodilator function. Of even greater importance, they demonstrate that this beneficial effect of cocoa flavanol supplementation can suppress the harmful exacerbation of pressor responses to exercise in obese individuals, and chronically lower BP in borderline hypertensives. Furthermore, the results suggest that there is a threshold dose of flavanols required to elicit these benefits.

An initial comparison of cardiometabolic risk factors was made between obese and normal weight sedentary men and women. The key finding from this study is the apparent differential effect of adiposity and basal aerobic fitness on arterial function, i.e. obesity has the greatest influence on endothelial function and aerobic fitness on arterial stiffness. This study is the first to directly test the relative influence of fitness and fatness on these parameters while controlling for the effect of physical activity. It provides an interesting insight into the potential mechanism by which specific diet and exercise interventions may improve arterial function and therefore decrease cardiovascular risk. Moreover it suggests that interventions aimed at only weight reduction or fitness improvements may not be as effective as those that attempt to address both aspects jointly. This is supported by a recent study demonstrating both aerobic fitness and abdominal obesity are independent predictors of cardiovascular risk factors [238]. However, further investigation is warranted to provide further insight into the mechanisms underlying these associations.

The significantly lower FMD in the obese group compared to those with a healthy BMI supports a potential causal effect of obesity given the close matching of other potential influences such as physical activity and smoking status. Chronic dietary influences were not controlled for however acute dietary influences were alleviated by a minimum 12 hour fast prior to all testing. Additionally, fasting values for total cholesterol and glucose were not significantly different between groups. A proposed mechanism for obesity induced endothelial dysfunction is thought to be through inflammatory mediators and adipokines released from adipose cells as described in chapter 1 section 1.1d [258]. Therefore interventions that can either attenuate the production or release of these adipokines or protect the vascular endothelium may be able to interrupt this process.

The results of this study provide a clear hypothesis for a need to independently improve the obesity related endothelial dysfunction and fitness related arterial stiffness. Furthermore this study confirms the previously reported suppressed endothelial function as measured by FMD in an otherwise healthy obese population.

Based on the results of this study and on the existing body of evidence (outlined in Chapter1) of the benefits of cocoa flavanol consumption on vascular health, a study was conducted to examine the role that consumption of flavanols can play in the management of obesity related cardiometabolic dysfunction in a sedentary obese population (chapter 4).

The results of this study provide significant evidence for the capacity of cocoa to improve cardiometabolic function including FMD, blood pressure and insulin sensitivity, in an overweight/obese population. With daily cocoa consumption of 902mg of cocoa flavanols FMD increased to approximately 140% of that seen at baseline in a fasted state (i.e. not

following acute consumption). This improvement was present at 6 weeks and remained until the cessation of the trial at 12 weeks. Compared to the results seen in the lean group in chapter 3, this would represent a complete reversal of the suppression of FMD in the obese group (i.e. a recovery to that seen in a comparable non-obese population). Although the direct reduction in CV risk cannot yet be quantified, it is widely accepted that an improvement in FMD would lead to reduced CV and metabolic risk [46]. Considering that weight loss interventions in obese individuals, including caloric restriction or gastric bypass, resulting in loss of 5-9% of body mass have been reported to have no impact on FMD [295, 296], it is of particular interest that FMD in this group was significantly enhanced in the absence of any weight loss.

In the proposed pathway from obesity to cardiometabolic dysfunction, hypertension and insulin resistance are likely to be both caused by and contributing to endothelial dysfunction. This is supported by the observation of impaired FMD in obese compared to lean population seen in chapter 3 (4.1 vs 5.7% respectively) yet essentially normal BP and insulin sensitivity. This may also explain the lesser magnitude of change in BP and insulin sensitivity with cocoa supplementation.

When the exercise groups were pooled, there was a significant reduction in the percentage of fat tissue in the abdominal region but no change in total body fat content. This result suggests that while the total amount of fat remained constant the distribution of fat storage was modified by the exercise training. This is consistent with previous results as exercise has been shown to preferentially utilise visceral fat stores [297]. It is however, difficult to explain why in the context of increased energy expenditure the small amount of fat lost from the abdominal region would be replaced with additional stores elsewhere rather than resulting in an overall loss of fat tissue. It may be that the total fat loss was not

of a sufficient magnitude to be detected from normal measurement error of the DEXA analysis within the volume of the entire body, but could be picked up within the smaller volume of the abdominal regional analysis.

It was also anticipated from previous studies that exercise would improve endothelial function and arterial compliance [110]. This was not the case with no additive effect on FMD seen in the HF cocoa plus exercise group and no overall effect seen on either FMD or SI in the pooled exercise group. While some studies have reported improvements in endothelial function with exercise training in overweight or obese subjects, others have not. The lack of effect in this instance is consistent with that seen previously with a similar population (obese with 1 metabolic syndrome characteristics) and an identical exercise intervention [114]. With respect to AC however, this study by Hill et al found the exercise intervention to significantly improve arterial compliance. A possible explanation for this is the different techniques used to assess arterial stiffness in the two studies. Hill et al [114] used the HDI cardiovascular profiler and found an improvement in the „Small Artery Elasticity Index" value. The present study used the *Pulsetrace* technique to derive a Stiffness Index which is more indicative of aortic stiffness. It may be the case that a 12 week exercise program can improve peripheral resistance vessel stiffness by improving endothelial function without reversing the more structurally defined central vessel stiffness.

It was hypothesised that cocoa supplementation may enhance the metabolic effects of exercise in the same way as seen with fish oil supplementation [114]. It was thought that this may be mediated through improvements in endothelial function and therefore increased perfusion of muscle tissue and delivery of oxygen and metabolic substrates. We found no evidence for this, although the improved markers of insulin sensitivity possibly

suggest some improvement in substrate delivery if only during rest. There was no effect of cocoa supplementation on body composition and no effect on fat oxidation during exercise. The effects of increased endothelial function on blood flow during exercise are not yet clear with conflicting reports of the role of the various vasodilator pathways during exercise [260]. There is evidence however of reduced dilation during exercise in some groups with suppressed endothelial function [260].

To further explore the relationship between improvements in endothelial function and exercise hemodynamics an investigation was designed as an extension of previous intervention to directly measure the acute effects of cocoa flavanols on the blood pressure response to exercise in an overweight/obese population (as presented in chapter 5). HF cocoa attenuated the rise in BP with exercise compared to LF cocoa, supporting the notion that that endothelial function is integral in the regulation of blood flow during exercise. As discussed briefly in Chapter 5, this supports the hypothesis that improving endothelial function where some degree of dysfunction is present can modify blood flow hemodynamics during exercise. As there was no difference in heart rate between HF and LF cocoa conditions it can be assumed that cardiac output was similar. Therefore the lower blood pressure seen with HF cocoa is indicative of increased peripheral vasodilatation during exercise. This supports the potential for improved endothelial function to enhance the blood flow to muscles, thereby increasing the delivery of oxygen and substrates to working muscles. Confirmation of this effect is required however as these results do not explain whether the reduced peripheral resistance, and subsequently lower BP, is due to enhanced vasodilatation to muscle tissue or is in fact suppressing the vasoconstriction to viscera and other non-active tissue.

Another important implication for the results seen in this acute experiment is simply the capacity for exercise BP to be influenced by endothelial function. The systemic BP response to exercise represents a more common physiological challenge than the tissue occlusion procedure used with FMD. Both FMD and finger plethysmography have been used to assess vasodilatation during exercise however there are significant technical challenges to acquiring sufficient quality samples due to movement during exercise that often necessitates brief cessation of exercise for sampling [298, 299]. The cessation of exercise will immediately alter the hemodynamics thereby reducing the integrity of the results. Additionally, these techniques only provide information on local vessels rather than an indication of systemic changes. The continuous measurement of BP during exercise overcomes these issues so presents a novel means of further investigating the implications of endothelial dysfunction during exercise. The greatest promise is the potential integration of this technique with markers of local vessel function to produce a more complete picture of exercise hemodynamics, and the implications of both dysfunction and therapeutic interventions.

The final study (presented in chapter 6) addressed the disparity in the literature between the effects of cocoa flavanols on BP with chocolate consumption compared to the lower energy beverage products. As discussed in detail within this chapter, there appears to be disparity in the literature when identifying an effective dose of flavanols to lower BP when consumed in chocolate compared to a lower fat beverage medium. Based on the acute improvements in vascular function this study sought to determine if there was a sustained effect of cocoa consumption on blood pressure in untreated hypertensives. The rationale for this was the abundance of evidence for antihypertensive effects of HF chocolate in this population. Because of the diversity of reported effective doses with HF

chocolate and the single result with HF powdered drink obtained in the experiment described in Chapter 4 of this thesis, a dose response trial was performed. The most compelling BP reduction with cocoa was in an untreated hypertensive population by Grassi at al where 15 days of HF chocolate reduced 24 hour ABP by approximately 12 mmHg systolic and 8mmHg diastolic [20]. To directly address this question it was necessary to recruit a similar population and assess BP in the same manner (i.e. 24 hour ambulatory monitoring). The results of this study confirmed that the effective dose of cocoa flavanols for BP reduction is somewhere in excess of 702mg and likely to be around 900mg/day. Even at this high dose (double that used by Grassi et al), the reduction seen was far less than those reported by Grassi et al [20].

The first possible explanation for this discrepancy is that the effect reported with chocolate is a false one that is entirely attributable to a placebo effect through lack of adequate treatment blinding. This seems unlikely in the case of the work of Grassi and colleagues [20]. The magnitude of response is higher than would be expected from a placebo, particularly when ambulatory monitoring is used. Asmar and colleages [300] specifically investigated the potential for placebo effect on BP and found significant reductions in both clinic (-5.4 mmHg in mean arterial pressure) and 24 hour ambulatory pressure (-2.5 mmHg in mean arterial pressure) compared to no treatment. The magnitudes seen here were markedly less than those reported by Grassi et al [20], suggesting that the placebo effect is unlikely to entirely explain the effect seen in these studies. The mechanism for placebo effects on non-voluntary physiological processes is not yet known so the potential for this effect to explain these reductions cannot be ruled out.

Another potential explanation is that there is a genuine difference in the physiological effects of the same dose of CF when delivered as chocolate compared to a non-chocolate form. This may be due to a difference in the bioavailability of the flavanols and/or a synergistic action with another ingredient of chocolate on BP. A recent study used an *in vitro* digestion model to compare the bioavailability of flavanols in a 50% fat liquor matrix to a 15% fat powder matrix [301]. They found a significant reduction in the accessible flavanols in the powder compared to the liquor however this needs to be confirmed *in vivo*. There is little evidence to support the possibility of a synergistic action with another nutrient and no suggestions to date what this nutrient may be. A similar situation exists with green tea where the active components are known to be flavanols yet results of supplementation with green tea flavanol extracts consistently show reduced efficacy compared to green tea itself [21, 130].

7.2 Limitations

Further insight into cardiometabolic effects of CF have been obtained by additional blood marker analyses. Samples were obtained to determine acute and chronic blood flavanol concentrations, as well as C reactive protein (CRP), an inflammatory mediator, and adiponectin, which a key adipokine in obesity. Blood flavanol levels could have provided further indications of the bioavailability of the products and basal levels at chronic assessments. Measures of CRP and adiponectin may have provided some further insight into the mechanism of action of the flavanols in reversing the endothelial dysfunction in obesity. While the blood samples obtained were taken and shipped to UC Davis, USA for analysis, the industry partner did not commission these analyses as previously agreed. Having already shipped the samples it was not possible to arrange alternative means of analysis.

For consistency, it would have been preferable to use a single technique to assess arterial stiffness. Unfortunately the HDI profiler was committed to another study during the conduct of the main intervention trial for this body of work. Therefore an alternative device, the Pulsetrace was made available for this study. While both methods assess arterial stiffness/compliance, the two techniques do not show good agreement (r=0.31) [224]. Despite the HDI pulsewave deriving indecies of both small and large arterial elasticity, both tend to be more indicative of smaller resistance vessel stiffness [224]. Comparatively the Pulsetrace „Stiffness Index" is more closely related to direct measures of central compliance such as PWV [224].

7.3 Future direction

There is work still to be done in the extension of these results to a practical intervention for improving cardiometabolic function and risk in overweight or obese individuals. Firstly, the underlying reason for the discrepancy between the apparent effective doses of cocoa flavanols when consumed in chocolate or non-chocolate foods needs to be investigated further. The difficulty with providing a suitable control for comparison with dark chocolate that effectively blinds the research subject from treatment remains a challenge. Until this can be achieved it is difficult to conclusively evaluate the effectiveness of a dark chocolate based intervention. Conducting a dose response trial similar to that presented in chapter 6 whereby various amounts of flavanol rich dark chocolate is combined with flavanol poor chocolate to produce a range of flavanol doses would go some way to address this issue. This would minimise the placebo effect between doses because some degree of blinding would be achieved through the inclusion of some dark (active) chocolate across doses, and based on previous results (i.e. significant reduction in BP with 30mg flavanol/day [206]) all doses are potentially an

effective dose. Comparing these results to those seen here may provide further insight into the dose response curve. Further to this it may be of value to investigate the dose response curve with the non-chocolate product at doses beyond 1052mg/day. It may be that, due to the different digestive effects of non-chocolate cocoa products, a higher dose of flavanols is required when delivered in this manner. The challenge will be to then determine the optimal food matrix to deliver the greatest benefit with the least impact on overall energy balance or saturated fat intake.

The second challenge is to conduct a longer term trial to see whether the beneficial changes in endothelial function can be sustained indefinitely, and furthermore if these changes in fact lead to reduced cardiovascular and metabolic disease endpoints. There is some evidence that the improvements in FMD seen with short term exercise are not sustained long term with maintenance of the exercise habits [260]. This is however, attributed to vascular remodelling to increase the basal diameter such that the same degree of vasodilatation is not warranted [260]. While the likely mechanistic differences between the effects of cocoa supplementation and exercise training mean this type of remodelling is less likely, it is still necessary to investigate the long term response to flavanol supplementation.

7.4 Conclusion

Obesity is significantly associated with suppressed endothelial function and this may play a causal role in the progression from obesity to the pathologies associated with the metabolic syndrome. This highlights the potential for interventions aimed at improving endothelial function to interrupt this process. The consumption of a HF cocoa drink can acutely improve endothelial function both at rest and during exercise in individuals who

are overweight or obese. The continued daily supplementation of HF cocoa leads to a sustained (non-acute) improvement in endothelial function, blood pressure and insulin sensitivity, with or without concurrent exercise training. Three days per week of exercise training can reduce the percentage of fat tissue in the abdominal region with or without the cocoa supplementation. Furthermore, a dose of 1052mg can significant lower BP in untreated borderline to mildly hypertensive individuals. Collectively, these results provide a compelling case for the consideration of HF cocoa supplementation in the management of obesity and/or hypertension, however further work is warranted to establish the optimal food matrix to deliver it.

Appendix 1

List of tables and figures

Appendix 2

List of abbreviations

ABP – Ambulatory Blood Pressure

ABPM – Ambulatory Blood Pressure Monitoring

AC – Arterial Compliance

ADMA – Asymmetrical dimethyl-arginine

ADP – Adenosine diphosphate

AF – Arterial Function

ANOVA – Analysis of Variance

ANCOVA – Analysis of Covariance

ATPIII - Adult Treatment Panel III

BMI – Body Mass Index

BP – Blood Pressure

CF – Cocoa Flavanols

cGMP – Cyclic guanosine monophosphate

CRF – Cardiorespiratory fitness

CV – Cardiovascular

CVD – Cardiovascular Disease

DBP – Diastolic Blood Pressure

DEXA - Dual energy X-ray absorptiometry

DVP – Digital volume pulse

EDHF – Endothelium derived hyperpolarising factor

eNOS – Endothelial nitric oxide synthase

FG – Fasting glucose

FFM – Fat free mass

FMD – Flow-mediated Dilatation

HF – High flavanol

HR – Heart Rate

HOMA – Homeostasis model of Insulin Assessment

HOMA2 – Homeostasis model of Insulin Assessment (2nd Version)

HOMA2 (IR) – Homeostasis model of Insulin Assessment (2nd Version) – Insulin resistance

HOMA2 (%B) – Homeostasis model of Insulin Assessment (2nd Version) – Beta cell function

HOMA2 (%S) – Homeostasis model of Insulin Assessment (2nd Version) - Sensitivity

ICAM-1 – Intracellular adhesion molecule-1

IDF – International Diabetes Federation

IL-6 – Interleukin 6

LAEI – Large Artery Elasticity Index

LDL – Low density lipoprotein

LF – Low flavanol

MAP – Mean Arterial Pressure

MAPK – Mitogen-activated protein kinase

MES-WAT – Mesenteric white adipose tissue

MetS – Metabolic Syndrome

MPO – Myeloperoxidases

NO – Nitric oxide

PI3K - Phosphoinositide 3-kinases

PWA – Pulse wave analysis

ROS – Reactive oxygen species

SAEI – Small Artery Elasticity Index

SBP – Systolic Blood Pressure

SEM – Standard error of the mean

SI – Stiffness Index

T2D – Type 2 Diabetes

TAG – Triacylglycerol

TChol – Total cholesterol

TNF-α – Tumor necrosis factor alpha

VCAM-1 – Vascular cell adhesion molecule-1

$\dot{V}O_2$ max – Maximal oxygen consumption

$\dot{V}O_2$max (R-TM) – Predicted maximal oxygen uptake (relative to total mass)

$\dot{V}O_2$max (R-FFM) - Predicted maximal oxygen uptake (relative to fat-free mass)

WC – Waist Circumference

WHO – World Health Organisation

%ABF – Percentage of fat as mass of abdominal region of interest

%BF – Percentage of fat as total mass

%FMD – Percentage flow mediated dilatation

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