The Effect of High-Fat Meals and Exercise on Endothelial Function and Triacylglycerol Concentrations in Adolescent Boys

By

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Abstract

The thesis investigated the effect of exercise on endothelial function (measured as flow-mediated dilation (FMD)) and triacylglycerol concentrations following the ingestion of a high-fat breakfast and lunch in adolescent boys. The validity of measuring lipid and lipoprotein concentrations from a capillary blood sample, and the reproducibility of the postprandial FMD and triacylglycerol concentration responses to the high-fat meals, was established. The effects of prior continuous moderate-intensity exercise (60 min walking at 60% $\dot{V}O_2$peak), repeated very short duration sprints (40 x 6 s maximal effort cycle sprints) and accumulated moderate-intensity exercise (6 x 10 min running at 70% $\dot{V}O_2$peak) on endothelial function and triacylglycerol concentrations in adolescent boys were then established across three studies, each consisting of two, 2-day main trials (control and exercise). On day 1, participants were either inactive or completed the prescribed exercise. On day 2, FMD and triacylglycerol concentrations were measured prior to, and following, ingestion of a high-fat breakfast and lunch. In each control trial FMD was reduced (signifying endothelial dysfunction), compared to fasting, by 20-32% and 24-33% following the high-fat breakfast and lunch. Following continuous moderate-intensity exercise, repeated very-short duration sprints and accumulated moderate-intensity exercise these reductions were only 8% and 10% (main effect trial, $P = 0.002$; main effect time, $P = 0.023$; interaction effect trial x time, $P = 0.088$), 2% and 5% (main effect trial, $P = 0.012$; main effect time, $P = 0.004$; interaction effect trial x time, $P = 0.003$) and 1% and 3% (main effect trial, $P = 0.020$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.014$) respectively. The continuous moderate-intensity exercise and repeated very short duration sprints also significantly reduced the total area under the triacylglycerol concentration versus time curve by 22% (Control vs. Exercise; 12.68 (sem 1.37) vs. 9.84 (sem 0.75) $mmol \cdot L^{-1} \cdot 6.5h$, $P = 0.018$) and 13% (Exercise vs. Control: 8.65 (sem 0.97) vs. 9.92 (sem 1.16) $mmol \cdot L^{-1} \cdot 6.5h$, $P = 0.023$). The accumulated moderate-intensity exercise also reduced the total area under the triacylglycerol concentration versus time curve by 11%, but this reduction was not significant (Control vs. Exercise: 10.71 (sem 0.94) vs. 9.56 (sem 0.67) $mmol \cdot L^{-1} \cdot 6.5h$, respectively, $P = 0.183$). The experimental evidence from these studies emphasise that exercise might offer an acceptable, non-pharmacological means of influencing CHD risk when individuals are young. The results of these studies can help shape future physical activity guidelines.

Key Words

Flow-mediated dilation
Postprandial lipaemia
Coronary heart disease risk
Young people
Sprint exercise
Lipids
Lipoproteins
Accumulated exercise
Physical activity

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Unless otherwise indicated by acknowledgement or reference to published literature the work contained herein is that of the author and has not previously been submitted for another degree to this or any other university.

The findings of some of the studies contained in this thesis have been published.

Published papers

Sedgwick MJ, Morris JG, Nevill ME, Tolfrey K, Nevill A and Barrett LA (2013). Effect of exercise on postprandial endothelial function in adolescent boys. *British Journal of Nutrition.* **110**, 301-9.

Sedgwick MJ, Morris JG, Nevill ME and Barrett LA (In Press). The accumulation of exercise and postprandial endothelial function in boys. *Scandinavian Journal Medicine and Science in Sports.*

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Sedgwick MJ, Morris JG, Nevill ME and Barrett LA (2012). Accumulating short bouts of exercise negates the postprandial impairment of endothelial function in adolescent boys. Med Sci Sports Ex, 44, s884.

Table of contents

List of tables

List of figures

Figure **Page**

Figure 8.2. Plasma (a) triacylglycerol (b) glucose (c) insulin concentrations. B, breakfast; L, lunch. Values are means (sem) (n 14). Data were analysed using a mixed-effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay; triacylglycerol data were Ln transformed prior to the analysis. (a) Triacylglycerol: main effect trial, $P = 0.385$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.091$. (b) Glucose: main effect trial, $P = 0.916$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.066$. (c) Insulin: main effect trial, $P = 0.435$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.515$.

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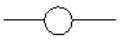
Control  Exercise 

Chapter 1: Introduction

Cardiovascular disease (a group of heart and blood vessel disorders) is the leading cause of mortality in western societies, accounting for 200,000 (31%) deaths per year in the United Kingdom (Townsend et al., 2012). Coronary heart disease (CHD) is the most prevalent of these cardiovascular diseases and accounts for 17% and 12% of all deaths of males and females in the United Kingdom (Townsend et al., 2012). Furthermore, in the United Kingdom 17% and 8% of premature mortality (death before the age of 75 years) in males and females was a result of CHD (Townsend et al., 2012). Although the mortality rate associated with CHD has decreased in western societies over the past 30 years, the morbidity rate has increased over this time (Townsend et al., 2012). Current data suggests that 6.5% of males and 4.0% of females in the United Kingdom suffer from CHD (Health Survey for England, 2006). These figures may seem low, but the prevalence of CHD progressively increases with age (Figure 1.1) with 29% of males and 19% of females suffering from CHD when aged 75 years or older (Health Surveys for England, 2006). Along with being a major cause of mortality and morbidity in western societies CHD also has substantial economic implications, with the annual economic cost in the United Kingdom in 2009 estimated to be £6.7 billion; £1.8 billion covering healthcare costs and an additional £4.9 billion as a result of production losses due to death and illness in those of working age and due to the informal care of people with the disease (Townsend et al., 2012). Therefore, interventions that can reduce CHD could have significant benefits for both the health of the population and for the economy.

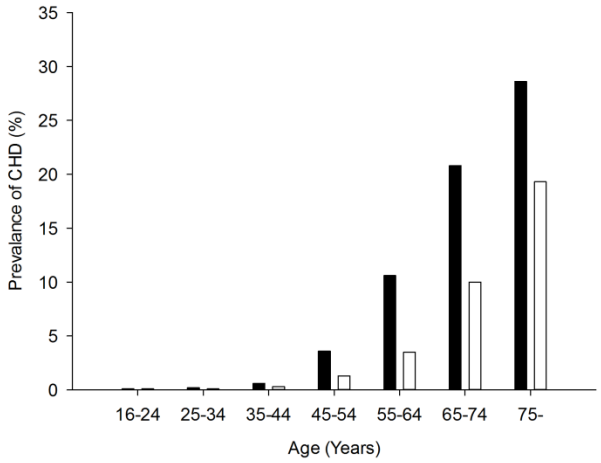


Figure 1.1. Prevalence of coronary heart disease (CHD) in the United Kingdom for males (filled bars) and females (non-filled bars) (Health Survey for England, 2006).

A physically active lifestyle was identified as cardio-protective in a landmark study conducted by Morris and colleagues (1953). Morris and colleagues (1953) observed a higher incidence of CHD in London bus drivers (assumed to have low physical activity level) than the bus conductors (assumed to have high physical activity levels). The concept of physical activity reducing CHD risk was further substantiated by Paffenbarger and colleagues when they observed a dose-dependent relationship between physical activity levels and CHD risk where those with high physical activity levels had a lower CHD risk than those with low physical activity levels, who in turn had lower CHD risk than the sedentary (Paffenbarger et al., 1970; Paffenbarger & Hale, 1975; Paffenbarger et al., 1978; Brand et al., 1979). Since these seminal studies, multiple studies of a comparable design have reported similar observations and when the results from these studies are considered as a whole, physical activity levels appear to be inversely and causally related to the incidence of CHD (Blair et al., 2001).

Although CHD typically occurs late in life (Figure 1.1), it is the clinical manifestation of atherosclerosis (Solberg & Strong, 1983), a chronic progressive disease that leads to the thickening and narrowing of artery walls. Autopsy examinations have ascertained that atherosclerosis has its origins in childhood, as a result of fatty streaks (lipid depositions under the intima) which are the initial atherosclerotic lesions being found in the arteries of children and adolescents (Newman et al., 1991; Pathobiological Determinants of Atherosclerosis Research Group, 1990; Berenson et al., 1998). Histological evidence indicates that not all of these fatty streaks will develop into raised lesions but that over time they can form, dissolve and reform (Stary, 2000). Over time these fatty streaks can thicken, by accumulating lipids and connective tissue, and develop into advanced atherosclerotic lesions before undergoing calcification and vascularisation (Figure 1.2; McGill et al., 2000). It is these complicated, advanced, atherosclerotic lesions that occlude arteries, reduce blood supply to cardiac muscle and ultimately result in the clinical manifestation of CHD.

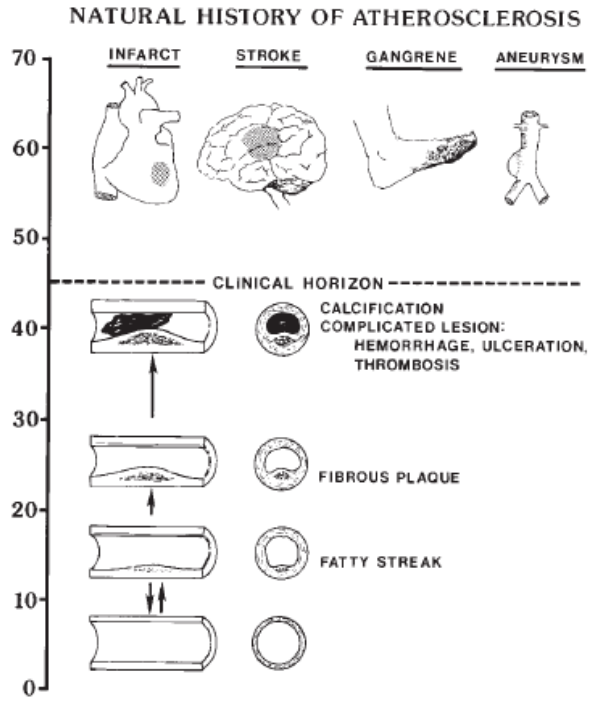


Figure 1.2. The progression of atherosclerosis (depicted as a reversible process) to a clinical horizon where symptoms of atherosclerosis become evident (reproduced from McGill et al., 2000).

As atherosclerosis which leads to CHD has its origins in childhood, CHD should be considered a "major paediatric problem" (Berenson et al., 1987). An understanding of the factors which augment or attenuate the development of the disease, even in the young, could therefore have important clinical implications, particularly when it has been argued that the greatest benefit from a treatment occurs the earlier it is initiated (Magnussen et al., 2012). In adults many of the interventions used to combat CHD involve pharmacological therapies which, while often effective, can be costly and have numerous side effects, especially if undertaken for a prolonged period (Steinberg, 2010). Furthermore, the safety and efficacy of many pharmacological treatments has not been established in young people. Physical activity might offer an acceptable, effective and safe non-pharmacological means of influencing CHD risk in the young (children and adolescents).

Despite clear evidence that physical activity undertaken during adulthood can reduce CHD risk, the evidence available that physical activity undertaken during childhood and adolescence can reduce CHD risk is not as compelling. This is due to a paucity of research as a consequence of the logistical issues involved in conducting a study to establish a direct relationship between physical activity in the young with an outcome (CHD) that

occurs over 40 years in the future (Rowland, 2009). For this reason, research designed to establish the relationship between physical activity and CHD in the young has focused on investigating the effect of physical activity on CHD risk factors.

Epidemiological studies have identified a number of factors, in addition to physical (in)activity, that may increase an individual's risk of CHD. Some of these CHD risk factors, such as age, sex and genetics, are fixed and non-modifiable. Fortunately many risk factors for CHD can be modified. An adult that smokes and has abnormal blood lipid concentrations, hypertension, diabetes, abdominal obesity, low physical activity levels, low daily fruit and vegetable consumption, alcohol overconsumption, and poor psychosocial index is 90% more likely to suffer from CHD (Yusuf et al., 2004). These are all risk factors that can be modified. As CHD is the endpoint of atherosclerosis, risk factors increase the risk of CHD by speeding up the development and / or progression of atherosclerosis. This theory is supported by both the "Bogalusa Heart Study" (Berenson et al., 1992; McGill et al., 2000) and the "Pathobiological Determinants of Atherosclerosis in Youth Study" (Wissler, 1991) where the number and severity of CHD risk factors were directly related to the percentage of the coronary artery surface occupied by "fatty streaks" and "fibrous plaques".

Although physical activity has been identified as an independent CHD risk factor, it is believed that the benefits of being physically active are due to its modifying effects on other risk factors, with a physically active lifestyle being associated with a more beneficial lipid profile (Durstine & Haskell, 1994; Leon & Sanchez, 2001), lower blood pressure (Arroll & Beaglehole, 1992; Whelton et al., 2002) and improved glycaemic control (Lindgarde & Saltin, 1981; Mayer-Davis et al., 1998; Kelley & Goodpaster, 1999). However, only 36% of this lowering in risk can be explained by changes in traditional CHD risk factors (Mora et al., 2007) with other unknown factors explaining the remainder of the lowering in risk. The link between traditional CHD risk factors and physical activity in the young, unlike in adults, is not robust often suggesting a minimal effect or no effect of physical activity (Despres et al., 1990; Rowland, 2009). Therefore, future research in young people should consider the effect of physical activity on measures that may better represent the total daily exposure to a risk factor or risk factors that have a strong theoretical link to atherosclerotic development and / or progression.

CHD risk factors have traditionally been assessed in the fasted state; however, some researchers hypothesise that atherosclerosis is a postprandial phenomenon (Zilversmit, 1979). It has been argued that the postprandial (fed) state, not the traditionally assessed fasting state, may better represent an individual's "normal" physiological state in western societies (Gill & Hardman, 2003), as multiple meals are consumed each day resulting in the majority of the waking day being spent in a postprandial state (Williams, 1997). In addition, a number of CHD risk factors are either directly involved in metabolism e.g. lipids and lipoproteins, glucose and insulin or related to metabolism e.g. inflammation and oxidative stress. The measurement of these factors in the fasting state fails to reflect the dynamic metabolic state experienced on a daily basis. Therefore, postprandial measures of CHD risk factors may be more revealing indicators of an individual's CHD risk.

The endothelium is a single layer of cells that lines the interior surface of blood vessels. The endothelium was initially thought to be an inert barrier that simply separated the vascular walls from the circulating blood. However, in recent years the endothelium has been identified as an active tissue with autocrine, endocrine and paracrine properties (Bonetti et al., 2003) implicated in the maintenance of vascular homeostasis through a variety of functions including the regulation of vascular tone, cellular adhesions, thrombogenesis, smooth muscle cell proliferation and inflammation (Celermajer, 1997). Many of the actions of a healthy endothelium (normal endothelial function) could be considered to be anti-atherogenic i.e. protect against the development and progression of atherosclerosis (Table 1.1). A state of endothelial dysfunction is present when the endothelium can't maintain vascular homeostasis and adopts a phenotype that facilitates inflammation, thrombosis, vasoconstriction and atherosclerotic lesion formation. Endothelial dysfunction has been identified as an independent CHD risk factor (Yeboah et al., 2009; Inaba et al., 2010).

A state of endothelial dysfunction has been observed in adults following the ingestion of high-fat mixed meals (Vogel et al., 1997; Marchesi et al., 2000; Tushuizen et al., 2006; Wallace et al., 2010). Along with endothelial dysfunction being an independent CHD risk factor, it has been implicated in the early stages of atherogenesis (Ross, 1986) with recent data suggesting that even in the presence of traditional CHD risk factors atherosclerotic development and progression will only occur when endothelial dysfunction is also present

(Juonala et al., 2004). This fact makes endothelial (dys)function a particularly important measure of CHD risk in the young. When combined with the fact that the young commonly consume high-fat meals, interventions that attenuate or prevent postprandial endothelial dysfunction occurring in the young could have important clinical benefits. To date no one has investigated the effect of the ingestion of high-fat mixed meals containing substantial quantities of fat on endothelial function in the young.

Table 1.1. Actions of a healthy endothelium (Bonetti et al., 2003)

Normal endothelial function
Promotes vasodilatation
Inhibits leukocyte adhesion and migration
Inhibits smooth muscle cell proliferation and migration
Inhibits platelet aggregation and adhesion
Anti-inflammatory effects
Anticoagulant effects
Profibrinolytic effects

The magnitude of postprandial endothelial dysfunction has been shown to be directly associated with postprandial triacylglycerol concentrations (Vogel et al., 1997; Marchesi et al., 2000; Wallace et al., 2010). High postprandial triacylglycerol concentrations have also been identified as an independent CHD risk factor in adults (Bansal et al., 2007; Nordestgaard et al., 2007) and importantly triacylglycerol concentrations in the young have been reported to predict future CHD events (Morrisson et al., 2009). Interventions that lower postprandial triacylglycerol concentrations may directly lower CHD risk but also indirectly lower risk by attenuating postprandial endothelial dysfunction, as postprandial endothelial dysfunction and triacylglycerol concentration have been found to be associated.

Physical activity has been shown to lower postprandial triacylglycerol concentrations in adults (Sady et al., 1988; Cohen et al., 1989; Merrill et al., 1989; Hartung et al., 1993; Podl et al., 1994; Miyashita et al., 2011) with this effect found to be acute and related to the last bout of activity (Hardman et al., 1998; Herd et al., 1998). The majority of research conducted investigating the effect of physical activity on postprandial endothelial function

and triacylglycerol concentrations has focused on the effect of a prior, single bout of continuous moderate-intensity exercise. It has been shown that a prior bout of continuous moderate-intensity exercise can lower postprandial triacylglycerol concentrations in both adults (Gill & Hardman, 2003; Petitt and Cureton, 2003) and adolescents (Barrett et al., 2007; Tolfrey et al., 2008; MacEneaney et al., 2009; Tolfrey et al., 2012a) and attenuate postprandial endothelial dysfunction in adults (Gill et al., 2004; Tyldum et al., 2009). The effect of exercise on postprandial endothelial function in the young has yet to be investigated.

It is important to establish the effect of other modalities and patterns of exercise on CHD risk as exercise modality preference is individual, with some preferring the typically recommended continuous moderate intensity exercise whilst others preferring low-intensity, high-intensity intermittent, sprint or resistance exercise. It may be particularly appealing and practical to establish the effect of exercise patterns which could be structured around daily commitments (e.g. work or school). In addition to continuous moderate-intensity exercise, accumulated short-duration moderate-intensity exercise (Gill et al., 1998; Miyashita et al., 2006; Miyashita, 2008; Miyashita et al., 2008), high-intensity intermittent exercise (Barrett et al., 2006; Freese et al., 2011; Gabriel et al., 2012) and resistance exercise (Petitt et al., 2003; Burns et al., 2007; Zafeiridis et al., 2007; Pafili et al., 2009; Singhal et al., 2009) have been shown to lower postprandial triacylglycerol concentrations in adults; however, similar research in the young is limited to continuous moderate-intensity exercise (Barrett et al., 2007; Tolfrey et al., 2008; MacEneaney et al., 2009; Tolfrey et al., 2012a; Tolfrey et al., 2012b) and high-intensity intermittent exercise (Barrett et al., 2007). Although research is available establishing the effect of different modes and patterns of exercise on postprandial triacylglycerol concentrations in adults, it should not be assumed that the young will show similar responses to this exercise; because, the young are known to experience different metabolic and hormonal responses to exercise than adults (Riddell, 2008). Therefore it is important to investigate the effect of different exercise modes and patterns in the young.

Therefore the primary purpose of the studies conducted in this thesis was to investigate the effect of different modes of exercise on postprandial endothelial function and triacylglycerol concentrations in adolescents. The exercise modes employed in the

chapters of this thesis were selected to represent the traditional mode of exercise recommended for good health (continuous moderate-intensity exercise), an exercise mode that may be attractive and enjoyable to the young (repeated sprint exercise) and an exercise pattern recommended in current physical activity guidelines which could be structured around the school day (accumulating bouts of moderate-intensity exercise). All studies were conducted in adolescent boys as additional female supervision, required if adolescent girls were used, could not be guaranteed for every visit. It was hypothesised that the ingestion of high-fat mixed meals would induce a state of endothelial dysfunction, proportional to the increase in triacylglycerol concentrations, in the adolescent boys. It was also hypothesised that each mode and pattern of exercise would attenuate this high-fat meal induced endothelial dysfunction by lowering postprandial triacylglycerol concentrations.

The remainder of this thesis is presented in a further 8 main chapters:

- Chapter 2 presents a review of the literature which provides an overview of the role of endothelial function and triacylglycerol concentrations in the development of atherosclerosis and CHD, examines the effect of exercise on postprandial triacylglycerol concentrations, the effect of food (primarily high-fat meals) ingestion and exercise on endothelial function.
- Chapter 3 presents the general procedures, equipment and methods of analysis used during the experimental studies described in this thesis.
- Chapter 4 presents a study establishing the validity of using capillary blood samples to determine lipid and lipoprotein concentrations.
- Chapter 5 presents a study establishing the reproducibility of endothelial function and triacylglycerol concentration measurements following the consumption of high-fat meals.
- Chapter 6 presents a study investigating the effect of continuous moderate-intensity exercise on postprandial endothelial function and triacylglycerol concentrations in adolescent boys.
- Chapter 7 presents a study investigating the effect of repeated very short duration sprints on postprandial endothelial function and triacylglycerol concentrations in adolescent boys.

- Chapter 8 presents a study investigating the effect of accumulating short-duration bouts of moderate-intensity exercise on postprandial endothelial function and triacylglycerol concentrations in adolescent boys.
- Chapter 9 amalgamates the findings of the studies conducted as part of this thesis; discussing the implications of the findings, identifying the limitation of the studies and providing future directions of research.

Chapter 2: Review of literature

2.1 Introduction

This chapter aims to establish the scientific rationale for the experimental studies described later in this thesis. This literature review first establishes the role of endothelial function and triacylglycerol concentrations in the development of atherosclerosis and CHD, then examines the effect of food consumption (particularly high fat meals) on endothelial function and explores the possible mechanisms associated with these effects. The next section examines the effect of exercise (particularly acute exercise) on postprandial triacylglycerol concentrations and explores possible mechanisms associated with these effects. The final section examines the effect of exercise on postprandial endothelial function and explores possible mechanisms associated with these effects.

2.2 Endothelial function, atherosclerosis and coronary heart disease

A role of the endothelium in atherosclerosis and CHD was postulated in the "response to injury" hypothesis which proposed endothelial denudation to be the first stage in atherogenesis (Ross and Glomset, 1973). This "response to injury" hypothesis has since been updated with endothelial dysfunction replacing endothelial denudation (Ross, 1986). Support for this hypothesis has come from the Cardiovascular Risk in Young Finns Study where carotid intima media thickness (a non-invasive measure of atherosclerosis) was larger in adults with endothelial dysfunction and the presence of other CHD risk factors compared to adults with normal endothelial function and the presence of other CHD risk factors (Juonala et al., 2004). No differences in carotid intima media thickness were apparent between adults with normal endothelial function and the presence of other CHD risk factors, adults with normal endothelial function and no other CHD risk factors, and adults with endothelial dysfunction and no other CHD risk factors. This observation suggests that endothelial dysfunction is implicit in, and may even be a pre-requisite for, the development and / or progression of atherosclerosis. In addition endothelial dysfunction has been identified as an independent risk factor for CHD in a number of prospective studies (Yeboah et al., 2009; Inaba et al., 2010).

The actions of the endothelium are regulated by a multitude of substances released from the endothelial cells (Celermajer, 1997; Widlansky et al., 2003). One such substance, endothelium-derived relaxing factor, was identified by Furchgott and Zawadzki (1980) in a landmark study where isolated aortic rings were exposed to acetylcholine. When the endothelium was intact the aortic rings dilated, but they constricted when the endothelium was denudated. This endothelium-derived relaxing factor has since been identified as nitric oxide due to its identical biological activity, stability, and susceptibility to an inhibitor and potentiator (Palmer et al. 1987).

The majority of measures of endothelial function are based on the measurement of endothelial-dependent dilation, where the dilatory response to a mechanical (blood flow) or pharmacological (acetylcholine) stimulus, known to result in endothelial nitric oxide production, is quantified and taken to represent endothelial function (for an overview of the different techniques used to measure endothelial function see: Alam et al., 2005; Kasprzak et al., 2006, Hogas et al., 2010; McCall et al., 2011; Roustit & Cracowski, 2012).

Quantitative coronary angiography following intra-arterial infusion of an endothethelial-dependent dilator (e.g. acetylcholine or bradykanin) is considered to be the clinical gold standard for the assessment of endothelial function in coronary arteries. However, the invasive nature of this proceedure caries a strong risk of myocardial infarction and therefore should not be performed unless coronary angiography becomes clinically necessary. The dilatory response to infused acetylcholine in peripheral arteries (most commonly infused through the brachial artery) has also been studied by measuring changes in blood flow (typically the forearm) using strain gauge plethysmography. Although acetylcholine infusion is through a peripheral conduit artery it exerts its effect on downstream vessels and is consequently a measure of resistance vessel or microvascular fuction. This technique is also invasive as it involves the catheterisation of arteries for the acetylcholine infusion, and therefore may not be suitable for use in healthy and / or young individuals. An alternative, less invasive, approach to pharmacological induced dilation of resistance vessels involves transdermal acetylcholine delivery by iontophoresis, with laser Doppler imaging or flowmetry being used to quantify the changes in skin blood flow. This technique has been shown to be reliable providing the measurement site is standardised (Kubli et al., 2000); however, the applied current may confound the measurement as it can

independently induce vasodilatation (Durand et al., 2002). Each of these techniques are thought to be mediated by the respective stimulus increasing endothelial nitric oxide production; however, N^G-monomethyl-$_L$-arginine, an inhibitor of nitric oxide production, does not fully prevent the increases in blood flow suggesting factors other than nitric oxide also mediate the vasodilation in resistance vessels (Shimokawa et al., 1996; Newby et al., 1997).

In recent years flow-mediated dilation (FMD) has been the most widely used method to quantify endothelial function. Flow-mediated dilation is a non-invasive measure of conduit artery endothelial function, making it particularly convenient for use in young and healthy populations. The technique was first utilised by Celermajer and colleagues (1992) and many guidelines have since been published clarifying and standardising the measurement (Corretti et al., 2002; Pyke & Tschakovsky, 2005; Harris et al., 2010; Thijssen et al., 2011). Flow-mediated dilation is based on the artery dilating in response to an increase in shear stress, induced by a period of ischemia; with larger post-occlusion dilation representing greater endothelial function. Flow-mediated dilation is traditionally reported as a percentage of dilation relative to the basal, pre-occlusion diameter. As the dilation is dependent on the post-occlusion shear rates, approaches have also been taken to normalise FMD to this initiating shear rate stimulus (Pyke & Tschakovsky, 2005; Pyke & Tschakovsky, 2007; Harris et al., 2010; Thijssen et al., 2011). Unlike the techniques used in determining resistance vessel endothelial function, FMD is dependent on the experiance of the researcher which requires at least six months of training before implementing this technique (Corretti et al., 2002). Importantly, the post-occlusion dilation has been shown to be almost entirely dependent on nitric oxide, as no dilation was evident when N^G-monomethyl-$_L$-arginine was infused (Joannides et al., 1995; Leeson et al., 1997; Doshi et al., 2001). Ideally endothelial function of the coronary arteries, not peripheral conduit arteries, would be assessed because atherosclerotic lesions do not typically manifest in peripheral conduit arteries. However, significant associations have been reported between brachial artery FMD and endothelial function of coronary vessels measured using quantitative coronary angiography (Anderson et al., 1995). Therefore, brachial artery FMD can be considered to represent whole body, and importantly endothelial function in the coronary arteries.

With endothelial (dys)function implicated in the development and / or progression of atherosclerosis and given that many of the measures of endothelial function are surrogate markers of nitric oxide bioavailability, it seems that nitric oxide is anti-atherosclerotic. No data supporting the anti-atherosclerotic properties of nitric oxide appear to be available in humans. However, in a study conducted by Cayette and colleagues (1994) where rabbits were fed a high cholesterol diet, lower endothelium-dependent dilation and a greater neointimal lesion area were observed following five weeks of endothelial nitric oxide synthase inhibition (preventing the formation of nitric oxide from endothelial cells), compared to those who did not receive the endothelial nitric oxide synthase inhibition. One interesting finding from this study was that rabbits fed a normal diet and who received the nitric oxide synthase inhibitors showed no increase in neointimal lesion area, suggesting that nitric oxide synthase inhibition, and by implication a low bioavailability of nitric oxide, only accelerates atherosclerosis in the presence of an atherogenic diet.

In adults endothelial dysfunction has been shown to be associated with many traditional and novel CHD risk factors, including: body composition, lipid and lipoprotein concentrations, glucose tolerance and insulin sensitivity, physical activity levels, diet and smoking habits (for review see Glasser et al., 1996; Bonetti et al., 2003; Grover-Paez & Zavalza-Gomez, 2009). It is not clear if endothelial dysfunction clusters with CHD risk factors or if these risk factors induce endothelial dysfunction with Bonetti and colleagues (2003) referring to endothelial dysfunction as the ultimate risk of risk factors. Endothelial dysfunction is not limited to adults and has been observed in the young. The young were first shown to exhibit endothelial dysfunction in a study conducted by Celermajer and colleagues (1992) when they observed that children (aged 8 to 16 years) with familial hypercholesterolemia demonstrated lower FMD than their healthy peers (0% vs. 9% respectively). A plethora of additional studies have since observed endothelial dysfunction in young people who exhibit other CHD risk factors including; obesity (Watts et al., 2004a; Watts et al., 2004b; Meyer et al., 2006a; Meyer et al., 2006b; Akinci et al., 2008; Hopkins et al., 2009), low physical activity (Abbott et al., 2002; Hopkins et al., 2009; Pahkala et al., 2009), low physical fitness (Meyer et al., 2009b), elevated C-reactive protein (Jarvisalo et al., 2002), diabetes and poor glucose handling (Khan et al., 2003; Jarvisalo et al., 2004; Pena et al., 2006) and hypertension (Meyer et al., 2009b). Importantly research has shown that this endothelial dysfunction is not permanent and can be attenuated by strategies

known to reduce CHD risk (Vita & Keaney, 2000; Widlansky et al., 2003; Gielen & Hambrecht, 2004) such as diet (Stein et al., 1999; Goodfellow et al., 2000; Raitakari et al., 2004), pharmacological agents (Leung et al., 1993; Prasad et al., 2001) and physical activity (Green et al., 2003; Hambrecht et al., 2003; Watts et al., 2004a; Watts et al., 2004b),.

In summary, endothelial dysfunction is an independent CHD risk factor and it has been hypothesised to be implicit in the early stages of atherosclerosis. In fact experimental evidence in both humans and animals suggests that endothelial dysfunction is a prerequisite for the development and / or progression of atherosclerosis, making it an important risk factor to monitor and manage from a young age.

2.3 Triacylglycerol concentrations, atherosclerosis and coronary heart disease

A link between high fasting triacylglycerol concentration and CHD events was first established in the 1950s (Gofman et al., 1953). Since this initial study, an association between high fasting triacylglycerol concentration and CHD events has repeatedly been observed in both case-control and prospective studies (for review see Austin et al., 1991; Hokanson & Austin, 1996; Austin et al., 1998). However, the association between fasting triacylglycerol concentration and CHD events is weakened or removed in multivariate analysis including high-density lipoprotein (HDL) cholesterol concentrations (Austin, 1991; Hokanson and Austin, 1996), as a result fasting triacylglycerol concentration is not classified as an independent CHD risk factor (Hulley et al., 1980).

This apparently weak association between fasting triacylglycerol concentration and CHD is somewhat surprising, given that HDL-cholesterol concentration demonstrates such a strong relationship with CHD (Griffin, 1999), and that the later concentration is chiefly determined by the efficiency of triacylglycerol-rich lipoprotein metabolism. The weak association may be a consequence of the substantial biological variation observed in fasting triacylglycerol concentration (Durrington, 1990; Tolfrey et al., 1999) as this would reduce its predictive power in multivariate analysis (Durrington, 1998). In addition, fasting triacylglycerol concentrations may not be a true reflection of the stressed, dynamic metabolic state experienced during a normal day. Thus, postprandial triacylglycerol

concentrations may be a more accurate, sensitive and revealing indicator of an individual's triacylglycerol-rich lipoprotein metabolism efficiency. This is supported by the observation that individuals who have similar fasting triacylglycerol concentrations can have very different postprandial triacylglycerol concentrations (Patsch et al., 1983; Schrezenmeir et al., 1992; Lopez-Miranda et al., 2003; Corella & Ordovas, 2005) indicating that postprandial triacylglycerol concentrations are not simply a reflection of fasting triacylglycerol concentrations.

Postprandial triacylglycerol concentrations have been associated with CHD; however, unlike with fasting triacylglycerol concentrations this association remains following multivariate analysis (Patsch et al., 1992; Stensvold et al., 1993; Stampfer et al., 1996; Nordestgaard et al., 2007; Bansal et al., 2007; Langsted et al., 2011; Nordestgaard & Freiberg, 2011). In fact, Patsch and colleagues (1992) reported a stronger association between postprandial triacylglycerol concentrations and CHD than HDL-cholesterol in a case-control study and Stampfer and colleagues (1996) identified that the inclusion of postprandial triacylglycerol concentration in multivariate analysis resulted in the elimination of HDL-cholesterol as an independent CHD risk factor in a prospective study. Therefore unlike fasting triacylglycerol concentration, postprandial triacylglycerol concentrations appear to be an independent CHD risk factor. Importantly when considering the need to monitor and manage CHD risk factors from a young age, postprandial triacylglycerol concentrations early in life have been shown to predict future CHD (Morrison et al., 2009).

Differences in postprandial triacylglycerol concentrations have been observed in the young in the presence of other CHD risk factors (Kolovou et al., 2011) indicating that exaggerated postprandial triacylglycerol concentrations can exist in the young. Higher postprandial triacylglycerol concentrations have been reported in adolescents with type 2 diabetes (Umpaichitra et al., 2004), children with familial hypercholesterolemia (Reiber et al., 2003) and central obesity (Moreno et al., 2001) compared to healthy asymptomatic control participants. Further to this, Couch and colleagues (2000) reported postprandial triacylglycerol concentrations to be directly correlated to fasting triacylglycerol concentrations and indirectly correlated to high-density lipoprotein cholesterol

concentrations. The authors concluded that the predictors of postprandial triacylglycerol concentrations may be similar in the young and adults.

The mechanisms through which the metabolism of triacylglycerol-rich lipoproteins, and consequently postprandial triacylglycerol concentrations, are implicated in the pathogenesis of atherosclerosis have yet to be fully elucidated. Zilversmit (1979) hypothesised that atherogenesis was a postprandial phenomenon, where remnant lipoproteins would penetrate the arterial wall and the cholesterol laden particles then contribute to the formation of atherosclerotic lesions. This hypothesis has received some support with studies observing chylomicron remnant accumulation in the intima of individuals with lipid disorders and normolimidemic individuals with coronary artery disease (Mamo et al., 1998). Apolipoprotein E containing lipoproteins have similarly been found in the human aortic intima (Yla-Herttuala et al., 1988). The metabolism of triacylglycerol-rich lipoproteins also directly affects the concentration and characteristics of all lipoproteins (for overview see Griffin & Zampelas, 1995; Ebenbichler et al., 1995; Frayn, 2010). Specifically, an inefficient metabolism of triacylglycerol-rich lipoproteins, evidenced by high postprandial triacylglycerol concentrations, results in low HDL-cholesterol concentrations and a preponderance of small, dense low-density lipoproteins (LDL) (Griffin & Zampelas, 1995; Austin & Edwards, 1996). This phenotype is associated with an increased risk of CHD (Austin et al., 1990; Griffin & Zampelas, 1995; Superko, 1996) and is commonly referred to as the "atherogenic lipoprotein phenotype".

In summary, postprandial triacylglycerol concentration, unlike fasting triacylglycerol concentration, is an independent risk factor for CHD and has been linked to the development of atherosclerosis. Therefore, it seems pertinent that postprandial triacylglycerol concentrations be kept low to delay, slow or prevent the development of atherosclerosis and ultimately CHD. With evidence that childhood and adolescent triacylglycerol concentrations predict later CHD, the monitoring and management of triacylglycerol concentrations should start early in life.

2.4 Exercise and postprandial triacylglycerol concentrations

Initial evidence that physical activity and exercise can have a favourable effect on postprandial triacylglycerol concentrations comes from a series of cross-sectional studies

that contrasted sedentary adults with endurance-trained adults. These cross-sectional studies observed that the endurance-trained adults experience lower triacylglycerol concentrations following the consumption of a high-fat mixed meal (Cohen et al., 1989; Merrill et al., 1989; Hartung et al., 1993) and had an increased clearance rate of an intravenous triacylglycerol infusion (Sady et al., 1988; Cohen et al., 1989; Podl et al., 1994) when compared to sedentary controls.

As endurance-trained individuals will invariably exercise unless they are specifically instructed not to, these cross-sectional studies may be confounded as a result of recent exercise in the endurance-trained group. In order to overcome this limitation, two studies, one investigating the effect of a period of detraining in endurance-trained adults (Hardman et al., 1998) and the other investigating the effect of a period of training and detraining in sedentary adults (Herd et al., 1998) were conducted. In the study conducted by Hardman and colleagues (1998), ten endurance-trained adults were instructed to refrain from training for seven days with triacylglycerol concentrations measured following the ingestion of a high-fat mixed meal 15 h, 60 h and 6.5 d after the cessation of exercise. The study observed higher postprandial triacylglycerol concentrations at 60 h (35%) and 6.5 d (42%) when compared with those at 15 h. Similar observations were reported by Herd and colleagues (1998), with lower postprandial triacylglycerol concentrations evident 15 h, but not 60 h and 9.5 d, following the cessation of a 13 week exercise training programme when compared with pre-training postprandial triacylglycerol concentrations. These studies suggest that the effect of exercise on postprandial triacylglycerol concentrations is an acute effect and not an adaptation to regular exercise.

Although the weight of available evidence suggests that the effect of exercise in lowering postprandial triacylglycerol concentrations is acute and lost without the presence of a recent exercise bout, there are studies that have identified a chronic effect (Tsetsonis et al., 1997; Miyashita et al., 2011). Miyashita and colleagues (2011) observed that physically active older adults (age = 69.3 (sem 1.1) yr; >150 min of moderate-to-vigorous physical activity per week) experienced lower (38%) postprandial triacylglycerol concentrations, following two days of physical inactivity, than physically inactive older adults (age = 70.4 (sem 1.2) yr; <150 min of moderate-to-vigorous physical activity per week). The lower postprandial triacylglycerol concentrations experienced by the physically active

individuals may be related to the fact that the physically active had lower body mass than the physically inactive (76.9 (sem 2.1) kg vs. 85.5 (sem 2.0) kg) with a significant correlation reported between body mass and the total area under the triacylglycerol versus time curve ($r = 0.731$, $P < 0.0005$). Although no information on body fat levels was given in this study it seems logical to assume that the lower body mass observed in the active participants represents lower body fat levels. In addition, Tsetsonis and colleagues (1997) suggested that regular exercise may amplify the ability of a bout of exercise to lower postprandial triacylglycerol concentrations, when they observed that 90 min of walking at 60% of their maximum oxygen uptake ($\dot{V}O_2max$) resulted in a greater reduction in postprandial triacylglycerol concentrations in endurance-trained than sedentary adults (30% vs. 16%, respectively).

Due to the overwhelming evidence that the effect of exercise on postprandial triacylglycerol concentrations is acute, research has focused on investigating the effect of a single bout of exercise rather than the effect of training. Early work established that exercise performed following the ingestion of a high-fat mixed meal can lower postprandial triacylglycerol concentrations in adults (Cohen and Goldberg, 1960; Maruhama et al., 1977; Hardman and Aldred, 1995). However, as evidenced in the detraining studies the effect of exercise lasts many hours after its cessation, leading to the majority of research conducted since the early 1990s investigating the effect of exercise on postprandial triacylglycerol concentrations focusing on the effect of exercise performed 12 to 16 h before the ingestion of a high-fat mixed meal. A recent review identified that the ability of exercise to lower postprandial triacylglycerol concentrations was more consistent when the exercise was performed 12 to 16 h before the ingestion of the high-fat meal rather than when exercise was performed in the postprandial period (Peddie et al., 2012).

Aldred and colleagues (1994) conducted what appears to be the first study to investigate the effect of a single session of continuous, moderate-intensity exercise, completed many hours prior to the ingestion of a high-fat mixed meal, on postprandial triacylglycerol concentrations. The authors reported that 120 min of walking at 30% $\dot{V}O_2max$ completed 15 h before the ingestion of a high-fat mixed meal, resulted in the subsequent postprandial triacylglycerol concentrations being lowered by 31% in young healthy adults. Since this initial study, a plethora of studies have investigated the effect of moderate-intensity

exercise on postprandial triacylglycerol concentrations in adults, utilising a similar design; where exercise is performed late in the afternoon of day 1, and on day 2 a high-fat meal is consumed and the subsequent postprandial triacylglycerol concentrations measured. These studies have consistently observed that prior moderate-intensity exercise lowers postprandial triacylglycerol concentrations (for review see: Gill & Hardman, 2003; Maraki & Sidossis, 2010; Peddie et al., 2012) with a meta-analysis reporting the effect of continuous, moderate-intensity exercise to be moderate (Petitt and Cureton, 2003).

Not all studies have reported that a bout of moderate-intensity exercise performed 12 to 16 h prior to the consumption of a high-fat meal lowers the resulting postprandial triacylglycerol concentrations (Tsetsonis and Hardman, 1996a; Dalgaard et al., 2004). One of these studies, conducted by Tsetsonis and Hardman (1996a) reported that 90 min of walking at 30% $\dot{V}O_2$max did not lower postprandial triacylglycerol concentrations, but 90 min of walking at 60% $\dot{V}O_2$max did. This study may suggest that exercise intensity influences the effect of exercise on postprandial triacylglycerol concentrations; however, altering exercise intensity but not exercise duration also resulted in differing energy expenditures, confounding any conclusions made on the effect of exercise intensity. A follow-up study, designed such that exercise intensity was different but energy expenditure was equal across exercise trials (90 min of walking at 60% $\dot{V}O_2$max and 180 min of walking at 30% $\dot{V}O_2$max), observed that both intensities of exercise resulted in similar reductions in postprandial triacylglycerol concentrations (32% vs. 33%, respectively) (Tsetsonis and Hardman, 1996b). Taken together these studies indicate that the ability of moderate-intensity exercise to lower postprandial triacylglycerol concentrations is dependent on the energy expended during the exercise.

The influence of energy expenditure on the effect of exercise was further highlighted in a study conducted by Gill and colleagues (2002) when it was observed that 120 min of walking at 50% $\dot{V}O_2$max lowered postprandial triacylglycerol concentrations to a greater extent than 60 min of walking at 50% $\dot{V}O_2$max (9% vs. 23%, respectively). The authors concluded that a dose-dependent relationship existed between the energy expended through exercise and the later reduction in postprandial triacylglycerol concentrations. This dose-response relationship was also apparent in a meta-analysis conducted by Petitt and Cureton (2003), with a positive relationship between energy expenditure during

moderate-intensity exercise and the magnitude by which exercise lowered postprandial triacylglycerol concentrations evident ($r = 0.62$, $P = 0.02$). It has been argued that in addition to this relationship, exercise will only exert an effect if the energy expenditure is above 2.0 MJ (Maraki and Sidossis, 2010). However, this 2000 kJ threshold must be viewed sceptically as a number of studies have observed an effect of exercise on postprandial triacylglycerol concentrations when the exercise induced an energy expenditure of less than 2000 kJ (Miyashita, 2008; Miyashita & Tokuyama, 2008; Miyashita et al., 2008; Peddie et al., 2012).

This proposed dose-response relationship has been based on changes in the total area under the triacylglycerol concentration vs. time curve; a variable which incorporates changes in fasting triacylglycerol concentrations. The effect of exercise on fasting triacylglycerol concentration is beyond the scope of this review and thesis; however, it should be pointed out that exercise, particularly that with high energy expenditure, has been shown to lower fasting triacylglycerol concentrations (for review see Pronk, 1993; Durstine et al., 2001). Incremental area under the triacylglycerol vs. time curve (a quantification of postprandial triacylglycerol concentrations after adjusting for fasting triacylglycerol concentrations) has been argued by some to be a more important quantification of postprandial triacylglycerol concentrations because of the positive correlations between fasting triacylglycerol and the total area under the triacylglycerol vs. time curve (Nestel, 1964; Weiss et al., 2008b). A relationship between the energy expended during moderate-intensity exercise and the change in incremental area under the triacylglycerol vs. time curve has yet to be established (Peddie et al., 2012). It is important to remember that absolute postprandial triacylglycerol concentration and not the change in triacylglycerol concentration from fasting is the identified risk factor for CHD.

Research investigating the effect of moderate-intensity exercise on postprandial triacylglycerol concentrations is limited in young people. However the limited number of studies that have been undertaken have reported similar findings to those in adults; in that a session of prior moderate-intensity exercise, completed 12 to 16 h prior to the consumption of a high-fat mixed meal, lowers postprandial triacylglycerol concentrations in both healthy adolescent boys (Barrett et al., 2007; Tolfrey et al., 2008; MacEneaney et al., 2009; Tolfrey et al., 2012a) and girls (Tolfrey at al., 2012b) and in overweight

adolescent boys (MacEneaney et al., 2009). However, unlike the observations in adults, a dose-response relationship between the energy expended during exercise and the resulting change in postprandial triacylglycerol concentrations has yet to be established. Studies have observed similar reductions in postprandial triacylglycerol concentrations in adolescent boys following 60 min of running at 53% of their peak oxygen uptake ($\dot{V}O_2$peak) or 75% $\dot{V}O_2$peak (24% vs. 21%, respectively) (Tolfrey et al., 2008) and 30 min or 60 min of running at 55% $\dot{V}O_2$peak (13% vs. 16%, respectively) (Tolfrey et al., 2012a), despite large differences in energy expenditures evident between the exercise conditions.

Since the mid 1990s physical activity guidelines have indicated that the recommended volume of daily physical activity need not be completed in a single bout but can be accumulated through a series of multiple short-duration bouts (Pate et al., 1995; American College of Sports Medicine, 1998; Department of Health, 2004). This concept of accumulating physical activity for health remains in current physical guidelines for both adults and the young (World Health Organization, 2010; Department of Health 2011). Such a concept may be particularly appealing to those who struggle to complete prolonged sessions of activity or those whose daily commitments prevent a single prolonged session of activity being completed. The effect of accumulating physical activity, the day before the ingestion of high-fat mixed meals, on postprandial triacylglycerol concentrations was first investigated by Gill and colleagues (1998). In this study it was observed that 90 min of walking at 60% $\dot{V}O_2$max completed as a single bout (at 15:30 h) or in three 30 min bouts (at 09:30, 12:45 and 17:00 h) resulted in similar reductions in postprandial triacylglycerol concentrations in adults. With observations that 30 min of continuous moderate-intensity exercise alone can lower postprandial triacylglycerol concentrations (Miyashita et al., 2006) Miyashita and colleagues followed on from this work by performing a series of studies investigating the effect of accumulating a total of 30 min of moderate-intensity exercise. These studies observed that accumulating 30 min of moderate-intensity exercise in multiple bouts of 3 min was as effective as a single 30 min bout of exercise at lowering postprandial triacylglycerol concentrations (Miyashita et al., 2006; Miyashita, 2008; Miyashita et al., 2008). The effect of accumulating exercise on postprandial triacylglycerol concentrations in the young has yet to be investigated.

Moderate-intensity exercise has traditionally been the mode of exercise recommended for good health and fitness; however, over recent years other modes of exercise have been recommended in physical activity guidelines to have positive effects on health including high-intensity exercise and resistance exercise (World Health Organization, 2010; Department of Health 2011).

High-intensity or sprint interval training for health is a contemporary issue with research observing similar health benefits following a period of "traditional" continuous, moderate-intensity exercise and repeated 30 s maximal sprints (Gibala et al., 2012). Regarding the effect of sprint exercise on postprandial triacylglycerol concentrations; a session of repeated 30 s maximal sprints completed 14 to 18 h before the ingestion of a high-fat mixed meal has been shown to lower the subsequent postprandial triacylglycerol concentrations in adults (Freese et al., 2011; Gabriel et al., 2012). In addition, simulated games activity (intermittent high-intensity exercise), which included repeated very short-duration sprints, has been demonstrated to lower postprandial triacylglycerol concentrations in both adults (Barrett et al., 2006) and adolescents (Barrett et al., 2007). Notably three of these studies have indicated that the effect of high-intensity intermittent exercise in lowering postprandial triacylglycerol concentrations may be greater than that of moderate-intensity exercise (Barrett et al., 2006; Barrett et al., 2007; Gabriel et al., 2012).

A session of resistance exercise has also been observed to lower postprandial triacylglycerol concentrations in adults (Petitt et al., 2003; Burns et al., 2007; Zafeiridis et al., 2007; Pafili et al., 2009; Singhal et al 2009); however, other studies have observed no effect of prior resistance exercise on postprandial triacylglycerol concentrations (Burns et al., 2005; Shannon et al., 2005). Research has yet to investigate the effect of resistance exercise on postprandial triacylglycerol concentrations in the young. As these sprint and resistance exercise sessions were associated with low energy expenditures, it may be somewhat surprising that lower postprandial triacylglycerol concentrations have been observed when prior sprint or resistance exercise is performed given the strong evidence of a relationship between the reduction in postprandial triacylglycerol concentrations and energy expenditure. It would therefore appear that such a dose-response relationship may only be apparent for low- to moderate-intensity exercise at least in adults. However, any

effect of energy expenditure on postprandial triacylglycerol concentrations may be hidden through the difficulties of measuring energy expenditure during sprinting.

In summary endurance trained adults have been observed to experience lower postprandial triacylglycerol concentrations than their sedentary peers, but this effect appears to be an effect of the last bout of exercise lasting for up to 60 h. A bout of moderate-intensity exercise performed 12 to 18 h before the ingestion of a test breakfast, can lower postprandial triacylglycerol concentrations in adults and adolescents. Research has also identified that prior sprint exercise and resistance exercise can lower postprandial triacylglycerol concentrations; however, research investigating the effect of these exercise modalities in the young is lacking.

2.4.1 Exercise and postprandial triacylglycerol concentrations: Mechanisms

The mechanisms through which the exercise induced reduction in postprandial triacylglycerol concentrations are exerted are not fully understood, but lower circulating postprandial triacylglycerol concentrations will be a result of a decreased appearance of triacylglycerol in to the circulation and / or an increased triacylglycerol clearance from the circulation.

Prior exercise has not been shown to delay the time to peak postprandial chylomicron-triacylglycerol concentration (Gill et al., 2001a) indicating that exercise does not affect the rate of chylomicron-triacylglycerol appearance from the intestine. In support of this is evidence that exercise completed 16 h before the ingestion of food does not affect gastric emptying (Gill et al., 2001a). Therefore, it seems that the appearance of exogenous triacylglycerol is not affected by exercise and does not account for the exercise-induced reduction in postprandial triacylglycerol concentrations.

Although exercise may not affect chylomicron-triacylglycerol appearance, research has reported that exercise may reduce the hepatic secretion rate of very-low density lipoprotein (VLDL) triacylglycerol (Gill et al., 2001a; Tsekouras et al., 2008; Bellou et al., 2013). This is likely to be related to an increase in hepatic fatty acid oxidation as evidenced by prior exercise increasing hepatic ketone body production (Malkova et al., 2000; Gill et al.,

2007). Therefore, it appears that some effect of the exercise induced reduction in postprandial triacylglycerol concentrations may be through exercise altering the partitioning of fatty acids in the liver from esterification to oxidation resulting in less hepatic triacylglycerol synthesis and secretion.

It has been identified that prior exercise can increase the clearance of triacylglycerol in some studies (Dufaux et al., 1981; Sady et al., 1986; Annuzzi et al., 1987) but not all (Annuzzi et al., 1987; Malkova et al., 2000; Gill et al., 2001b). Perhaps the most common mechanism proposed to explain this enhanced clearance rate of triacylglycerol from the circulation is that exercise induces an upregulation of lipoprotein lipase activity. Lipoprotein lipase is an enzyme located on the endothelium which is responsible for the hydrolysis of triacylglycerol in chylomicrons and VLDL allowing the resulting free fatty acids to cross the endothelium into muscle or adipose tissue (Goldberg & Merkel, 2001). In support of this proposal is the observation of a positive correlation between plasma lipoprotein lipase activity and the clearance rate of an intravenous triacylglycerol infusion in males (Sady et al., 1988) and females (Podl et al., 1994). A single bout of exercise has been shown to result in a delayed increase in lipoprotein lipase activity in some (Sady et al., 1986; Kantor et al., 1987; Ferguson et al., 1988; Gordon et al., 1994) but not all (Ferguson et al., 1988; Herd et al., 2001; Thomas et al., 2001; Miyashita & Tokuyama, 2008) studies. Ferguson and colleagues (1998) indicated that the effect of exercise on lipoprotein lipase activity may be dependent on the energy expended through exercise, with energy expenditures of 4.61, 5.44 and 6.28 MJ resulting in increased lipoprotein lipase activity but not 3.35 MJ. In this study the exercise trial which induced an energy expenditure of 3.35 MJ lowered triacylglycerol concentrations despite no increase in lipoprotein lipase activity. In fact, many other studies have demonstrated exercise to lower postprandial triacylglycerol concentrations but not increase lipoprotein lipase activity (Herd et al., 2001; Gill et al., 2003b, Miyashita & Tokuyama, 2008). It therefore seems unlikely that exercise induced changes in lipoprotein lipase is the primary mechanism through which exercise lowers postprandial triacylglycerol concentrations. However, exercise induced alterations in lipoprotein lipase activity may account for some of the large intra-individual variance in the postprandial triacylglycerol concentrations response to exercise, with those who do experience the largest increase in lipoprotein lipase in response to moderate-intensity

exercise also experiencing the greatest reduction in postprandial triacylglycerol concentrations (Herd et al., 2001; Gill et al., 2003b).

Along with research identifying that prior exercise can reduce the hepatic secretion of VLDL-triacylglycerol it also appears that exercise can alter the composition of the secreted VLDL particles. Receeent studies identified that exercise can result in larger, more triacylglycerol enriched, VLDL particles (Gill et al., 2006; Magkos et al., 2006; Tsekouras et al., 2007; Al-Shayji et al., 2012). These triacylglycerol rich VLDL particles are hydrolysed faster by lipoprotein lipase (Streja, 1979; Fisher et al., 1995) resulting in their enhanced clearance from the circulation.

Therefore, it appears that there are multiple, potentially complementary, pathways through which exercise can lower postprandial triacylglycerol concentrations with evidence available to support a decrease in hepatic triacylglycerol secretion, mediated by a shift to hepatic fatty acid oxidation, and an increase in the clearance of triacylglycerol from the circulation, mediated by an increase in lipoprotein lipase activity and alterations in lipoprotein particle composition.

2.5 Postprandial endothelial (dys)function

A transient state of postprandial endothelial dysfunction was first reported in a study conducted by Vogel and colleagues (1997). In this study a transient decline in FMD, indicating endothelial dysfunction, occurred following the ingestion of a high-fat mixed meal (50 g fat, 3.7 MJ) in adult males and females (39 (sd 10) years). The greatest decline in FMD occurred between 2 and 4 h following the ingestion of the meal. No decline in FMD was evident when participants consumed an isoenergetic meal containing no fat. Since this initial study, a plethora of studies have consistently observed a transient decline in FMD following the ingestion of a meal containing substantial quantities of fat (Plotnick et al., 1997; Ong et al., 1999; Marchesi et al., 2000; Anderson et al., 2001; Bae et al., 2001; Gokce et al., 2001; Liu et al., 2002; Bae et al., 2003; Plotnick et al., 2003; Maggi et al., 2004; Cortes et al., 2006; Padilla et al., 2006; Tushizen et al., 2006; Lin et al., 2008; Tyldum et al., 2009; Ramirez-Velez, 2011; Xiang et al., 2012). This transient endothelial dysfunction has also been observed when it was assessed in the microvasculature using

laser Doppler imaging with iontophoresis (Gill et al., 2003a; Gill et al., 2004) but when assessed using acetylcholine infusion and plethysmography the results are equivocal (Gudmundsson et al., 2000; Muntwyler et al., 2001; Schinkovitz et al., 2001). As endothelial dysfunction signifies an increased susceptibility to atherosclerotic development and progression, the observation of endothelial dysfunction in the postprandial state supports the hypothesis that atherosclerosis is a postprandial phenomenon (Zilversmit, 1979). No study has yet to investigate the effect of such meals on endothelial function in the young.

Although the transient endothelial dysfunction evident following the ingestion of a high-fat mixed meal is a common observation, it has not been observed in all studies (Raitakari et al., 2000; Gaenzer et al., 2001; Giannattasio et al., 2004; West et al., 2005; Cortes et al., 2006; Berry et al., 2008; Silvestre et al., 2008; Muniyappa et al., 2012). This may be due to methodological issues, such as a lack of a control trial, the timing of the postprandial measures, and the characteristics of the test meals.

Gaezner and colleagues (2001) reported an increase in FMD, indicating an enhancement of endothelial function, following the ingestion of a high-fat mixed meal (65 g fat, 730 kcal per square meter of body surface area); however, they observed a greater increase in FMD over the same time period when no food was consumed. Other studies have also reported increased FMD following the ingestion of high-fat meals (West et al., 2004; Muniyappa et al., 2012). This postprandial improvement in endothelial function could be a result of a circadian rhythm, as endothelial function has been found to increase over the waking day (Gaenzer et al., 2000; Otto et al., 2004). The work of Gaezner and colleagues (2001) may highlight the need for a fasting control trial to account for any circadian rhythm. On the other hand, many studies have not observed such circadian rhythms (Etsuda et al., 1999; Harris et al., 2005; Tushiezen et al., 2006). Marchesi and colleagues (2000) clearly demonstrated a postprandial decline in FMD following the ingestion of a high-fat mixed meal, relative to a fasting measure, suggesting that a control trial may not be required to identify any postprandial endothelial dysfunction.

The timing of the postprandial endothelial dysfunction measurements may also impact on the observations of the study. Giannattsio and colleagues (2004) observed no effect of a

high-fat mixed meal on endothelial function in healthy adults. In this study FMD was only measured at 0 and 6 h. Previous research has reported that the postprandial endothelial dysfunction following the ingestion of a meal in healthy adults peaks between 2 and 4 h, and this perturbation is restored within 6 h (Plotnick et al., 1997; Vogel et al., 1997; Marchesi et al., 2000; Maggi et al., 2004). Therefore, Giannattsio and colleagues (2004) potentially missed any postprandial endothelial dysfunction in the healthy adults as a consequence of their measurement times. However, postprandial endothelial dysfunction was evident six hours following the ingestion of the same meal in hypercholesterolemic adults. This is in agreement with other studies where high-fat meal induced postprandial endothelial dysfunction has been shown to be exacerbated and prolonged in adults with CHD risk factors (Ceriello et al., 2002).

The effect of high-fat mixed meals on endothelial function may be dependent on the characteristics of the fat in the meal. Many of the studies investigating the effect of high-fat mixed meals on endothelial function have used a test meal from a global fast food chain which has consistently been shown to induce postprandial endothelial dysfunction (Plotnick et al., 1997; Vogel et al., 1997; Tsai et al., 2004; Padilla et al., 2006; Rudolph et al., 2007; Lin et al., 2008). This postprandial endothelial dysfunction may be a result of the cooking fat used in these high-fat meals with one study identifying that only meals containing used cooking fat (which is typically contained in fast food meals and is high in lipid peroxides and free fatty acids) induce endothelial dysfunction (Williams et al., 1999). Meals containing unused (or unmodified) fat did not induce postprandial endothelial dysfunction. The authors concluded that only heat-modified fats are associated with postprandial endothelial dysfunction; however, other studies have reported endothelial dysfunction in meals that do not contain heat-modified fats (Ong et al., 1999; Anderson et al., 2001; Bae et al., 2001; Gaenzer et al., 2001; Bae et al., 2003; Berry et al., 2008; Tyldum et al., 2009).

It has been argued that investigating the effect of ingesting these high-fat meals lacks external validity and research should consider meals more typical of daily macronutrient intakes; however, it must be noted that the ingestion of meals containing high quantities of fat is not uncommon in western societies, as evidenced by data indicating that 60% of teenagers in the United States of America eat “fast food” (which is typically high in fat

content) at least once a week (Taveras et al., 2005) and 30% eat such foods every day (Schmidt et al., 2005). Research has observed that ingestion of meals containing more moderate quantities of fat (26 or 34 g fat, 2.9 or 3.8 MJ) still induce a transient state of endothelial dysfunction (Steer et al., 2003) but the postprandial endothelial dysfunction associated with these moderate-fat meals is less than that observed following the high-fat meals. The nature of the relationship between the amount of fat in the meal and the subsequent endothelial dysfunction is not clear and limited to a single study (Steer et al., 2003).

The majority of research investigating the ingestion of food on endothelial function has focused on meals containing substantial quantities of fat; however, research has also shown that a transient state of endothelial dysfunction occurs following an oral glucose tolerance test in adults (Title et al., 2000; Zhu et al., 2007; Weiss et al., 2008a; Lavi et al., 2009; Mah et al., 2011; Watanabe et al., 2011). A single study has been conducted in children and adolescents, and observed no effect of an oral glucose tolerance test on endothelial function (Metzig et al., 2011). These observations are in contrast to studies investigating the effect of high-fat meals on endothelial function, where they have indicated that a meal containing little or no fat and therefore high amounts of carbohydrate has no effect on endothelial function (Plotnick et al., 1997; Vogel et al., 1997; Bae et al., 2001; Gokce et al., 2001; Bae et al., 2003; Padilla et al., 2006). The postprandial endothelial dysfunction associated with an oral glucose tolerance test appears to be more acute than with high-fat mixed meals, evidenced by the peak endothelial dysfunction occurring 1 h following the ingestion of the glucose load (Zhu et al., 2007; Weiss et al., 2008) and the perturbation restored within 3 h (Title et al., 2000; Zhu et al., 2007; Weiss et al., 2008; Mah et al., 2011; Watanabe et al., 2011). With the measurement of endothelial function occurring hourly at best in studies investigating the effect of high-fat meals on endothelial function, any effect of the carbohydrate in the low-fat meals may have been concealed. The fact that the postprandial endothelial dysfunction induced by fat and glucose follow different time courses may suggest that the endothelial dysfunction could be induced through different pathways. Lavi and colleagues (2009) also indicated that only carbohydrate with a high glycaemic index induced endothelial dysfunction. The low-fat meals provided in the studies investigating the effect of a high-fat mixed meal may

have been of a low glycaemic index providing another explanation as to why these studies did not observe an effect of carbohydrate.

2.5.1 High-fat meal induced endothelial dysfunction: Triacylglycerol concentrations and oxidative stress

The magnitude of endothelial dysfunction associated with the ingestion of a meal containing substantial quantities of fat has been shown to be directly related to postprandial triacylglycerol concentrations (for review see Wallace et al., 2010). This relationship was first observed in a study conducted by Vogel and colleagues (1997) where the mean change in FMD at 2, 3 and 4 h correlated with the change in triacylglycerol concentration at 2 h with a Pearson Product Moment Coefficient (r) of -0.51 ($P < 0.02$). Since this initial study a number of other studies have reported a significant correlation between some quantification of postprandial endothelial dysfunction and postprandial triacylglycerol concentrations, with Pearson Product Moment Coefficients (r) ranging from -0.31 (Bae et al., 2001) to -0.70 (Marchesi et al., 2000). It must be highlighted that the correlations performed across these studies are not consistent as variables have been quantified in a number of different ways, including; the absolute postprandial values, postprandial changes, total area under the variable versus time curve and incremental area under the variable versus time curve. Despite this, these correlations suggest a role of postprandial triacylglycerol in high-fat meal induced endothelial dysfunction.

This association between postprandial endothelial function and triacylglycerol concentrations was further shaped by the study conducted by Gaezner and colleagues (2001). The authors observed a correlation between postprandial endothelial function and postprandial triacylglycerol concentrations. When the authors examined the data they identified two clear groups, based on postprandial triacylglycerol concentrations (low postprandial triacylglycerol concentrations, <800 $mg \cdot dL^{-1} \cdot 8h$ and high postprandial triacylglycerol concentrations, >950$\cdot mg \cdot dL^{-1} \cdot 8h$), in the study population. Post-hoc subgroup analysis identified that only those with high-postprandial triacylglycerol concentrations experienced postprandial endothelial dysfunction. No differences between the groups in participant characteristics and fasting lipid and lipoprotein concentrations were evident. The findings of this study suggest that a "threshold" exists which

postprandial triacylglycerol concentrations must exceed in order for endothelial dysfunction to be present.

This "threshold" concept was utilised in a study conducted by Berry and colleagues (2008). They investigated the effect of two high-fat meals on endothelial function, one rich in stearic fatty acids (50 g fat, 3.57 MJ, 26.7 g stearic fatty acid, 16.6 g oleic fatty acid and 4.5 g linoleic fatty acid) and the other rich in oleic fatty acids (50 g fat, 3.57 MJ, 0.8 g stearic fatty acid, 42.5 g oleic fatty acid and 4.0 g linoleic fatty acid). Stearic fatty acids are known to result in smaller increases in triacylglycerol concentrations compared to other fatty acids (Sanders et al., 2000; Tholstrup et al., 2001). In this study the stearic fatty acid rich meal led to significantly lower postprandial triacylglycerol concentrations than the oleic fatty acid rich meal. Only the oleic fatty acid rich meal induced endothelial dysfunction. Therefore, the low postprandial triacylglycerol concentrations following the stearic acid rich meal may have prevented any endothelial dysfunction. Although it is possible that the oleic-acid could promote- or stearic-acid prevent endothelial dysfunction directly rather than through their effect on triacylglycerol concentrations.

Although it seems attractive to suggest that triacylglycerol is responsible for the postprandial endothelial dysfunction, when an intravenous triacylglycerol emulsion was infused in adults no decline in FMD was reported (Gokce et al., 2001). However, Lundman and colleagues (1997) did observe a decline in FMD following the infusion of a triglyceride emulsion. Therefore, the postprandial endothelial dysfunction observed in these studies may not be solely down to the postprandial increase in triacylglycerol concentrations but related to the complex and interrelated metabolic responses to the ingestion of a meal containing a mixture of fat, glucose and protein. The fact that a triacylglycerol infusion does not initiate an insulinaemic response but ingestion of a mixed meal does, may indicate some role of insulin in postprandial endothelial dysfunction.

A transient increase in oxidative stress (an imbalance between oxidant and antioxidant levels such that oxidant production exceeds the antioxidant defences) is also evident following the ingestion of meals containing a substantial quantity of fat (Sies et al., 2005; Bloomer et al., 2009; Fisher-Wellman & Bloomer, 2010; Gabriel et al., 2012; McCarthy et al., 2013). This oxidative stress is likely to be related to the increased flux of non-

esterified fatty acids into the muscle, adipose and hepatic tissues and the endothelial cells, during the postprandial period, resulting in increased mitochondrial fat oxidation (Brownlee, 2005; Goldberg et al., 2009). Increased fat oxidation can result in the overproduction of NADH and $FADH_2$ which overloads the electron transport chain and in response superoxide anions (a reactive oxygen species) are formed. Tsai and colleagues (2004) observed a postprandial increase in 8-epi-postaglandin F2α, a stable marker of oxidative stress, and a decrease in glutathione peroxidise, an endogenous antioxidant enzyme, which followed the same time course as the postprandial rise in triacylglycerol concentrations. Furthermore, the magnitude of postprandial oxidative stress has been shown to correlate with postprandial triacylglycerol concentrations and postprandial endothelial dysfunction (for review see Wallace et al., 2010). However, these correlations may be dependent on the marker used to indicate oxidative stress as no significant correlations are evident when malondialdehyde and thiobarbituric acid reactive substances were used to quantify oxidative stress (Anderson et al., 2001; Bae et al., 2003; Tushuizen et al., 2006; Wallace et al., 2010). This triangle of correlations (postprandial triacyglycerol concentrations, oxidative stress and endothelial function) was clearly demonstrated in a study by Bae and colleagues (2001). In this study they reported an increase in leukocyte superoxide anion production following the ingestion of a high-fat meal which was found to be directly related to the change in triglyceride concentrations ($r = 0.798$, $P < 0.001$); and they also reported correlations between the increase in leukocyte superoxide anion production and the change in endothelial function ($r = -0.784$, $P < 0.001$) and between the increase in triacylglycerol concentrations and the change in endothelial function ($r = -0.488$, $P = 0.040$).

Some role of oxidative stress in high-fat meal induced endothelial dysfunction is also apparent through a second line of evidence where endothelial function is assessed following the ingestion of a high-fat meal with and without the coingestion of antioxidant vitamins. Plotnick and colleagues (1997) observed that the co-ingestion of vitamin C and vitamin E, antioxidant vitamins, with a high-fat meal prevented the postprandial decline in FMD which was apparent when only the high-fat meal was ingested. These vitamins had no effect on endothelial function following the ingestion of low-fat meals. Since this initial study others have also observed that the co-ingestion of antioxidant vitamins (Ling

et al., 2002; Bae et al., 2003) or foods rich in antioxidants (Vogel et al., 2000; Burton-Freeman et al., 2012) can attenuate high-fat meal induced endothelial dysfunction.

Oxidative stress has a firm mechanistic link to endothelial dysfunction. As endothelial function is essentially a measure of nitric oxide bioavailability, either a decrease in the production of nitric oxide and / or an increase in the deactivation of nitric oxide could theoretically lead to endothelial dysfunction. Nitric oxide is a free radical and will readily react with other reactive oxygen species leading to the destruction of nitric oxide and the formation of peroxynitrate. In addition, these reactive oxygen species can also oxidise tetrahydrobiopterin, a cofactor in the reaction which synthesises L-argenine to nitric oxide, which leads to endothelial nitric oxide uncoupling. Uncoupled endothelial nitric oxide synthase results in the synthesis of superoxide anions (a reactive oxygen species) instead of nitric oxide. Therefore, an increase in oxidative stress can increase the deactivation of nitric oxide and decrease the production of nitric oxide, resulting in endothelial dysfunction. Research has in fact identified a decrease in the production of endothelium derived nitric oxide in rats following the ingestion of a meal containing substantial quantities of fat (Ishii et al., 1991; Magne et al., 2009).

Therefore interventions that reduce postprandial triacylglycerol concentrations should be sought as they would be expected to concomitantly attenuate postprandial endothelial dysfunction either directly or through reducing the postprandial rise in oxidative stress.

2.5.2 High-fat meal induced endothelial dysfunction: Vascular tone

It has been observed by many that following the ingestion of meals containing a substantial quantity of fat there is an increase in vascular tone, resulting in increased basal diameters (Djousse et al., 1999; Fard et al., 2000; Raitakari et al., 2000; Gokce et al., 2001; Maggi et al., 2004). Some researchers have argued that endothelial dysfunction induced by a high-fat meal, evidenced by the postprandial decline in FMD, is merely an artefact resulting from this increased vascular tone (Raitakari et al., 2000; Gokce et al., 2001).

It is well established that basal artery diameter is inversely correlated to FMD, such that arteries with large diameters have been shown to exhibit lower FMD than arteries with

smaller diameters (Herrington et al., 2001; Silber et al., 2001; Pyke et al., 2004; Thijssen et al., 2008). This may simply be a result of the mathematical calculation of FMD, where for a large artery to produce a given FMD, it must dilate by a greater absolute distance than a smaller artery. In addition, larger arteries experience lower post-occlusion shear rates, the stimulus for the post-occlusion dilation, resulting in reduced dilation, because shear rate is inversely related to the diameter of the artery (Herrington et al., 2001; Pyke et al., 2004). Normalisation of FMD, for the post-occlusion shear rates (Harris et al., 2010), removes any association with basal diameter (Pyke et al., 2004; Thijssen et al., 2008) and this normalised FMD has also been observed to decline following the ingestion of high-fat meals (Tyldum et al., 2009). In addition, the postprandial change in basal diameter and FMD has been reported not to correlate (Maggi et al., 2004). Such a correlation would be expected if the increased vascular tone was responsible for any decline in FMD.

It has also been suggested that the post-occlusion diameter is at its peak in the fasted state and therefore any increase in basal diameter following meal ingestion, will result in lower postprandial dilation. Such an argument would seem flawed as dilation in response to a nitric oxide donor results in much larger peak artery diameters than those following occlusion (Fard et al., 2000; Anderson et al., 2001; Gaenzer et al., 2001; Liu et al., 2002). Studies have also reported unchanged FMD following triacylglycerol infusion despite observing an increased basal diameter (Gokce et al., 2001) which indicates that post-occlusion diameters must also have increased. These points suggest that postprandial post-occlusion diameters can exceed those observed in the fasting state.

An increase in basal diameter following the ingestion of a high-fat meal may explain some of the postprandial endothelial dysfunction observed when measured as FMD. However, the lack of correlations between the change in basal diameter and FMD, and that a similar decline is evident even after FMD has been normalised for the post-occlusion shear rate, suggest that postprandial endothelial dysfunction is not simply an artefact of a postprandial increase in basal diameter.

2.6 Exercise and high-fat meal induced endothelial dysfunction

As exercise has been shown to lower postprandial triacylglycerol concentrations it has been hypothesised that exercise will also attenuate high-fat meal induced endothelial dysfunction (Chandrruangphen and Collin, 2002; Gill et al., 2004). Research investigating the effect of physical activity and exercise on high-fat meal induced endothelial dysfunction is limited to six studies all conducted in adults (Gill et al., 2003a, Gill et al., 2004; Padilla et al., 2006; Silvestre et al., 2008; Tyldum et al., 2009; Johnson et al., 2011).

Physically active adults (three or more sessions of moderate or greater intensity physical activity, each lasting at least 30 min, per week) have been shown not to experience endothelial dysfunction 4 h after the consumption of a high-fat meal, whilst their physically inactive peers (less than 30 min of moderate or greater intensity physical activity per week) did experience postprandial endothelial dysfunction (Johnson et al., 2011). As with the effect of exercise on postprandial triacylglycerol concentrations, the effect of exercise on postprandial endothelial (dys)function also appears to be acute when Gill and colleagues (2004) observed a bout of moderate-intensity exercise (90 min of walking at 50% $\dot{V}O_2max$), completed 16 to 18 h prior to the ingestion of high-fat mixed meal, attenuated the postprandial endothelial dysfunction which was evident in a control trial. Tyldum and colleagues (2009) have also observed that an acute bout of moderate-intensity exercise (47 min of walking at 60 – 70% maximum heart rate) performed 16 to 18 h prior to the ingestion of a high-fat mixed meal attenuated postprandial endothelial dysfunction. This study also identified that high-intensity intermittent exercise (10 min walking at 50 – 60% maximum heart rate, followed by 4 x 4 min of walking at 85 – 95% maximum heart rate with each block separated by 3 min of walking at 50 - 60% maximum heart rate and finishing with 5 min of walking at 50 - 60% maximum heart rate) prevented any postprandial endothelial dysfunction, suggesting a mediating role of exercise intensity on the effect of exercise on postprandial endothelial dysfunction. Despite these studies showing positive effects of exercise, one study observed no effect of exercise (combination of resistance exercise and moderate-intensity running), completed 4 h or 16 h before the ingestion of a meal containing a substantial quantity of fat, on postprandial endothelial function (Silvestre et al., 2008). The results of this study may be confounded by the

extremely small mean values reported for FMD (< 3 %) and due to the fact that no postprandial decline in FMD was apparent in the control trial.

Although Tyldum and colleagues (2009) observed that moderate-intensity exercise could attenuate, and in the case of high-intensity intermittent exercise prevent, high-fat meal induced endothelial dysfunction, they did not observe any effect of exercise on postprandial triacylglycerol concentrations. This is somewhat surprising given the plethora of research that has consistently reported exercise similar to that used by Tyldum and colleagues (2009) to lower postprandial triacylglycerol concentrations. However, this study does highlight the fact that changes in postprandial triacylglycerol concentrations may not be required in order to see an attenuation in postprandial endothelial dysfunction. This potential dissociation between postprandial triacylglycerol concentrations and endothelial dysfunction was further highlighted by Gill and colleagues (2003a) when they observed that following seven days of detraining postprandial triacylglycerol concentrations had increased, but postprandial endothelial (dys)function was unchanged. Therefore, it appears that exercise, performed 12 to 18 hours before the ingestion of a meal containing substantial quantities of fat can attenuate postprandial endothelial dysfunction, but this effect may not be dependent on changes in postprandial triacylglycerol concentrations.

Padilla and colleagues (2006) have also identified that moderate-intensity exercise (45 min of walking at 60% $\dot{V}O_2$max), performed 2 h following the ingestion of a meal containing substantial quantities of fat, can attenuate postprandial endothelial dysfunction. It must be noted that the validity of research investigating the effect of exercise on endothelial function immediately following the cessation of exercise has been questioned, as many of the effects may simply be related to the increased peripheral blood flow and vasodilation that occurs following the end of exercise (Padilla et al., 2007).

The acute effect of prior exercise on postprandial endothelial function may be related to acute changes in postprandial oxidative stress. Immediately following a session of exercise an increase in oxidative stress is evident, although this typically only remains for 0.5 h to 1 h (Powers & Jackson, 2008; Fisher-Wellman & Bloomer, 2009). In the aftermath of this, exercise induced increase in oxidative stress, the body responds by

increasing its antioxidant defences (Ji, 2002; Haram et al., 2006; Tyldum et al., 2009), and it is this response to exercise which may reduce postprandial oxidative stress and in turn attenuate postprandial endothelial dysfunction. Tyldum and colleagues (2009) observed that both high-intensity intermittent and moderate intensity exercise, completed 16 to 18 h before the ingestion of a meal containing substantial quantities of fat, resulted in a greater fasting and postprandial total antioxidant status. Their observations also identified high-intensity intermittent exercise to be more effective at increasing total antioxidant status than the moderate-intensity exercise. Complementing this, Gabriel and colleagues (2012) observed that repeated sprint exercise (5 x 30 s maximal cycle sprints), completed 18 h before the ingestion of a high-fat mixed meal, could attenuate a postprandial rise in protein carbonyls and thiobarbituric acid-reactive substances, indicating a reduction in postprandial oxidative stress; however, no effect of moderate-intensity exercise (30 min brisk walk) was apparent. Therefore, these results demonstrate that prior exercise can increase the antioxidant capacity of the body and attenuate postprandial oxidative stress, with more intense exercise appearing to have greater effects. This may explain why Tyldum and colleagues (2009) observed a greater effect of high-intensity intermittent exercise on postprandial endothelial dysfunction compared to moderate-intensity exercise.

2.7 Summary

Elevated postprandial triacylglycerol concentrations and the subsequent endothelial dysfunction appear to play a role in the development and progression of atherosclerosis and CHD, and thus these risk factors should be managed from a young age. Exercise completed 12 to 16 h before the consumption of a high-fat mixed meal can lower postprandial triacylglycerol concentrations in adults but there is very limited data available for the young. The ingestion of food, particularly food high in fat, induces a transient state of endothelial dysfunction and that this dysfunction may be mediated by the postprandial increases in triacylglycerol concentrations and oxidative stress. It must be noted that the effect of these meals on endothelial function in the young has not been investigated. Finally as exercise lowers postprandial triacylglycerol concentrations it can be hypothesised that exercise will also attenuate any postprandial endothelial dysfunction. There is limited data in adults supporting a positive effect of exercise on both postprandial

triacylglycerol concentrations and endothelial dysfunction; however, the changes may not be dependent on one another.

Therefore, due to the limited research conducted in the young the research conducted in this thesis sought to establish the acute effect of high-fat meals and exercise on endothelial function and triacylglycerol concentrations with a particular focus on considering the effect of different modes and patterns of exercise. Three modes and patterns of exercise were investigated in this thesis:

- ***Continuous, moderate-intensity exercise***. This is a mode of exercise that has been a part of physical activity guidelines for decades and has been shown to lower postprandial triacylglycerol concentrations in the young but the effect on postprandial endothelial function has yet to be investigated.
- ***Repeated very short duration sprints***. This exercise has been considered to be time-efficient and research suggests that it may be a particularly attractive mode of exercise for the young.
- ***Accumulated, moderate-intensity exercise*.** This pattern of exercise allows the exercise to be structured around the school day and the short duration bouts may make it appealing to the young.

Chapter 3: General methods

3.1 Introduction

This chapter describes the study design, methodological procedures and equipment employed in the experimental studies described in this thesis. All of the laboratory tests in this thesis were carried out in the laboratory facilities of the School of Sport, Exercise and Health Sciences at Loughborough University. All studies were conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the University Ethical Advisory Committee prior to participant recruitment.

3.2 Participant recruitment

The adult participants who volunteered to take part in the studies described in chapters 4 and 5 were recruited from within Loughborough University using poster advertising. The adolescent participants who volunteered to take part in the studies described in chapters 6, 7 and 8 were recruited from local schools in Loughborough.

All participants, and additionally in the case of adolescents their parents/guardians/care givers, were given written information explaining the study rationale, the requirements of the study, the procedures and techniques involved, and any possible risks and discomforts participation could entail (for example see Appendix 1). This information was then explained verbally and individuals were encouraged to seek clarification if necessary. Prior to any testing taking place adult participants signed a statement of informed consent (for example see Appendix 2) and adolescent participants signed a statement of informed assent (for example see Appendix 3) with their parent/guardian/caregiver providing a signed statement of informed consent (for example see Appendix 4). Participants also completed a health history questionnaire in the presence of an investigator who provided any clarification and assistance that was needed (for example see Appendix 5). Additionally this health screen was also completed on behalf of the child by their parent/guardian/care giver.

Any participant with a history of cardiovascular disease, coagulation/bleeding disorders, metabolic disease or food allergies were excluded from involvement in a study. Participants taking medications that affected metabolism were also excluded.

3.3 Study design

In all of the studies described in this thesis participants visited the laboratory for a single preliminary session before commencement of the main trials. In the study described in chapter 4 preliminary sessions took place at least one day prior to the main trial. In the studies described in chapters 5, 6, 7 and 8 preliminary sessions took place at least seven days prior to the main trials. These preliminary sessions involved anthropometric measurements, self-assessment of maturity (adolescent participants only), exercise familiarisation or tests and familiarisation with the measurement of FMD.

In the study described in chapter 4 participants completed a single 1-day main trial. This single 1-day trial involved an oral fat tolerance test consisting of a high-fat breakfast. In the studies described in chapters 5, 6, 7 and 8 each participant took part in two, 2-day main trials each separated by at least seven days. In the study described in chapter 5 participants completed two identical control (rest) trials. In the studies described in chapters 6, 7 and 8 each participant completed a control and an exercise trial, in a randomised, counter-balanced cross-over design. Trial order was allocated prior to any testing using restricted randomisation with a block size of 2, and computer generated random numbers were used to construct the block allocation sequence (Altman and Bland, 1999). Day 1 of each main trial involved either refraining from physical activity or completing the prescribed exercise being investigated in the study. Day 2 of each main trial involved an oral fat tolerance test consisting of a high-fat breakfast and lunch.

For the studies described in chapters 5, 6, 7 and 8 participants were instructed not to consume alcohol or supplements in the seven days before and throughout each main trial. Participants recorded all food and drinks consumed during the day before and on day 1 of the first main trial and were asked to replicate this diet prior to the subsequent trial. Participants were also instructed to refrain from physical activity during the day before and throughout each main trial.

3.4 Anthropometric measurements and self-assessment of maturity

Anthropometric measurements were taken following the protocols of the International Society for the Advancement of Kinanthropometry (Stewart et al., 2011).

3.4.1 Measurements of height, sitting height and body mass

Participants' standing height and sitting height were measured using a Holtain fixed-wall stadiometer (Holtain Ltd., Crymych, U.K.). For measurements of standing height participants were instructed to stand with their back, buttock and heels against the stadiometer; this was checked by the investigator. For measurements of sitting height participants were instructed to sit, with their hands on their thighs, on a box of known height with their back against the stadiometer. When in the correct position the head was then placed in the Frankfort Plane and participants were instructed to inhale deeply whilst the investigator applied a gentle upward lift through the mastoid process. At the same time as this, the stadiometer headboard was lowered onto the top of the head with sufficient pressure to compress the hair. Height was then measured to the nearest 0.1 cm. Body mass was measured on a balance beam scale (Avery Industrial Ltd., Leicester, U.K.) to the nearest 0.05 kg. All measures of standing height, sitting height and body mass were made with the participant wearing light clothing without shoes.

Standing height and body mass measurements were used to calculate body mass index in all studies described in this thesis. In the studies with adolescents, age from peak height velocity (a marker of biological age and maturity) was estimated using standing height, sitting height and body mass (Mirwald et al., 2002).

3.4.2 Skinfold thickness measurements

Skinfold thickness measurements were made in private using Harpenden skinfold callipers (Baty International, Burgess Hill, England) at the following four sites on the right side of the body:

1. Biceps – This skinfold measurement was taken from a fold running parallel to the long axis of the arm at the biceps skinfold site. The biceps skinfold site was located on the anterior surface of the arm, in the mid-line, at the level of the midpoint between the acromion process and the olecranon process. This skinfold measurement was taken with the participant standing in a relaxed position with the right arm relaxed, the shoulder joint externally rotated and the elbow extended by the side of the body.

2. Triceps – This skinfold measurement was taken from a fold running parallel to the long axis of the arm at the triceps skinfold site. The triceps skinfold site was located on the posterior surface of the arm, in the mid-line, at the level of the midpoint between the acromion process and the olecranon process. This skinfold measurement was taken with the participant standing in a relaxed position with the right arm relaxed, the shoulder joint externally rotated to the mid-prone position and the elbow extended by the side of the body.

3. Subscapular – This skinfold measurement was taken from a fold running obliquely downwards at the side of the subscapular skinfold site. The subscapular skinfold site was located at a site 2 cm along a line running laterally and obliquely downward from the tip of the inferior angle of the scapula. This skinfold measurement was taken with the participant standing in a relaxed position with both arms hanging by their sides.

4. Suprailliac – This skinfold measurement was taken at a near horizontal fold located immediately above the point on the iliac crest which lies on a line drawn from the mid-axilla, on the longitudinal axis of the body, to the illium. This skinfold measurement was taken with the participant standing in a relaxed position with the right arm abducted.

Each skinfold was lifted between the thumb and the index finger of the investigators left hand, 1 cm above the site of measurement. The skinfold calliper was then applied level with the area marked and the thickness read 2 s after the full pressure of the calliper was applied.

Three skinfold thickness measurements were taken at each site and the median taken as the skinfold thickness. The sum of the four skinfold thicknesses was reported as an indicator of adiposity.

3.4.3 Circumference measurements

Waist and hip circumference were measured using a flexible, non-elastic, tape. Three measures of each circumference were made with the median taken to represent the circumference. Waist circumference was measured in the horizontal plane midway between the costal margin and iliac crest with the abdominal muscles relaxed and the participant breathing normally. Hip circumference was also measured in the horizontal plane but around the maximum circumference of the buttocks.

3.4.4 Self-assessment of maturity

All adolescent participants completed a self-assessment of maturity based on secondary sexual characteristics (Tanner, 1962). The participants were asked to assess their genital and pubic hair development using a five-point scale with a rating of 1 being pre-pubescent and 5 being adult. Briefly, participants were asked, by an investigator of the same sex, to view photographs and read the description of the different stages of genitalia and pubic hair development during puberty and mark with an "A" the stage they believed they were closest to and "B" to the next closest stage. This test was performed in an enclosed, lockable room, which contained a full length mirror allowing participants to compare their stage of development with the photographs. The participant sealed their assessment in an envelope which was only opened at the end of the study. Participants were reminded that their data was confidential and for the use of the study only and of their right to refuse assent.

3.5 Exercise tests and sessions

3.5.1 Exercise equipment

All treadmill based exercise tests and sessions described in this thesis (chapters 6 and 8) were performed on the same treadmill (Runner Galaxy M.J.C., MTC Climb 2000, Bianchiah and Draghetti Sac, Cavezzo, Italy). Prior to each study, in which the treadmill was used, the velocity of the unloaded treadmill belt was checked against the digital reading given on the treadmill display over a range of speeds expected for the study. The treadmill belt length was initially measured and the time for the belt to complete 100 revolutions was recorded. This allowed the actual belt speed to be calculated.

A WattBike Pro cycle ergometer (WattBike Pro, Nottingham, U.K.) was used to perform the cycle exercise described in this thesis (chapter 7). Prior to each exercise session the cycle ergometer was set to zero which ensured the electronics were stabilised and that there was no feedback within the electrical circuits.

3.5.2 Treadmill familiarisation

Prior to the commencement of the treadmill exercise tests all participants were familiarised with treadmill walking (chapter 6) or running (chapter 8). The familiarisation began with participants walking on the treadmill until they felt comfortable and at ease. The speed of the treadmill was then increased to either a brisk walk (chapter 6) or 7 $km \cdot h^{-1}$ (chapter 8) and participants continued to exercise until they felt comfortable. The participants were then given instructions on how to perform an "emergency stop". Participants were then fitted with a nose-clip and mouthpiece and familiarised with the rating of perceived exertion scale (Borg, 1973). Participants then practiced fitting and removing the nose-clip and mouth piece whilst walking or jogging. Finally the treadmill gradient was increased to 3% whilst the participant was exercising so that they could experience the change in incline, the participant then completed a final "emergency stop". This familiarisation period was not of a set duration and lasted as long as necessary for the participant to feel competent exercising on the treadmill and to become familiar with the procedures required during the exercise tests.

3.5.3 Exercise tests

3.5.3.1 Submaximal incremental treadmill test

In the study described in chapter 8 each participant completed a continuous, incremental treadmill test that consisted of 4 min stages of increasing exercise intensity to determine the oxygen uptake responses of each participant. Participants began jogging at an initial speed of 7.0 $km \cdot h^{-1}$ with an incline of 1.0%. The speed of the treadmill belt increased by 1.0 $km \cdot h^{-1}$ at each subsequent stage.

Heart rate was monitored and recorded during the last minute of each stage using short-range telemetry (Polar, Kempele, Finland) and the participant's ratings of perceived exertion was recorded midway during this final minute. Finally, expired air samples were also collected, and analysed, during the final minute of each stage using the Douglas bag method (Williams & Nute, 1983). The exercise test was stopped if the participant's ratings of perceived exertion was >17 and heart rate was > 180 $beats \cdot min^{-1}$.

3.5.3.2 Peak oxygen uptake tests

In the study described in chapter 6, $\dot{V}O_2peak$ was determined using a continuous, progressive, incremental uphill walking test to volitional exhaustion. The test began with participants walking at a belt speed selected by the participant that elicited a brisk walk at an incline of 5%. Each stage of the test lasted for 3 min. The belt speed remained constant throughout the test with the incline increasing by 4% at the end of each stage. Heart rate was monitored; ratings of perceived exertion recorded and an expired air sample collected between 1 min 45 s and 2 min 45 s of each stage. Participants kept exercising until they felt they could only continue for one further minute. When they indicated this last minute a final expired air sample was collected. This test was used to set the initial speed and gradient that the participants walked at during the main trials by assuming a linear relationship between treadmill incline and oxygen uptake.

In the study described in chapter 8, $\dot{V}O_2peak$ was determined using a similar continuous, progressive, incremental uphill running test to volitional exhaustion. The test protocol

began at least 60 min after the end of the sub-maximal treadmill test at a speed chosen by the investigator that elicited a heart rate of 170 beats·min^{-1} in the sub-maximal treadmill test and at an initial incline of 3%. Each stage of the test lasted for 3 min. The belt speed remained constant throughout the test with the incline increasing by 2% at the end of each stage. Heart rate was monitored, ratings of perceived exertion recorded and an expired air sample collected between 1 min 45 s and 2 min 45 s of each stage and in the minute after the participant indicated that they could only continue for a further minute. This test was used in combination with the sub-maximal treadmill test to set the initial speed that the participants ran at during the main trials by assuming a linear relationship between treadmill belt speed and oxygen uptake.

Strong verbal encouragement was given throughout these exercise tests and the highest oxygen uptake value achieved during the tests was taken as the participant's $\dot{V}O_2$peak (usually the final collection).

3.5.4 Exercise sessions

On day 1 of the control trial participants continued to refrain from physical activity whereas during the exercise trials participants reported to the laboratory and completed the prescribed exercise. The prescribed exercise is described in detail in the respective chapter (chapters 6, 7 and 8). During the exercise, heart rate and ratings of perceived exertion were recorded and expired air was collected and analysed to determine exercise intensity and energy expenditure using indirect calorimetry (Frayn, 1983).

3.5.5 Expired air analysis

Participants breathed through a low resistance respiratory valve and 30 mm wide bore, low resistance, Falconia flexible ducting, tubing (Woodhouse and Taylor Ltd., Macclesfield, U.K.), whilst wearing a nose-clip, into a previously evacuated 150 litre capacity Douglas bags (Plysu Protection Systems, Milton Keynes, U.K.). The percentage of oxygen and carbon dioxide in the expired air samples was measured using a paramagnetic oxygen analyser and an infrared carbon dioxide analyser (Servomex, Crowborough, U.K.). The analyser was calibrated before each experiment using a certified reference nitrogen gas and

certified reference oxygen – carbon dioxide gas. The volume and temperature of the expired air was then determined using a dry gas meter (Harvard Apparatus, Edenbridge, U.K.) fitted with an electronic thermometer. Inspired gas volumes were calculated using the Haldane transformation, and oxygen uptake, carbon dioxide uptake and the respiratory exchange ratio were all subsequently calculated. All gas volumes were corrected to standard temperature and pressure for a dry gas.

3.6 Oral fat tolerance tests

Participants arrived in the laboratory at 08:00 following an overnight fast. Following the assessment of FMD and collection of a blood sample participants were given a high-fat breakfast. A clock was started on the commencement of breakfast. After breakfast participants remained in the laboratory and were required to rest (read, watch television, work at a computer) for 3 h (chapter 4), 6.5 h (chapter 6, 7 and 8) or 7 h (chapter 5). In the studies described in chapters 5, 6, 7 and 8 a high-fat lunch was also provided to the participants at 3.5 hours. Further assessments of FMD were made (chapters 5, 6, 7 and 8) and additional blood samples collected (chapters 4, 5, 6, 7 and 8) at regular intervals throughout the day.

3.6.1 Breakfast

Breakfast consisted of croissant, chocolate spread and a chocolate milkshake (made up of chocolate powder, whole milk (80% volume) and double cream (20% volume)). The mass of each constituent was based on each participant's body mass and derived as the manufacturers "typical serving" divided by the average body mass of the study population (obtained from previous studies in our laboratory). The mass of each constituent received per kilogram of body mass is shown in Table 3.1 and the energy content and macronutrient content per kilogram of body mass is shown in Table 3.2. Participants were given 15 min to consume breakfast.

3.6.2 Lunch

Lunch was based on a previously used high-fat mixed meal (Burns et al., 2006; Miyashita et al., 2006) and consisted of a cheese sandwich (made up of white bread, butter and cheese), ready salted crisps and a chocolate milkshake (made up of chocolate powder and whole milk). The mass of each constituent was based on each participant's body mass. In the study using an adult population (chapter 5) servings matched those previously published. In studies using adolescent populations (chapters 6, 7 and 8) the amount of food per kg body mass was increased, using the average weight of the adolescent study population, to ensure that these participants received an adequate amount of food. The mass of each constituent received per kilogram of body mass is shown in Table 3.1 and the energy content and macronutrient content of the meal per kilogram of body mass is shown in Table 3.2. Participants were given 20 min to consume lunch.

Table 3.1. Mass of each food provided based on participant's body mass

Ingredient	Quantity ($g \cdot kg^{-1}$ body mass)	
	Adult	Adolescent
Breakfast		
Tesco Finest croissant	1.52	2.53
Cadbury's chocolate spread	0.40	0.67
Nesquik chocolate powder	0.20	0.33
Whole milk	2.13	3.56
Double cream	0.53	0.89
Lunch		
Tesco sliced white bread	1.43	2.22
Anchor spreadable butter	0.23	0.36
Tesco mature cheddar cheese	0.61	0.96
Tesco ready salted crisps	0.56	0.87
Nesquik chocolate powder	0.21	0.33
Whole milk	2.86	4.44

Table 3.2. Energy and macronutrient content of breakfast and lunch based on participant's body mass

	Quantity (kg^{-1} body mass)			
	Breakfast		Lunch	
	Adult	Adolescent	Adult	Adolescent
Energy (kJ)	55.6	92.8	54.3	84.4
Fat (g)	0.9	1.5	0.7	1.1
Carbohydrate (g)	1.1	1.8	1.2	1.9
Protein (g)	0.2	0.4	0.4	0.6
Energy from fat (%)	60		50	
Energy from carbohydrate (%)	33		37	
Energy from protein (%)	7		13	

3.7 Flow-mediated dilation

Flow-mediated dilation measurements were made using previously published guidelines and recommendations aimed at making the measurement dependent on endothelial nitric oxide (Corretti et al., 2002; Pyke & Tschakovsky, 2005; Harris et al., 2010).

For each FMD measurement a pneumatic blood pressure cuff (Hokanson, WA, USA) was placed on the participant's right arm immediately distal to the elbow. Participants then rested in a supine position for 20 min, with their right arm extended at approximately 90˚ from their torso. The brachial artery was imaged longitudinally in the distal third of the upper arm using ultrasound (11 MHz linear array transducer, attached to a high-resolution ultrasound machine Power Vision 6000, Toshiba, Tochigi, Japan). A baseline scan of the artery was conducted over 20 consecutive cardiac cycles. Then the blood pressure cuff was inflated to 200 mmHg for 5 min. Further images were captured continuously 30 s prior to and 3 min following cuff release. Images were captured on the r-wave of each cardiac cycle (Vascular Imager, version 4.1.3; Medical Imaging Applications LLC, Iowa City, IA).

Ultrasound settings (gain, focus zone and depth) were set to optimise longitudinal B-mode images of the lumen-arterial wall interface. Pulsed wave Doppler was used, simultaneously with B-mode imaging, to assess blood flow velocity; data was collected at

an insonation angle ≤70° with the sample volume minimised and positioned in the centre of the artery. Ultrasound settings were standardised, for each participant, for every subsequent measurement. Measurement location was also standardised for each participant based on anatomical landmarks, snapshot images and an ink marker placed on the skin that was maintained between trials.

Recorded images were later digitised using specialised, validated (Mancini et al., 2002), edge-detection and wall-tracking software (Brachial analyzer, version 4.1.3; Medical Imaging Applications LLC, Iowa City, IA) with the investigator blind to the scans trial and time. Analysis provided the artery diameter and blood flow velocity for each captured frame.

Baseline diameter was determined as the mean diameter over the 20 collected cardiac cycles. Post-occlusion diameters were smoothed using a three-point moving average and this peak diameter was utilised in the calculation of FMD (Harris et al., 2010); time to peak dilation was also determined. Flow-mediated dilation was calculated as the percentage change in diameter relative to the baseline diameter.

*FMD (%) = ((Peak diameter – Baseline diameter) / Baseline diameter)*100*

It has been recommended that FMD be expressed after accounting for the shear rate stimulus responsible for the dilation (Pyke & Tschakovsky, 2005). Shear rate was determined for each frame and then the area under the shear rate versus time curve, between cuff release and peak dilation, was calculated using the trapezium rule. The area under the shear rate versus time curve was used to normalise FMD (Harris et al., 2010).

*Shear rate = (Mean blood flow velocity [cm.s^{-1}] * 4) / Artery diameter [cm]*

Normalised FMD = FMD [%] / Area under the shear rate versus time curve

3.8 Blood collection, treatment and storage

Venous blood samples were collected in the studies with adults (chapter 4 and 5). In the study described in chapter 4 venous blood samples were collected using the venepuncture technique from the anticubital vein. In the study described in chapter 5 venous blood samples were obtained via an indwelling cannula inserted into the anticubital vein. The cannula was connected to a three way stop-valve. The cannula was kept patent by flushing with a non-heparinised saline solution. All blood samples were obtained once the participant had been in a supine position for at least 10 min as changes in posture can influence plasma volume (Rowell, 1993). Blood samples were collected directly into a pre-cooled 9-mL potassium-EDTA-coated monovette (Sarstedt, Leicester, U.K.), samples were kept on ice until centrifugation.

Capillary blood samples were collected in the study described in chapter 4 and in all studies in adolescents (chapters 6, 7 and 8). Before each blood sample, the whole hand was warmed for 5 min in water heated to 40 C° while the participant remained in a seated position. The hand was dried and cleaned with an alcohol swab before the finger was pierced with a single use disposable lancet (Sarstedt, Leicester, U.K.). The first drop of blood was discarded and 500 µl – 1000 µl of whole blood was collected into potassium-EDTA-coated 500 µl microvettes (Sarstedt, Leicester, U.K.), samples were kept on ice until centrifugation.

All blood samples were centrifuged at 1500 x g for 10 min at 4 C° (Koolspin, Brukard Scientific Ltd., Uxbridge, U.K.) immediately after collection. The plasma was then removed and aliquotted into either a 1.5 ml eppendorf (venous plasma) or a 0.5 ml eppendorf (capillary plasma) and then stored at -80 C° until later analysis.

3.9 Blood analysis

3.9.1 Estimation of changes in plasma volume

Haematrocrit was determined in duplicate from aliquots of EDTA blood dispensed into heparinised micro-heamatocrit tubes. These tubes were centrifuged for 10 min in a micro-

haematocrit centrifuge (Hawksley and Sons Ltd., Lancing, U.K.) and the haematocrit value determined using a micro-haematocrit reader (Hawksley and Sons Ltd., Lancing, U.K.). Haemoglobin concentration was determined using spectrophotometric analysis (Cecil Instruments Ltd., Cambridge, U.K.) in duplicate 20 µL blood samples using the cyanmethemoglobin method (see Appendix 6). Changes in plasma volumes were estimated from changes in haematocrit and haemoglobin (Dill and Costill, 1974).

3.9.2 Spectrophotometric and enzyme-linked immunoabsorbent assays

Plasma triacylglycerol, glucose, total cholesterol, LDL-cholesterol and HDL-cholesterol concentrations in the collected plasma samples were determined by enzymatic, colorimetric methods (see Appendix 6; HORIBA ABX Diagnostics, Montpellier, France) with the use of an automated centrifugal analyser (Pentra 400, HORIBA ABX Diagnostics, Montpellier, France).

Plasma insulin concentration in the collected plasma samples was determined using a commercially available enzyme-linked immunosorbent assay (see Appendix 6; Mercodia, Uppsala, Sweden).

The accuracy of the assays described above was monitored using quality control sera provided by the respective manufacturers). The pipetting accuracy and reliability of the automated centrifugal analyser was monitored monthly and if it did not meet the standards outlined by the manufacture a service was performed on the analyser to remedy this (most often involving a change of syringes and needles). All samples for each individual participant were performed in the same analysis run (except for haematocrit and haemoglobin as they were analysed on each testing day).

Coefficients of variation for each analysis were determined through ten repeated measurements on a human blood sample. The coefficients of variation for each analyte analysed in each study are presented in Table 3.3.

Table 3.3. Coefficients of variation for analysis of triacylglycerol, glucose, insulin, total cholesterol, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol

	Coefficient of variation (%)				
	Chapter 4[a,b]	Chapter 5[a]	Chapter 6[b]	Chapter 7[b]	Chapter 8[b]
Triacylglycerol	1.0, 1.2	1.3	1.2	1.0	1.7
($mmol \cdot L^{-1}$)	(1.82, 1.89)	(1.94)	(1.78)	(1.35)	(1.98)
Glucose	0.6, 0.7	0.8	0.5	0.3	0.7
($mmol \cdot L^{-1}$)	(7.51, 7.41)	(5.77)	(5.29)	(6.74)	(6.49)
Insulin	7.0, 7.4	-	8.8	2.9	4.8
($pmol \cdot L^{-1}$)	(122.7, 164.6)		(68.8)	(117.3)	(186.5)
Total cholesterol	0.7, 0.9	-	-	-	-
($mmol \cdot L^{-1}$)	(4.79, 4.91)				
HDL-cholesterol	1.4, 1.3	-	-	-	-
($mmol \cdot L^{-1}$)	(0.87, 0.87)				
LDL-cholesterol	0.4, 0.6	-	-	-	-
($mmol \cdot L^{-1}$)	(2.72, 2.65)				

Data presented as coefficient of variation (mean concentration)

[a] Coefficients of variation calculated on analysis using plasma from a venous blood sample

[b] Coefficients of variation calculated on analysis using plasma from a capillary blood sample

3.10 Statistical analysis

A range of statistical procedures were utilised in this thesis. The specific statistics used in each study are outlined in the respective chapter.

Chapter 4: Validity of determining analyte concentrations from capillary blood samples

4.1 Introduction

Abnormal lipid and lipoprotein concentrations (high total cholesterol, LDL-cholesterol and triacylglycerol and low HDL-cholesterol concentrations) have been established as factors which increase the risk of developing CHD (Gofman et al., 1953; Castelli et al., 1992; Wilson, 1994; Levine et al., 1995; Hokonson & Austin, 1996; Superko, 1996; Griffin, 1999; Yusuf et al., 2004; Bansal et al., 2007). Autopsy studies have identified that the underlying cause of CHD, atherosclerosis, has its origins in childhood (McGill et al., 2000); thus it seems pertinent that lipid and lipoprotein concentrations are monitored and controlled from a young age (Magnussen et al., 2012).

Lipid and lipoprotein concentrations have traditionally been assessed from a venous blood sample. However, the collection of venous blood may be deemed to be inappropriate in young populations (particularly those who are healthy) due to its invasive nature. Also from a practical point of view, using venous blood sampling might make it less likely that children and adolescents would assent, or parents consent, to involvement in a study. Finger prick blood sampling may constitute an alternative, less invasive and more acceptable method to that of venous blood sampling. In addition capillary blood sampling requires less skill to perform making it particularly useful in large scale studies.

Research is available comparing the concentration of total cholesterol (Bachorik et al., 1990; Greenland et al., 1990; Kafonek et al., 1996; Stein et al., 2002; Rubin et al., 2003; Sblendorio et al., 2008), HDL-cholesterol (Warnick et al., 1993; Kafonek et al., 1996; Stein et al., 2002; Rubin et al., 2003) and triacylglycerol (Kafonek et al., 1996; Stein et al., 2002; Rubin et al., 2003; Mohanlal & Holman, 2004) measured from a venous and capillary blood sample; however, research is at best sparse comparing the concentrations of directly measured LDL-cholesterol in venous and capillary blood. Venous blood samples have been reported to contain different concentrations to a matched capillary blood samples for total cholesterol (Kupke et al., 1979; Bachorik et al., 1990; Greenland et al., 1990; Bachorik et al., 1991; Kafonek et al, 1996; Stein et al., 2002; Sblendorio et al.,

2008), HDL-cholesterol (Kupke et al., 1979; Warnick et al., 1993; Kafonek et al., 1996; Stein et al., 2002), and triacylglycerol (Kupke et al., 1979; Kafonek et al., 1996; Stein et al., 2002; Mohanlal & Holman, 2004). These differences between blood samples may be a consequence of the methods implemented within the studies, in that the treatment of blood samples (Greenland et al., 1990) and/or the analytical procedures utilised (Stein et al., 2002; Rubin et al., 2003) have not been consistent for both the venous and capillary blood sample. Therefore, it cannot be concluded from these studies that a biological difference exists between venous and capillary blood.

The purpose of this study was to establish the validity of measuring plasma total cholesterol, HDL-cholesterol, LDL-cholesterol and triacylglycerol concentrations from a capillary blood sample, treated in identical fashion, to a venous blood sample both analysed using wet-chemistry based techniques. Furthermore, the validity of plasma concentrations were established from blood samples collected in both the fasted and postprandial state, as one focus of the studies described in this thesis was to investigate the effect of exercise on postprandial triacylglycerol concentrations. The postprandial state may be more revealing of an individual's CHD risk with postprandial triacylglycerol concentrations being identified as stronger predictor of CHD than fasting triacylglycerol concentration (Stampfer et al., 1996). Importantly, only minimal changes in total cholesterol, HDL-cholesterol and LDL-cholesterol concentrations are evident between the fasted and postprandial state (Langsted et al., 2008) meaning these concentrations can be determined from either the fasted or postprandial state. Because the test meal provided contained fat, carbohydrate and protein the validity of measuring glucose and insulin concentrations were also reported and these responses to the test meals are included throughout this thesis.

4.2 Experimental methods

4.2.1 Participants

Eighteen apparently healthy adult males (n = 10) and females (n = 8) aged 20 - 60 years volunteered to participate and completed the study. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved

by Loughborough University Ethical Advisory Committee. Before any testing took place all participants had the procedures and risks associated with involvement in the study explained to them. Written consent from the participant was then obtained. The physical characteristics of the participants are presented in Table 4.1.

Table 4.1. Physical characteristics of participants

	Male (n 10)		Female (n 8)	
	Mean	sd	Mean	sd
Age (years)	27.0	8.2	27.7	12.5
Height (m)	1.78	0.07	1.69	0.06
Body mass (kg)	77.8	9.60	67.4	14.3
BMI ($kg.m^{-2}$)	24.7	4.0	23.5	4.0
Sum of four skinfolds (mm)[a]	43.8	20.2	62.5	24.7
Waist circumference (cm)	82.7	9.8	73.0	8.7
Hip circumference (cm)	100.3	6.0	102.4	8.0
Waist : Hip ratio	0.82	0.05	0.71	0.03

BMI, Body mass index
[a]Four skinfold sites were biceps, triceps, subscapular and suprailiac.

4.2.2 Study design

Participants completed preliminary tests and then undertook one 1-day main trial.

4.2.3 Preliminary tests

The day prior to the main trial, participants visited the laboratory for preliminary tests where measurements of height, body mass, skinfold thicknesses at four sites, waist circumference hip circumference were made (see section 3.4).

4.2.4 Main trials

Participants reported to the laboratory at 08:00 following an overnight fast. Participants rested quietly for 10 minutes before fasting (0 h) blood samples were obtained (venous blood samples were obtained first followed immediately by the capillary blood sample at

each time point) (see section 3.8). Participants then consumed a breakfast (see section 3.6.1) and a clock was started on commencement of the meal. After breakfast participants rested for 3 h. Further blood samples were taken at 0.5 h and 3 h.

Blood samples were centrifuged (see section 3.8) and the resulting plasma was later analysed to determine total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerol, glucose and insulin concentrations (see section 3.9).

4.2.5 Statistical analysis

Data was analysed using the PASW statistics software version 18.0 for Windows (SPSS Inc, Chicago, IL). Statistical significance was accepted at the $P < 0.05$ level. All participant characteristics are presented as population marginal means and standard deviations (sd) and experimental data are presented as population marginal means and standard error of the mean (sem).

A linear regression plot and correlations (Pearson product moment) were conducted to determine the relationship between the respective analyte concentrations in the venous and capillary plasma samples. Paired samples *t*-tests were performed on the respective analyte concentrations derived from the venous and capillary plasma samples to determine if a difference was evident between the two. It has been argued that these statistics do not determine the agreement between two methods (Atkinson & Nevill, 1998) and for this reason venous and capillary plasma analyte concentrations were also compared using Bland and Altman's 95% limits of agreement approach (Bland & Altman, 1986). Heteroscedasticity of the data utilised to determine the limits of agreement was assessed following previously outlined methods by examining the correlation between the absolute difference between the venous and capillary analyte concentration and the mean concentration (Atkinson & Nevill, 1998); if heteroscedasticity was present bias and limits of agreement were also calculated following $\log_e$ transformation. Bias and levels of agreement were independent of time for total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerol and insulin concentration and data is therefore presented pooled over the three time points. Results for glucose concentration were not independent of time and were analysed in two time groups, fasting (0 h) and postprandial (30 min and 3 h).

Outliers in the differences between venous and capillary concentrations were identified through a visual observation of the data set and suspected outliers were confirmed if the data point was more than three inter quartile ranges from the 25^{th} or 75^{th} percentile (this classification of an outlier is used by PASW statistics software version 18.0 for Windows). Bland and Altman 95% Limits of Agreement were calculated on data sets inclusive and exclusive of identified outliers.

The limits of agreement approach taken requires that analytical goals be set prior to analysis (Atkinson & Nevill, 1998), which if met would allow capillary blood samples to be accepted as a valid alternative to venous blood samples. The analytical goals for the lipid and lipoprotein concentrations in this study were based on an approach previously described by Stein and collegues (2002). As a result the pre-study target limits of agreement were set as the following; total cholesterol < 1.0 $mmol \cdot L^{-1}$, HDL-C < 0.5 $mmol \cdot L^{-1}$, LDL-C < 0.75 $mmol \cdot L^{-1}$ and triacylglycerol < 0.5 $mmol \cdot L^{-1}$.

4.3 Results

Descriptive data of venous and capillary plasma concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerol, glucose and insulin at each time point is presented in Table 4.2.

Using a paired sample t-test there was a significant difference between venous and capillary sample concentrations for total cholesterol (Table 4.3, $P < 0.001$), HDL-cholesterol (Table 4.3, $P < 0.001$), LDL-cholesterol (Table 4.3, $P < 0.001$) and postprandial glucose (Table 4.3, $P = 0.009$); however, no differences were evident for triacylglycerol (Table 4.3, $P = 0.128$), fasting glucose (Table 4.3, $P = 0.900$) and insulin (Table 4.3, $P = 0.457$). Strong, significant, Pearson product moment correlation coefficients were evident between venous and capillary samples for all analyte concentrations (Table 4.3, $P < 0.001$). The bias and 95% limits of agreement, before and after $\log_e$ transformation, for each analyte are also presented in Table 4.3. Two outliers were identified for triacylglycerol and one for postprandial glucose. Repeat analysis conducted on these data sets with outliers removed is presented in Table 4.4. Figures demonstrating the relationship between plasma concentrations obtained in venous and

capillary blood (including the line of equality) and the Bland and Altman plot can be found in Appendix 7.

Table 4.2. Plasma analyte concentrations in venous and capillary samples (n 18)

	Blood Sample	0 h		0.5 h		3 h	
		Mean	sem	Mean	sem	Mean	sem
Total cholesterol	Venous	4.22	0.20	4.23	0.20	4.18	0.22
($mmol.L^{-1}$)	Capillary	3.98	0.19	4.09	0.19	4.07	0.22
HDL-cholesterol	Venous	1.40	0.08	1.40	0.08	1.36	0.08
($mmol.L^{-1}$)	Capillary	1.29	0.09	1.36	0.09	1.30	0.09
LDL-cholesterol[a]	Venous	2.54	0.24	2.46	0.24	2.44	0.24
($mmol.L^{-1}$)	Capillary	2.28	0.20	2.40	0.24	2.30	0.22
Triacylglycerol	Venous	0.96	0.09	1.04	0.09	1.77	0.22
($mmol.L^{-1}$)	Capillary	0.93	0.09	1.06	0.09	1.71	0.22
Glucose	Venous	4.86	0.07	5.27	0.23	4.98	0.11
($mmol.L^{-1}$)	Capillary	4.86	0.09	5.69	0.16	5.11	0.13
Insulin	Venous	35.7	5.8	193.0	24.1	86.7	12.7
($pmol.L^{-1}$)	Capillary	40.7	6.8	201.3	21.1	89.1	16.8

[a] n 13

Table 4.3. Bias, 95% limits of agreement (LOA) and Pearsons product moment correlation coefficient (r) for lipid and lipoprotein concentrations from venous and capillary plasma

	Venous		Capillary		Bias	LOA -	LOA+	r	Intercept	Gradient
	Mean	sem	Mean	sem						
mmol·L^{-1}										
Total cholesterol	4.21	0.12	4.05	0.12	-0.16*	-0.52	0.19	0.978	-0.02	0.966
HDL-cholesterol	1.38	0.05	1.32	0.05	-0.07*	-0.26	0.12	0.964	-0.07	1.003
LDL-cholesterol	2.48	0.14	2.33	0.13	-0.15*	-0.70	0.40	0.945	0.14	0.883
Triacylglycerol	1.25	0.10	1.23	0.10	-0.02	-0.21	0.16	0.991	0.01	0.975
Fasting glucose	4.86	0.07	4.86	0.10	0.00	-0.57	0.56	0.701	0.41	0.914
Postprandial glucose	5.12	0.13	5.40	0.11	0.28*	-0.89	1.45	0.662	2.40	0.586
pmol·L^{-1}										
Insulin	105.1	7.3	110.4	7.3	5.2	-104.8	115.2	0.824	22.6	0.835
Log$_e$ transformed										
Total cholesterol					0.960*	0.883	1.044	0.980	0.999	1.013
HDL-cholesterol					0.945*	0.805	1.100	0.950	0.999	1.044
LDL-cholesterol					0.941*	0.760	1.165	0.948	1.000	0.918
Triacylglycerol					0.983	0.835	1.157	0.987	1.000	0.974
Fasting glucose					0.998	0.892	1.118	0.723	1.001	0.939
Postprandial glucose					1.059*	0.820	1.367	0.594	1.009	0.463
Insulin					1.042	0.471	2.304	0.908	1.004	0.913

LOA-, Lower limits of agreement; LOA+, Upper limits of agreement

* Students paired t-test been venous and capillary concentration, $P < 0.05$.

Table 4.4. Bias, 95% limits of agreement (LOA) and Pearsons product moment correlation coefficient (r) for triacylglycerol and postprandial glucose concentrations, following the removal of outliers, from venous and capillary plasma

	Venous		Capillary		Bias	LOA-	LOA+	r	Intercept	Gradient
	Mean	sem	Mean	sem						
$mmol \cdot L^{-1}$										
Triacylglycerol	1.26	0.10	1.25	0.10	-0.02*	-0.14	0.10	1.00	-0.001	0.979
Postprandial glucose	5.18	0.11	5.41	0.12	0.22*	-0.77	1.21	0.72	1.588	0.737
Log_e transformed										
Triacylglycerol					0.985*	0.898	1.081	0.99	1.000	0.992
Postprandial glucose					1.043*	0.861	1.264	0.69	1.006	0.684

LOA-, Lower limits of agreement; LOA+, Upper limits of agreement

* Students paired t-test been venous and capillary concentration, $P < 0.05$.

In order to interpret the bias and limits of agreement obtained from the $\log_e$ transformed data the bias and limits of agreement need to be "antilogged" and applied to data. Therefore, to interpret the data with respect to our analytical goals, the expected concentration of total cholesterol, HLD-cholesterol, LDL-cholesterol and triacylglycerol in a capillary blood was estimated when the venous concentration of the respective lipid or lipoprotein is set as the highest concentration between the "normal/optimal/desirable" and "high" categories described by the National Cholesterol Education Program (2002). The expected capillary concentration and the expected difference from the venous concentration, for each lipid or lipoprotein concentration from the venous sample are presented in Table 4.5.

Table 4.5. The expected lipid or lipoprotein concentration, and difference, in a capillary blood sample to a given venous blood sample based on the Limits of Agreement analysis on Log_e transformed data. The venous blood sample concentration was set as the highest concentration between the "normal/optimal/desirable" and "high" categories outlined by the National Cholesterol Education Program (2002)

		Expected Capillary Concentration (Difference)		
	Venous Concentration ($mmol \cdot L^{-1}$)	Capillary Concentration ($mmol \cdot L^{-1}$)	LOA- ($mmol \cdot L^{-1}$)	LOA+ ($mmol \cdot L^{-1}$)
Total cholesterol	6.20	5.95 (-0.25)	5.47 (-0.73)	6.47 (0.27)
HDL-cholesterol	1.55	1.46 (-0.09)	1.25 (-0.31)	1.71 (0.16)
LDL-cholesterol	3.36	3.16 (-0.20)	2.55 (-0.82)	3.91 (0.56)
Triacylglycerol *(with outliers)*	2.26	2.22 (-0.04)	1.89 (-0.38)	2.61 (0.36)
Triacylglycerol *(without outliers)*	2.26	2.23 (-0.03)	2.03 (-0.23)	2.44 (0.19)

LOA-, Lower limits of agreement; LOA+, Upper limits of agreement

4.4 Discussion

The purpose of the present study was to establish the validity of determining lipid and lipoprotein concentrations (total cholesterol, HDL-cholesterol, LDL-cholesterol and triacylglycerol concentrations) from a capillary blood sample. The validity of determining

glucose and insulin concentrations from a capillary blood sample were also reported. All lipid and lipoprotein concentrations were significantly lower, albeit by a small absolute concentration, in the capillary blood samples and the correlations between the venous and capillary blood samples for these analytes were strong and significant. As these statistical approaches may be flawed when determining the agreement between two methods (Bland & Altman, 1986; Atkinson & Nevill, 1998), a limits of agreement approach (Bland & Altman, 1986) was taken to determine validity in the present study. Such an approach requires that analytical goals be set prior to analysis (Atkinson & Nevill, 1998), which if met would allow capillary blood samples to be accepted as a valid alternative to venous blood samples. The analytical goals for the lipid and lipoprotein concentrations in this study were based on an approach previously described by Stein and colleagues (2002). That is for capillary blood to be accepted as valid, the limits of agreement had to be smaller than the difference between cut-offs in the diagnosis and treatment of the respective lipid or lipoprotein as outlined by the National Cholesterol Education Programme (2002). These targets were met for total cholesterol, HDL-cholesterol and triacylglycerol. LDL-cholesterol did not meet the pre-study targets.

The pre-analytical goals set in the present study were set so that 95 times out of 100, without using a correction factor to account for bias, an individual with normal "healthy" lipid or lipoprotein concentrations would not be diagnosed as having "at risk" concentrations that would result in the prescription of medication to control lipid and lipoprotein concentrations. The opposite is also true, individuals with "at risk" concentrations who would require medication would not be classified with normal "healthy" concentrations and not receive treatment. The worst that could happen is that an individual would be classified as "borderline" if they had "healthy" or "at risk" concentrations, in this case they would be monitored and further measurements would allow more accurate classification. The limits of agreement analysis on non-transformed data in the present study provided the largest absolute limit of agreement value of 0.52 $mmol \cdot L^{-1}$, 0.26 $mmol \cdot L^{-1}$, 0.70 $mmol \cdot L^{-1}$ and 0.21 $mmol \cdot L^{-1}$ for total cholesterol, HDL-cholesterol, LDL-cholesterol and triacylglycerol, respectively (Table 4.2). All these values met the pre-study analytical goals, resulting in capillary blood being accepted as a valid alternative to using venous blood samples when measuring these lipids and lipoproteins. However, heteroscedacity was evident for LDL-cholesterol and triacylglycerol indicating

that the limits would be wider at higher concentrations. When the limits of agreement were calculated using $\log_e$ transformed data, a more valid statistical model for these two analytes, and the results extrapolated to the clinical cut-off concentrations, the largest absolute limit of agreement was 0.82 $mmol \cdot L^{-1}$ and 0.38 $mmol \cdot L^{-1}$ for LDL-cholesterol and triacylglycerol, respectively (Table 4.2). This model resulted in triacylglycerol still falling within the pre-study analytical goals but LDL-C now falling outside.

The standard deviation of the bias, and therefore the limits of agreement, was substantially lower in the present study for each lipid and lipoprotein than those reported by Stein and colleagues (2000). In their study, venous blood samples were analysed using standard laboratory measures whilst the capillary blood samples were analysed using a portable, dry-chemistry analyser. Greenland and colleagues (1990) also reported a larger standard deviation of the bias for HDL-cholesterol, compared to the present study, with both the venous and capillary samples analysed using a portable, dry-chemistry analyser. These portable, dry-chemistry analysers have been associated with larger intra-assay variations compared to the standard laboratory based techniques (Bachorik et al., 1990; Kafonek et al., 1996). The narrower limits of agreement observed in the present study may therefore be related to using a less variable analytical technique on both blood samples. Importantly, the intra-assay variation for each analyte was similar in the present study (see section 3.9.2) suggesting that the analytical techniques utilised are reliable for determining concentrations in venous and capillary plasma.

The significant differences observed between blood samples (lower concentrations evident in the capillary blood samples) for total cholesterol, HDL-cholesterol and LDL-cholesterol concentrations in the present study is in agreement with previous studies (Kupke et al., 1979; Kafonek et al., 1996; Sblendorio et al., 2008). However, a number of studies have also reported higher concentrations in the capillary blood samples for total cholesterol (Bachorik et al., 1990; Greenland et al., 1990; Bachorik et al., 1991; Stein et al., 2002) and HDL-cholesterol (Warnick et al., 1993; Stein et al., 2002). The majority of these previous studies have utilised different analytical techniques on the two blood samples, with standard laboratory techniques used on the venous blood sample whilst portable, dry-chemistry analysers have been used on the capillary blood sample. These portable, dry-chemistry analysers have been shown to be a cause of bias, with research reporting

different concentrations from a single venous blood sample when analysed using a standard laboratory technique and a portable, dry-chemistry analyser (Warnick et al., 1993; Kafonek et al., 1996), potentially confounding any conclusions made on differences between analyte concentrations in venous and capillary blood. Warnick and colleagues (1993) also reported a bias between blood samples for HDL-cholesterol, with concentrations in venous blood samples lower than capillary blood sample, despite identical analytical techniques used on both samples. The authors identified that the EDTA concentration in the venous collection tubes was greater than that of the capillary collection tubes; concluding that a greater osmotic dilution, induced by the higher EDTA concentration, occurred in the venous blood sample which may account for the observed between sample differences. The EDTA concentrations were identical in both collection tubes in the present study.

A small bias, in favour of lower concentrations in capillary blood samples, was reported for triacylglycerol with and with-out the inclusions of outliers in the present study (1.5 % and 1.7 %, respectively); however, statistical significance was reached only when the outliers were excluded. The bias reported in previous studies appears equivocal, with lower (Stein et al., 2002; Mohanlal & Holman, 2004) and higher (Kupke et al., 1979; Kafonek et al., 1996) triacylglycerol concentrations in the capillary blood samples. Again the different analytical techniques used to determine triacylglycerol concentrations in the two blood samples could explain the bias observed in previous studies. However, in the study conducted by Mohanlal and Holman (2004) that used identical laboratory based techniques on both blood samples, triacylglycerol concentrations were on average 10 % higher in capillary blood. Capillary blood may better represent arterial blood than venous blood, and triacylglycerol concentrations in arterial blood have been shown to be higher, although only by approximately 0.1 $mmol \cdot L^{-1}$, than in venous blood (Malkova et al., 2000). The hand-warming implemented in the present study results in the arterialisation of venous blood (McGuire et al., 1976; van der Weerd et al., 2002) resulting in both venous and capillary blood being representative of arterial blood, potentially explaining the lack of any meaningful difference in triacylglycerol concentrations between venous and capillary blood samples in the present study. It is also plausible that the lower concentrations observed in the capillary blood, for all lipid and lipoproteins, could be a consequence of

the capillary blood sampling procedure diluting capillary blood with either lymphatic fluid or interstitial fluid; however, to prevent this care was taken not to "milk" the finger.

This appears to be the first study that has evaluated the validity of directly measuring LDL-cholesterol concentration in capillary blood samples; however, the validity of estimated LDL-cholesterol concentration using the Friedwald equation from a capillary blood sample has been investigated (Stein et al., 2002). In this study LDL-cholesterol concentrations were systematically lower in capillary blood a finding the authors attributed to the higher triacylglycerol concentrations they observed in capillary blood. Therefore if LDL-cholesterol is to be estimated from capillary blood samples the measures of total cholesterol, HDL-cholesterol and triacylglycerol from the blood sample need to be valid.
Higher glucose concentrations were observed in the capillary compared with venous blood samples during the postprandial, but not fasted, state in the present study; an observation reported by previous research (Larsson-Cohn, 1976; Kuwa et al., 2001; Kriijshoop et al., 2004). The higher plasma glucose concentration observed in the capillary blood samples is most likely caused by the uptake of glucose from the circulation, as it crosses the capillary bed, resulting in lower glucose concentrations entering the venous circulation. This proposition is supported by Kruijshoop and colleagues (2004) who observed a greater bias in healthy adults compared to adults with insulin resistance; insulin resistance would be expected to result in lower glucose uptake and extraction from the blood producing a smaller difference between capillary and venous measurements. The limits of agreement were greater for postprandial, compared to fasting, glucose concentrations in the present study and in previous work (Kruijshoop et al., 2004), suggesting that the postprandial period may be associated with a more variable uptake of glucose across the capillary bed than in the fasted state due to a large between participant variation in insulin responses to the meal and insulin resistance. These findings of similar glucose concentrations in the fasted, but different concentrations in the postprandial state, in venous and capillary blood have been integrated into the World Health Organizations diagnostic criteria by providing different glucose concentrations, depending on blood sampling site, required for diagnosis when samples are taken after, but not before, an oral glucose tolerance test (World Health Organization, 1999).

The present study is the first to evaluate the level of agreement between insulin concentrations in venous and capillary blood. There was no bias between insulin concentrations in venous and capillary blood, but the limits of agreement indicated that the differences in blood samples could vary by approximately 80% from the "true" insulin concentration.

Importantly, it has been argued by some that capillary blood sampling is preferential to venous when measuring postprandial glucose and insulin concentrations, because the concentrations determined in capillary blood are more sensitive to the glycaemic response to the meals and show a lower between subject variation (Wolever et al., 1991; Kuwa et al., 2001; Wolever, 2003; Brouns et al., 2005).

This study was not without its limitations. Firstly, the participant population had normal "healthy" lipid and lipoprotein concentrations which resulted in the limits of agreement having to be extrapolated to assess the analytical goals. Secondly, the different concentrations that were apparent between the two blood sampling sites may be a result of different plasma volumes. Although we did not measure plasma volumes, previous research has reported capillary blood to have a slightly lower (2.7 %) plasma volume than venous blood (Hutler et al., 2000); however, it must be noted this study did not use a hand-warming protocol.

4.5 Conclusions

Based on the pre-analytical goals that were set we conclude that total cholesterol, HDL-cholesterol and triacylglycerol concentrations obtained from a capillary blood sample is a valid alternative to those concentrations obtained from a venous sample; however, this is not the case for LDL-cholesterol. Despite acceptable agreement between the concentrations in venous and capillary blood samples it is best to standardise the sampling site used for repeat measures.

Chapter 5: The reproducibility of endothelial function and triacylglycerol concentration responses to two consecutive high-fat meals

5.1 Introduction

A transient state of endothelial dysfunction, which is implicated in the pathogenesis of atherosclerosis (Ross, 1999; Juonala et al., 2004), is an independent CHD risk factor (Yeboah et al., 2009) and is evident following the ingestion of high-fat mixed meals (Plotnick et al., 1997; Vogel et al., 1997; Marchesi et al., 2000; Bae et al., 2001; Tsai et al., 2004; Gill et al., 2004; Padilla et al., 2006; Tushuizen et al., 2006; Wallace et al., 2010). As the majority of every day is spent in a postprandial state in western societies, the transient endothelial dysfunction associated with the postprandial state may occur on a regular, if not daily, basis. Repeated exposure to this endothelial dysfunction could have profound, deleterious, and irreversible effects on the vasculature. Therefore, interventions that attenuate postprandial endothelial dysfunction appear to be pertinent as they may delay or prevent atherosclerotic development and ultimately the occurrence of CHD. Previous research has identified that exercise (Gill et al., 2004; Padilla et al., 2006; Tyldum et al., 2009), ingestion of anti-oxidant vitamins (Plotnick et al., 1997; Katz et al., 2001; Liu et al., 2002; Bae et al., 2003; Esposito et al., 2003; Anderson et al., 2006) and pharmacological treatments (Wilmink et al., 1999; Wilmink et al., 2001; Ceriello et al., 2002; Ceriello et al., 2005; Nagashima & Endo, 2011; Yunoki et al., 2011) have the potential to at least attenuate postprandial endothelial dysfunction. However, in order to determine if an intervention has a true affect on a given measure it is imperative to know the reproducibility of that respective measure (Atkinson & Nevill, 1998).

Flow-mediated dilation, a non-invasive measure of endothelial function, has previously been established to be a reproducible measure when assessed in the fasting state in a number of laboratories (Hijmering et al., 2001; Woodman et al., 2001; Craiem et al., 2007; Peretz et al., 2007). As the measurement of FMD is new to our laboratory it was important to establish that we could measure FMD reliably. In addition, despite the plethora of research investigating interventions designed to attenuate postprandial endothelial dysfunction, the reproducibility of postprandial FMD had not been established.

Previous studies have reported significant correlations between the magnitude of endothelial dysfunction evident following the ingestion of high-fat mixed meals and postprandial triacylglycerol concentrations (Plotnick et al., 1997; Marchesi et al., 2000; Wallace et al., 2010; Yunoki et al., 2011). Therefore, interventions that reduce postprandial triacylglycerol concentrations may concomitantly attenuate postprandial endothelial dysfunction. In addition, with postprandial triacylglycerol concentrations identified as an independent risk factor for CHD (Bansal et al., 2007), interventions that lower postprandial triacylglycerol concentrations may also have clinical importance.

The reproducibility of triacylglycerol concentrations in response to the consumption of a single meal containing substantial quantities of fat has previously been established (Gill and Hardman, 1998; Mohanlal & Holman, 2004; Gill et al., 2005; Weiss et al., 2008b) and it has been concluded that this response is reproducible providing sufficient control measures are followed (including diet and physical activity). However, with multiple meals consumed during the waking day the postprandial response to consecutive meals may provide a more reliable impression of an individual's daily risk. The reproducibility of the triacylglycerol concentration response to two consecutive high-fat meals has yet to be established.

Therefore, the purpose of this study was to establish the reproducibility of FMD and triacylglycerol concentrations following the consumption of two consecutive high-fat mixed meals (breakfast and lunch). With the breakfast and lunch consisting of carbohydrate, fat and protein, the reproducibility of postprandial glucose concentrations was also reported.

5.2 Experimental methods

5.2.1 Participants

Nine apparently healthy adult males volunteered to participate in and completed this study. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by Loughborough University Ethical Advisory Committee. Before any testing took place all participants had the procedures and risks associated with involvement in the study explained to them. Written consent from the

participant was then obtained. All participants were apparently healthy and not taking any drugs known to affect lipid or carbohydrate metabolism. The participants' physical characteristics are shown in Table 5.1.

Table 5.1. Physical characteristics of participants
(n 9)

	Mean	sd
Age (years)	27.1	7.7
Height (m)	1.80	0.06
Body mass (kg)	75.4	8.7
BMI ($kg.m^{-2}$)	23.2	2.3
Sum of four skin folds (mm)[a]	44.7	18.6
Waist circumference (cm)	79.2	5.1
Hip circumference (cm)	98.3	5.7
Waist : Hip ratio	0.80	0.02

BMI, Body mass index
[a] Four skinfold sites were biceps, triceps, subscapular and suprailiac.

5.2.2 Study design

Participants completed preliminary tests and then undertook two identical main trials which were separated by seven days.

5.2.3 Preliminary tests

Height, body mass, skinfold thicknesses (biceps, triceps, subscapular and suprailiac), waist circumference and hip circumference were measured (see section 3.4).

5.2.4 Main trials

Participants were instructed to refrain from physical activity for three days prior to each main trial. Participants also completed a weighed food diary during this period for the first main trial and were asked to replicate this diet prior to the subsequent trial. Participants were instructed not to consume alcohol or supplements for seven days prior to each main trial.

For each main trial participants reported to the laboratory at 08:00, following an overnight fast, where FMD was assessed (see section 3.7) and a venous blood sample collected (see section 3.8). Participants then ate breakfast (see section 3.6.1) and a clock was started on commencement of the meal. After breakfast participants rested for 7 h. Lunch (see section 3.6.2) was provided at 3.5 h. Flow-mediated dilation was again assessed at 3 and 7 h and additional blood samples collected at 0.5, 1, 2 3, 4, 5, 6 and 7 h.

Collected venous blood was centrifuged (see sections 3.8) and the resulting plasma was later analysed to determine plasma triacylglycerol and glucose concentrations (see section 3.9). Concentrations were not adjusted for plasma volume because plasma volumes were similar between trials and across time (main effect trial, $P = 0.899$; main effect time, $P = 0.718$; interaction effect trial x time, $P = 0.946$).

5.2.5 Statistical analysis

Data was analysed using the PASW statistics software version 18.0 for Windows (SPSS Inc, Chicago, IL). The total and incremental (after correcting for fasting concentrations) area under the plasma concentration versus time curves for triacylglycerol and glucose were calculated using the trapezium rule and represented the triacylglycerol and glucose concentration responses to the high-fat meals.

A number of statistical approaches were used to determine the reproducibility of the responses to the high-fat meals. Linear correlations (Pearson's product moment) were performed between trials for the following variables: basal diameter (at each time point), peak diameter (at each time point), FMD (at each time point), normalised FMD (at each time point), fasting triacylglycerol and glucose concentrations and the areas under the triacylglycerol and glucose concentration versus time curves. Student's paired t-tests were performed to compare between trials on FMD, normalised FMD, triacylglycerol and glucose concentrations and the areas under the triacylglycerol and glucose concentration versus time curves. A mixed effects general linear model with two fixed factors (trial and time) and one random factor (participant) was used to compare differences between trials over time, and any possible interactions between trial and time, for basal diameter, peak diameter, FMD, normalised FMD and plasma triacylglycerol and glucose concentration. For further information on this design and analysis see Winer (1971). Least significant

difference post-hoc analysis was used to identify where any significant effects of time lay. It has been argued that these statistics are not the most revealing tests to determine reproducibility (Bland & Altman, 1986; Atkinson & Nevill, 1998). For this reason reproducibility of all variables was also assessed using Bland and Altman's limits of agreement (Bland & Altman 1986) and within participant test-retest coefficients of variation (Bland & Altman, 1996).

As triacylglycerol concentrations and basal diameter are both suspected to influence endothelial function, Pearson's correlation coefficient was calculated in trial 1 and 2: for fasting FMD and plasma triacylglycerol concentration; for the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 3 h; for the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 7 h; for fasting FMD and basal diameter; for the change in FMD and basal diameter from 0 h to 3 h; and for the change in FMD and basal diameter from 0 h to 7 h (twelve correlations in total).

Statistical significance was accepted at the $P < 0.05$ level. All participant characteristics are presented as population marginal means (sd) and experimental data are presented as population marginal means (sem).

5.3 Results

5.3.1 Basal diameter, peak diameter, flow-mediated dilation and normalised flow-mediated dilation

Basal diameter, peak diameter, FMD and normalised FMD for each trial are presented in Figures 5.1(a), 5.1(b), 5.2(a) and 5.2(b) respectively.

No differences were observed between trials for fasting basal diameter (Figure 5.1(a); $P = 0.170$), peak diameter (Figure 5.1(b); $P = 0.362$), FMD (Figure 5.2(a); $P = 0.668$) or normalised FMD (Figure 5.2(b); $P = 0.162$).

Basal diameter increased following the ingestion of the high-fat meals, although this did not reach statistical significance, but no differences were evident between trials (Figure

5.1(a); main effect trial, P = 0.594; main effect time, P = 0.054; interaction effect time x trial, P = 0.123). Peak artery diameter was not different between trials and did not change over time (Figure 5.1(b); main effect trial, P = 0.588; main effect time, P = 0.986; interaction effect time x trial, P = 0.201). Flow-mediated dilation decreased following the ingestion of the high-fat meals but no differences were evident between trials both before (Figure 5.2(a); main effect trial, P = 0.421; main effect time, P = 0.002; interaction effect time x trial, P = 0.869) and after normalisation for the post-occlusion shear rate (Figure 5.2(b); main effect trial, P = 0.561; main effect time, P = 0.037; interaction effect time x trial, P = 0.219). Post hoc analysis revealed that FMD, both before and after normalisation for the post-occlusion shear rate, was significantly lower, compared to 0 h, at 3 h (P = 0.003 and P = 0.022, respectively) and 7 h (P = 0.008 and P = 0.043, respectively) but not between 3 h and 7 h (P = 0.470 and P = 0.934, respectively).

Strong significant correlations were reported between trials for basal diameter, peak diameter and FMD at 0, 3 and 7 h (Table 5.2; r = 0.939 – 0.998, all P < 0.001). Significant correlations were also reported between trials for normalised FMD at 0 h (r = 0.776, P = 0.012) and 3 h (r = 0.752, P = 0.019) but not 7 h (r = 0.534, P = 0.139).

Summary statistics for the assessment of reproducibility using coefficients of variation and Bland and Altman's Limits of Agreement for basal diameter, peak diameter, FMD and normalised FMD are presented in Table 5.2. As normalised FMD is dependent on both FMD and the area under the shear rate versus time curve, Table 5.2 also presents the summary statistics for the assessment of reproducibility using coefficients of variation and Bland and Altman's Limits of Agreement for the area under the shear rate versus time curve.

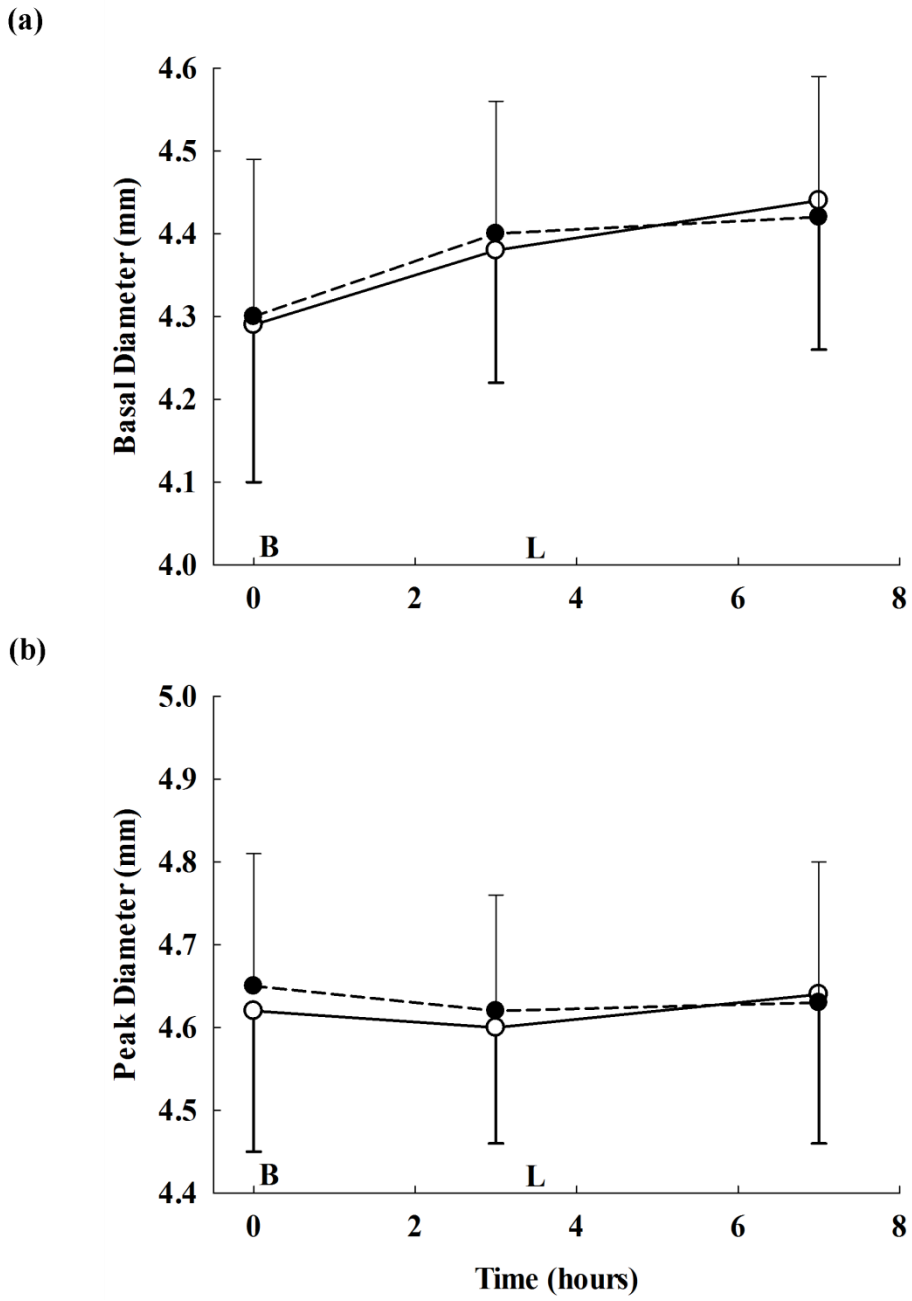


Figure 5.1. (a) Basal diameter and (b) Peak diameter. B, breakfast; L, lunch. Values are means (sem) (n 9). Data were analysed using a mixed-effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay. (a) Basal diameter: main effect trial, P = 0.594; main effect time, P = 0.054; interaction effect time x trial, P = 0.123. (b) Peak diameter: main effect trial, P = 0.588; main effect time, P = 0.986; interaction effect time x trial, P = 0.201.

Trial 1 ——○—— Trial 2 - - -●- - -

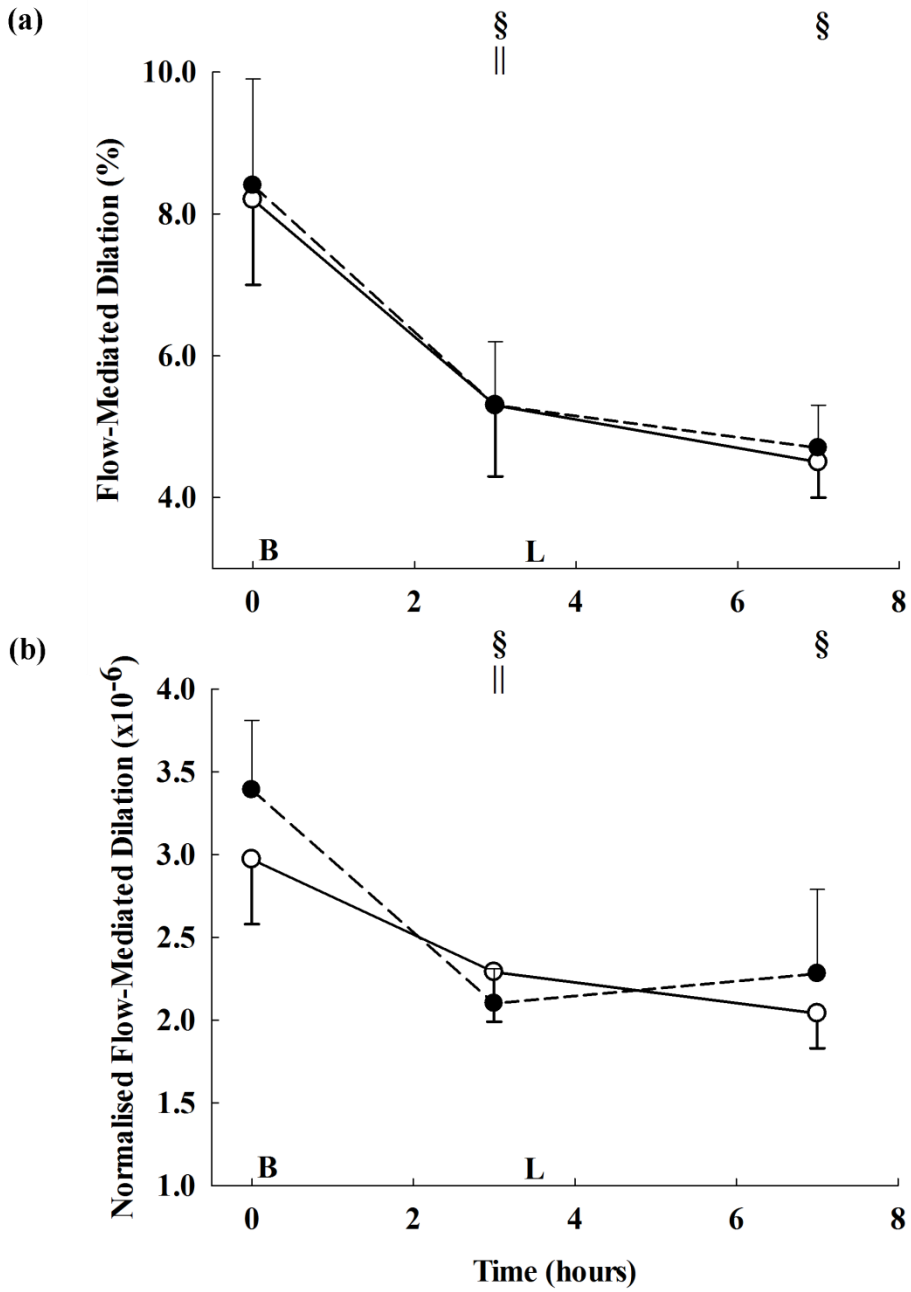


Figure 5.2. (a) Flow-mediated dilation and (b) normalised flow-mediated dilation. B, breakfast; L, lunch. Values are means (sem) (n 9). Data were analysed using a mixed-effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay. (a) Flow-mediated dilation: main effect trial, P = 0.421; main effect time, P = 0.002; interaction effect time x trial, P = 0.869. (b) Normalised flow-mediated dilation: main effect trial, P = 0.561; main effect time, P = 0.037; interaction effect time x trial, P = 0.219.

§ post-hoc analysis on main effect of time - different from 0 h, P < 0.05; || post-hoc analysis on main effect of time - different from previous time point, P < 0.05.

Trial 1 ——○—— Trial 2 - - -●- - -

Table 5.2. Bias, limits of agreement, Pearson product moment correlation and within-participant coefficient of variation for basal diameter, peak diameter, flow-mediated dilation, normalised flow-mediated dilation and the area under the shear rate versus time curve (AUC)
(n 9)

		Bias					
		Mean	sem	LOA -	LOA +	Pearsons' r	CV (%)
Basal Diameter (mm)	0 h	0.02	0.01	-0.05	0.09	0.998**	0.6
	3 h	0.02	0.01	-0.05	0.09	0.997**	0.5
	7 h	-0.02	0.02	-0.13	0.09	0.996**	0.9
Peak Diameter (mm)	0 h	0.02	0.02	-0.11	0.15	0.993**	1.1
	3 h	0.02	0.01	-0.02	0.06	0.995**	0.6
	7 h	-0.01	0.02	-0.15	0.13	0.993**	1.1
Flow-mediated dilation (%)	0 h	0.19	0.42	-2.28	2.66	0.979**	9.7
	3 h	0.01	0.24	-1.43	1.45	0.967**	9.6
	7 h	0.25	0.24	-1.14	1.64	0.939**	10.3
Normalised flow-mediated dilation (x 10^{-6})	0 h	0.42	0.27	-1.19	2.03	0.776 *	26.2
	3 h	-0.20	0.19	-1.35	0.95	0.752 *	22.0
	7 h	0.24	0.45	-2.38	2.86	0.534	29.8
Shear rate AUC (au)	0 h	3134	2988	-14434	20703	0.365	28.5
	3 h	-98	3168	-18720	18536	0.604	27.0
	7 h	-1451	2502	-16165	13263	0.523	24.2

LOA-, Lower limit of agreement; LOA +, Upper limit of agreement; CV, Within-participant coefficient of variation.
*P < 0.05 **P < 0.001

5.3.2 Triacylglycerol and glucose concentration

Triacylglycerol and glucose concentration during each trial are presented in Figures 5.3(a) and 5.3(b) respectively.

No difference was observed between trials for fasting triacylglycerol concentration (Figure 5.3(a), P = 0.17) or postprandial triacylglycerol concentrations (Figure 5.3(a), main effect of trial, P = 0.628; main effect time, P < 0.001; interaction effect time x trial, P = 0.111). No differences were observed between trials for the total area under the plasma triacylglycerol concentration versus time curve (Trial 1 vs. Trial 2: 10.92 (sem 0.82) vs. 10.46 (sem 0.88) mmol·L^{-1}·7h, respectively, P = 0.474) or the incremental area under the plasma triacylglycerol concentration versus time curve (Trial 1 vs. Trial 2: 6.49 (sem 0.77) vs. 5.24 (sem 0.66) mmol·L^{-1}·7h, respectively, P = 0.187).

No difference was observed between trials for fasting glucose concentration (Figure 5.3(b), P = 0.065) or postprandial glucose concentrations (Figure 5.3(b), main effect of trial, P = 0.654; main effect time, P = 0.002; interaction effect time x trial, P = 0.386). No differences were observed between trials for the total area under the plasma glucose concentration versus time curve (Trial 1 vs. Trial 2: 39.11 (sem 1.76) vs. 39.34 (sem 1.85) mmol·L^{-1}·7h, respectively, P = 0.889) or the incremental area under the plasma glucose concentration versus time curve (Trial 1 vs. Trial 2: 5.61 (sem 1.18) vs. 4.91 (sem 1.43) mmol·L^{-1}·7h, respectively, P = 0.562).

Summary statistics for assessment of reproducibility using coefficients of variation and Bland and Altman's Limits of Agreement are presented in Table 5.3.

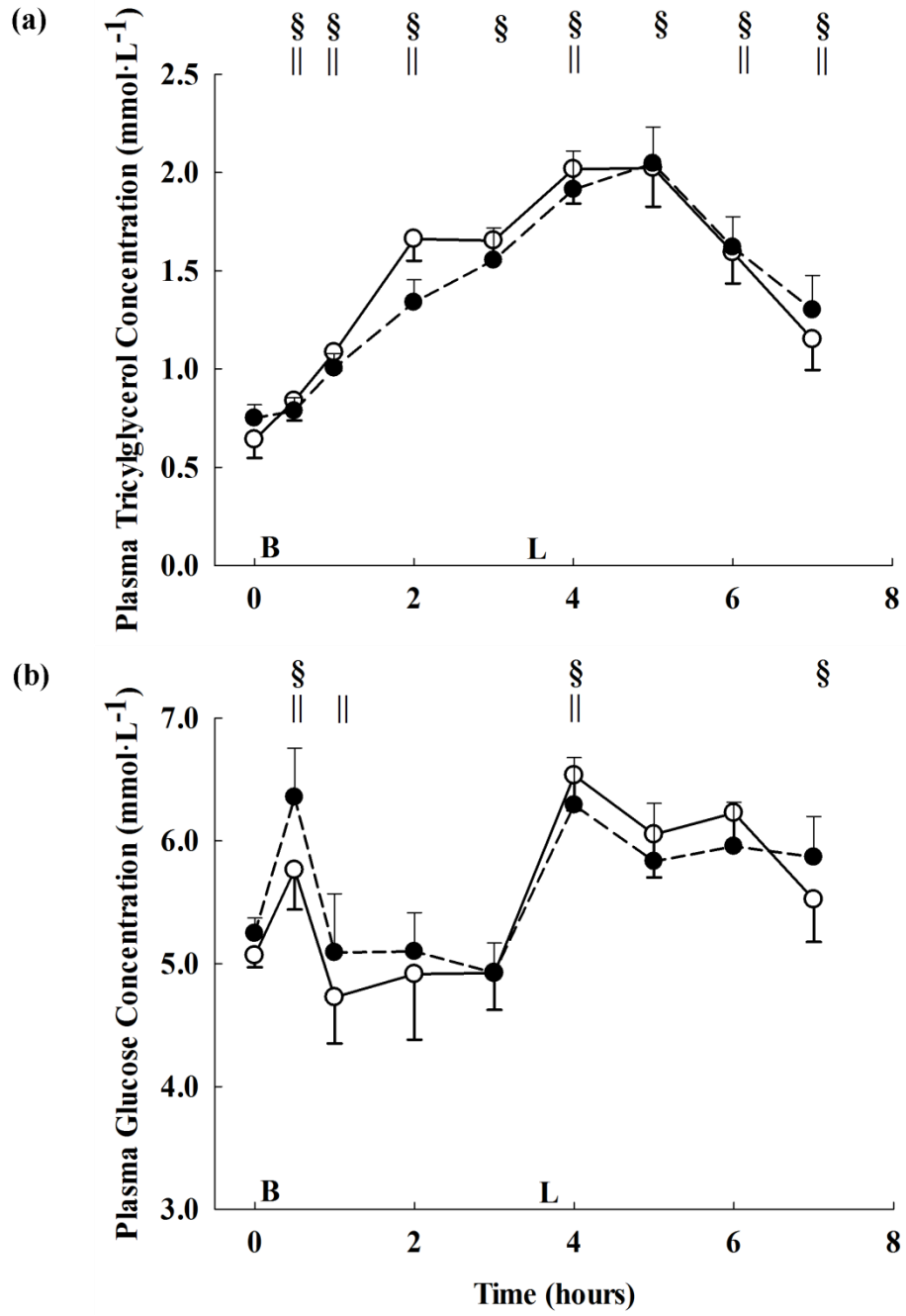


Figure 5.3. Plasma (a) triacylglycerol and (b) glucose concentrations. B, breakfast; L, lunch. Values are means (sem) (n 9). Data were analysed using a mixed-effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay. (a) Plasma triacylglycerol concentrations: main effect of trial, P = 0.628; main effect time, P < 0.001; interaction effect time x trial, P = 0.111. (b) Plasma glucose concentration: main effect of trial, P = 0.654; main effect time, P = 0.002; interaction effect time x trial, P = 0.386.

§ post-hoc analysis on main effect of time - different from 0 h, P < 0.05; || post-hoc analysis on main effect of time - different from previous time point, P < 0.05.

Trial 1 Trial 2

Table 5.3. Bias, limits of agreement, Pearson product moment correlation and within-participant coefficient of variation for fasting triacylglycerol and glucose concentration and the total and incremental area under the triacylglycerol and glucose concentration versus time curves (AUC)
(n 9)

	Bias					
	Mean	sem	LOA -	LOA +	Pearsons' r	CV (%)
Fasting triacylglycerol concentration ($mmol \cdot L^{-1}$)	0.11	0.07	-0.31	0.53	0.646	31.4
Triacylglycerol total AUC ($mmol \cdot L^{-1} \cdot 7h$)	-0.46	0.62	-4.09	3.17	0.701*	13.6
Triacylglycerol incremental AUC ($mmol \cdot L^{-1} \cdot 7h$)	1.26	0.87	-3.87	6.38	0.258	29.2
Fasting glucose concentration ($mmol \cdot L^{-1}$)	0.18	0.08	-0.31	0.67	0.745*	4.13
Glucose total AUC ($mmol \cdot L^{-1} \cdot 7h$)	0.24	1.64	-9.39	9.87	0.589	8.60
Glucose incremental AUC ($mmol \cdot L^{-1} \cdot 7h$)	0.61	1.24	-6.69	7.91	0.616	35.5

LOA-, Lower limit of agreement; LOA +, Upper limit of agreement; CV, Within-participant coefficient of variation.
*$P < 0.05$

5.3.3 Correlations between flow-mediated dilation and basal diameter and triacylglycerol concentrations

The correlations between fasting FMD and plasma triacylglycerol concentrations were: trial 1, r = 0.072, P = 0.855; trial 2, r = 0.183, P = 0.637. The correlations between the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 3 h, were: trial 1, r = -0.179, P = 0.644; trial 2, r = -0.228, P = 0.555. The correlations between the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 7 h, were: trial 1, r = 0.121, P = 0.757; trial 2, r = -0.222, P = 0.566.

The correlations between fasting FMD and basal diameter were: trial 1, r = -0.812, P = 0.008; trial 2, r = -0.856, P = 0.003. The correlations between the change in FMD and basal diameter from 0 h to 3 h, were: trial 1, r = -0.889, P = 0.001; trial 2, r = -0.875, P = 0.002. The correlations between the change in FMD and basal diameter from 0 h to 7 h, were: trial 1, r = -0.322, P = 0.398; trial 2, r = -0.313, P = 0.412.

5.4 Discussion

This study sought to establish the reproducibility of measuring FMD and triacylglycerol concentrations following the ingestion of a high-fat breakfast and lunch. In addition, this study augments previous studies investigating the effect of high-fat mixed meals on endothelial function.

5.4.1 Flow-mediated dilation and other brachial artery characteristics: Reproducibility

In the present study FMD and other brachial artery characteristics (basal diameter, peak diameter and normalised FMD) did not differ between two identical trials separated by seven days. Furthermore, significant and strong correlations between the two trials for FMD and other brachial artery characteristics (except normalised FMD) were evident (see Table 5.2).

The measurement of FMD is dependent on the ability to accurately and reproducibly measure artery diameter. The assessment of basal artery diameter has previously been

shown to be reproducible (De Roos et al., 2003) with a test-retest coefficient of variation of less than 2% reported (Hijmering et al., 2008). The corresponding coefficient of variation in the present study was 0.6% and the limits of agreement approach indicated that repeat measures could vary from the measured basal artery diameter, 95 times out of 100, by up to 0.09 mm. The greater reproducibility of basal diameter observed in the present study is likely due to a combination of stringent pre-trial control conditions and the use of automated edge detection wall tracking software which removes observer bias, reduces intra-observer variation and provides a higher resolution in the diameter measurements (Woodman et al., 2001).

The test-retest coefficient of variation for fasting FMD in the present study was 9.7%. As a result of personal communications with two research groups a test-retest coefficient of variation of 10% was targeted, and can be supported by past research which has typically reported test-retest coefficients of variation for fasting FMD to range between 9.6% and 14.7% (Hijmering et al., 2001; Woodman et al., 2001; Craiem et al., 2007; Peretz et al., 2007). Therefore, the reproducibility of FMD measurements made in the fasted state in this laboratory is in agreement with other laboratories that have vast experience in the measure and meets our predetermined target.

Although many studies have reported that their test-retest coefficients of variation for fasting flow mediated dilation lie between 9.6 and 14.7%, extremes to these exist with reports of coefficients below 2.5% (Celermajer et al., 1992; Sorensen et al., 1995; Uehata et al., 1997) and over 40% (Hardie et al., 1997; De Roos et al., 2003; Malik et al., 2004). Perhaps the poorest reproducibility reported for fasting flow-mediated dilation was in a study by Hardie and colleagues (1997) where they reported that the mean (sd) difference between two assessments of flow mediated dilation separated by three months was 0.57 (6.83)%, which equates to a test-retest coefficient of variation of over 1000%. The three month gap between measures is unlikely to be the reason for the large coefficient of variation reported by this group, as the month-to-month variation is similar to the day-to-day variation (Sorensen et al., 1995). Flawed methodology may account for the extremely poor reproducibility reported by Sorensen and colleagues (1995), including; images captured at random phases of the cardiac cycle, peak diameter measured at a single time point (60 seconds) and a lack of control of factors known to influence FMD including fed state, supplement and/or medication ingestion, time of day or recent physical activity

(Harris et al., 2010). Celermajer and colleagues (1992) and Sorensen and colleagues (1995) reported very good within participant, between days variability, < 2%, for FMD. However, what they termed FMD was calculated as the peak diameter divided by basal diameter, not the change in diameter divided by basal diameter; our test-retest coefficient of variation would have been 0.6% using this method. Furthermore the high level of reproducibility reported in these two studies is unexpected as stringent control conditions were not imposed on the participants.

Prior to the present study, no other study has established the reproducibility of postprandial measures of FMD. The test-retest coefficients of variation for the postprandial measures of FMD in the present study (9.6% and 10.3%) were similar to that of the fasting measure (9.7%). Furthermore, no differences in the reproducibility of basal diameter, peak diameter and normalised FMD were apparent between the fasted and postprandial state in the present study (see table 5.2). Therefore, this study suggests that providing that the assessment of FMD is performed at set time points after the ingestion of a test meal, the reproducibility is as good as that of fasting measures.

Although FMD showed good and acceptable reproducibility, normalised FMD was found to be less reproducible (see table 5.2). This lower reproducibility of normalised FMD is likely to be related to the large variability in the area under the shear rate versus time curve. Substantial measurement error may be evident in the estimation of shear rates. Shears rates are estimated from the measurements of blood flow velocity made using pulsed-wave Doppler velocity. Research suggests that an angle of insonation of <60 º is required for reasonable levels of measurement error in blood flow velocity (Polak, 2004) and that this measurement error increases exponentially when >60º (Oates, 2001; Kremkau, 2002; Thrush & Hartshorne. 2004). Despite this, an angle of insonation of 70º was employed in the present study as it was not possible to use an angle of insonation less than this using the ultrasound equipment available without impairing the acquisition of B-mode images required for the measurement of diameters integral to the primary outcome measure of this study and thesis (FMD). Optimal B-mode images are obtained when the angle of insonation is 90º (i.e. the artery is horizontal in the image); if this angle is changed the clarity and spatial resolution (the ability to differentiate between two adjacent high-contrast objects/lines) of the collected images is reduced. This technical limitation does not affect the primary outcome variable of this thesis "FMD", which shows good

reproducibility in both the fasted and postprandial state but it may affect a secondary outcome variable of this thesis "normalised FMD", which is dependent on accurate measurement of blood flow velocity.

5.4.2 Plasma triacylglycerol and glucose concentration: Reproducibility

As with FMD and other brachial artery characteristics, no significant differences were apparent for fasting or postprandial triacylglycerol and glucose concentrations between the two identical trials. The correlation coefficients were moderate for fasting and the total area under the concentration versus time curve for triacylglycerol and glucose concentrations (r = 0.589 – 0.745). Correlations for the incremental area under the concentration versus time curve were weak for triacylglycerol (r = 0.258) and moderate for glucose (r = 0.616). Previous research has reported correlation coefficients between repeated trials to be greater than 0.9 (Kaminsky et al. 1997; Gill & Hardman, 1998; Gill et al., 2005); however, correlation coefficients as low as 0.14 have also been observed (Aldred & Hardman, 1993).

Although comparison of means has been widely used to determine reproducibility it may not be the most revealing measure. Statistical techniques used to compare means are dependent on the spread of data: it is less likely to observe a statistically significant difference in data with a large variance even if the means of the two trials differ considerably (Bland & Altman, 1986; Atkinson & Nevill, 1998). In response to this Bland and Altman (1986) proposed a limits of agreement approach as a more valid assessment of reproducibility. To our knowledge only two studies have used this statistical approach (Gill & Hardman, 1998; Gill et al., 2005). Gill and Hardman (1998) reported a mean difference (± limits of agreement) in the total area under the triacylglycerol concentration versus time curve of 0.24 (2.37) mmol·L^{-1}·6h between repeated trials. Gill and colleagues (2005) reported a mean difference (± limits of agreement) in the total area under the triacylglycerol concentration versus time curve of 0.52 (1.57) mmol·L^{-1}·6h in males and 1.61 (3.39) mmol·L^{-1}·6h in females studied during different phases of the menstrual cycle. These previous findings are slightly lower than in the present study where the mean difference (± limits of agreement) for the seven hour total area under the triacylglycerol concentration versus time curve was 0.46 (3.63) mmol·L^{-1}·7h. A second statistical approach to establish reproducibility is to consider the within-participant test-retest

coefficient of variation. In the present study the coefficient of variation for total area under the triacylglycerol concentration versus time curve was 13.6% and was similar to that previously observed in adult males (8.1% (Weiss et al., 2008b) and 10.1% (Gill et al., 2005)). Gill and colleagues (2005) indicated that the reproducibility of the total area under the triacylglycerol versus time curve to be poorer in females when one trial was conducted during the follicular phase of the menstrual cycle and the second repeat trial conducted during the luteal phase curve with a coefficient of variation of 23.2%. These markers of reproducibility in the present study are slightly higher (poorer reproducibility) than previous studies in adult males. The slightly poorer reproducibility observed in the present study may be a result of the longer observation time (Weiss et al., 2008b) and the addition of a second meal.

The reproducibility of postprandial triacylglycerol concentrations when represented as the incremental area under the triacylglycerol concentration curve was less reproducible than the total area when the mean difference, limits of agreement or coefficient of variation were considered. Similarly, Weiss and colleagues (2008b) observed that the incremental area under the triacylglycerol concentration curve was less reproducible that the area under the triacylglycerol concentration curve. Fasting triacylglycerol is implicit to the calculation of incremental area under the concentration versus time curve. Fasting triacylglycerol concentration was found to have low reproducibility in the present study with a test-retest coefficient of variation of 31%. This high biological variability in fasting triacylglycerol concentration has also previously been observed by Sebastian-Gambaro and colleagues (1997) and Tolfrey and colleagues (1999) who reported test-retest coefficients of variation for fasting triacylglycerol concentration of 22% and 25%, respectively. It is this high biological variation that has resulted in fasting triacylglycerol being excluded as an independent risk factor for CHD (Hulley et al., 1980).

Fasting and postprandial glucose concentrations showed a greater level of reproducibility than triacylglycerol concentrations in the present study with test-retest coefficients of variation of 4.1% and 8.6% respectively. Other studies have also reported less variability in postprandial glucose concentrations (Gill and Hardman 1998; Gill et al., 2005) most likely as a result of the body controlling glucose concentrations more tightly than triacylglycerol concentrations.

5.4.3 Postprandial flow-mediated dilation

In addition to establishing the reproducibility of postprandial FMD and triacylglycerol concentrations, this study augments the growing literature investigating the effect of food ingestion on endothelial function. Compared to fasting, a 45% decline in FMD was evident 3 h following the ingestion of the high-fat breakfast in the present study indicating endothelial dysfunction. This observation is in line with previous research where a decline in FMD following the ingestion of a high-fat mixed meal has typically ranged between 30% and 50% (Vogel et al., 1997; Bae et al., 2001; Gokce et al., 2001; Tushuizen et al., 2006; Tyldum et al., 2009). The ingestion of the second consecutive high-fat mixed meal, lunch, maintained but did not further exacerbate the endothelial dysfunction induced by the high-fat breakfast. This is in contrast to the findings of the only other study to have investigated the effect of a second high-fat meal (Tushuizen et al., 2006), which observed that the second meal further reduced FMD. The postprandial decline in FMD reported in the present study remained once FMD was normalised for the area under the shear rate versus time curve.

Correlations between the postprandial change in FMD and triacylglycerol concentrations (such that greater postprandial declines in flow-mediated dilation are associated with higher postprandial triacylglycerol concentrations) have previously been reported (Plotnick et al., 1997; Vogel et al., 1997; Bae et al., 2001; Gaenzer et al., 2001; Bae et al., 2003; Wallace et al., 2010). However, these correlations have not been found it all studies (Williams et al., 1999; Steer et al., 2003; Tushuizen et al., 2006; Rudolph et al., 2007; Wallace et al., 2010; Johnson et al., 2011) including the present study. The fact that correlations are not seen in all studies may be a consequence of no consistent quantification of postprandial FMD or triacylglycerol concentrations (e.g. some studies have used absolute values, change from fasting or area under the variable versus time curve). In addition many studies that have previously reported significant correlations between the postprandial FMD and triacylglycerol concentration responses have pooled the data from a high-fat test meal trial and a low-fat test meal trial, by doing so the range of values within the data set was increased ultimately enhancing the chance of finding significant correlations (Bates et al., 1996). The small sample size in the current study may also explain the non-significant correlations observed.

A non-significant increase in basal diameter was evident following the ingestion of the high-fat meals in the present study (Figure 5.1(a); main effect time, P = 0.054). Other studies have also reported a postprandial increase in basal artery diameters (Djousse et al., 1999; Fard et al., 2000; Raitakari et al., 2000; Gokce et al., 2001; Maggi et al., 2004) and this increase is likely to be caused by the concomitant increase in cardiac output and peripheral blood flow (Hurren et al., 2011a). Normalised FMD, unlike FMD, is not associated with basal artery diameters (Pyke et al., 2004; Thijssen et al., 2008) and since similar postprandial responses were found for FMD and normalised it would suggest the effects are not simply an artefact of increased basal diameters. In support of this are the weak and non-significant correlations between the change in FMD and basal diameter, from fasting to 7 h following the ingestion of breakfast (and 3.5 h following the ingestion of lunch), in both trials. However, we did observe significant and strong correlations between the change in FMD and basal diameter, from fasting to 3 h following the ingestion of breakfast, in both trials. The presence of this correlation suggests that the postprandial decline in FMD may be related to the change in basal artery diameters. It is therefore unclear from these findings if the postprandial decline of FMD is merely an artefact of increases in basal diameter.

5.5 Conclusions

In conclusion our data suggests that the measurement of fasting FMD is reproducible in our laboratory and the reproducibility of FMD measured following the ingestion of a high-fat meal is comparable with fasting measures. The reproducibility of postprandial triacylglycerol concentrations in response to two consecutive high-fat meals is good providing diet, smoking and physical activity are controlled in the days preceding the ingestion of the test meals. The study also confirmed that the ingestion of the high-fat breakfast used throughout this thesis induces endothelial dysfunction and demonstrated that the ingestion of a second high-fat meal, lunch, maintains this dysfunction.

Chapter 6: Effect of continuous moderate-intensity exercise on postprandial endothelial function in adolescent boys

6.1 Introduction

The high-fat meal induced endothelial dysfunction observed in the study described in chapter 5 is in agreement with previous studies in adults (Vogel et al., 1997; Plotnick et al., 1997; Marchesi et al., 2000; Gill et al., 2004; Wallace et al., 2010); however, the effect of such meals on the young not been investigated.

Prior moderate-intensity exercise has been shown to attenuate the endothelial dysfunction (Gill et al., 2004; Tyldum et al., 2009) and the rise in triacylglycerol concentrations (Gill & Hardman, 2003) seen following the ingestion of a high-fat meal in adults. While exercise may induce similar responses in children and adolescents this is by no means certain, because as children and adolescents grow and mature their physiological responses to exercise may vary (Riddell, 2008). Recently it has been reported that exercise can reduce postprandial triacylglycerol concentrations in adolescents (Barrett et al., 2007; Tolfrey et al., 2008; MacEneaney et al., 2009; Tolfrey et al., 2012a), but no study has investigated the effect of exercise on postprandial endothelial function.

Therefore, the primary aim of this study was to investigate if a bout of moderate-intensity exercise influenced endothelial function following the ingestion of a high-fat breakfast and lunch in adolescent boys.

6.2 Experimental methods

6.2.1 Participants

Fifteen adolescent boys (aged 12.6 to 14.3 years, Tanner Stage 1 to 5 (median 4)) volunteered to participate, with thirteen boys completing the study. Non-completion of the study was due to either syncope during blood sampling or a participant's lack of time. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by Loughborough University Ethical Advisory

Committee. Before any testing took place all participants had the procedures and risks associated with involvement in the study explained to them. Written assent from each participant and written consent from a parent was then obtained. All participants were apparently healthy, and not taking any drugs known to affect lipid or carbohydrate metabolism; the physical characteristics of those who completed the study are shown in Table 6.1.

Table 6.1. Physical characteristics of participants
(n 13)

	Mean	sd
Age (years)	13.6	0.6
Height (m)	1.57	0.12
Body mass (kg)	56.2	17.1
BMI ($kg \cdot m^{-2}$)	22.3	4.2
Sum of four skin folds (mm)[a]	44.4	19.6
Waist circumference (cm)	71.6	9.2
Hip circumference (cm)	89.5	11.2
Waist : Hip ratio	0.80	0.02
Age from PHV (years)	-0.7	1.0
Tanner Stage: Genital[b]	4	1 - 5
Tanner Stage: Pubic Hair[b]	4	1 - 5
$\dot{V}O_2$peak ($mL \cdot kg^{-1} \cdot min^{-1}$)	48.1	7.5

BMI, Body mass index; PHV, Peak height velocity; $\dot{V}O_2$peak, Peak oxygen uptake.
[a] Four skinfold sites were biceps, triceps, subscapular and suprailiac.
[b] Values are median (range)

6.2.2 Study design

Participants completed preliminary tests and then undertook two, 2-day main trials which were separated by seven days (control trial and exercise trial) in a 2 x 2 replicated Latin square design.

6.2.3 Preliminary tests

Height, sitting height, body mass, skinfold thicknesses (biceps, triceps, subscapular and suprailiac), waist circumference and hip circumference were measured (see section 3.4). Each participant was asked to make a self-assessment of maturity based on secondary sexual characteristics (Tanner, 1989) (see section 3.4.4) and age from peak height velocity was also estimated (Mirwald et al., 2002) (see section 3.4.1). Finally $\dot{V}O_2$peak was measured and the relationship between oxygen uptake and treadmill incline was established (see section 3.5.3.2).

6.2.4 Main trials

Participants were instructed to refrain from physical activity for two days, and not to consume alcohol or supplements in the seven days, before the main trials. Participants also completed a food diary for two days before the first main trial and were asked to replicate this diet for the subsequent trial.

On day 1 of the control trial participants were physically inactive whereas in the exercise trial they reported to the laboratory at 16:00 and walked on a treadmill at 60% $\dot{V}O_2$peak for 60 min. The walking was divided into four, 15 min blocks interspersed with 3 min rest periods. Heart rate, ratings of perceived exertion (Borg et al., 1973) and expired air samples were collected in the last minute of each block (Williams & Nute, 1983). Energy expenditure was estimated using indirect calorimetry (Frayn, 1983).

On day 2 of each main trial participants reported to the laboratory at 08:00, following an overnight fast, and FMD was assessed (see section 3.7) and a capillary blood sample collected (see section 3.8). Participants then ate breakfast (see section 3.6.1) and a clock was started on commencement of the meal. After breakfast participants rested for 6.5 h. Lunch (see section 3.6.2) was provided at 3.5 h. Flow-mediated dilation was assessed again at 3 and 6.5 h and additional blood samples collected at 0.5, 1, 3, 4, 4.5 and 6.5 h.

Collected capillary blood was centrifuged (see sections 3.8) and the resulting plasma was later analysed to determine plasma triacylglycerol, glucose and insulin concentrations (see

section 3.9). Concentrations were not adjusted for plasma volume because plasma volumes were similar between trials and across time (main effect trial, P = 0.977; main effect time, P = 0.103; interaction effect trial x time, P = 0.300).

6.2.5 Statistical analysis

Based on previously published data and pilot research conducted in our laboratory the size of the effect of the ingestion of exercise on postprandial FMD and triacylglycerol concentrations was expected to be large (Cohen's d, ~0.9). Consequently, a priori power calculations (ES = 0.9; $\alpha = 0.05$, $\beta = 0.8$, two tailed) suggested that a sample size of 12 would be sufficient to discern differences in FMD when the fat content of the meals or the amount of exercise performed was manipulated. As a 20% drop out was deemed possible 15 participants were recruited to the study.

Data was analysed using the PASW statistics software version 18.0 for Windows (SPSS Inc, Chicago, IL). The total and incremental (after correcting for fasting concentrations) area under the plasma concentration versus time curves for triacylglycerol and the total area under the plasma concentration versus time curves for glucose and insulin were calculated using the trapezium rule. Insulin resistance was evaluated according to the homeostatic model assessment of insulin resistance (HOMA-IR) (Matthews et al., 1985). Residual errors were tested for normality using the Shapiro–Wilk test and where necessary data was logarithmically transformed prior to statistical analysis. Fasting basal diameter, peak diameter, FMD, normalised FMD and plasma triacylglycerol, glucose and insulin concentration, areas under the plasma concentration versus time curves and HOMA-IR were compared between trials using paired Student's *t*-test. A mixed effects general linear model with two fixed factors (trial and time) and one random factor (participant) was used to compare differences between trials over time, and any possible interactions between trial and time, for basal diameter, peak diameter, FMD, normalised FMD and plasma triacylglycerol, glucose and insulin concentration. For further information on this design and analysis see Winer (1971). Where there was a violation of compound symmetry, based on Mauchly's test, the degrees of freedom were adjusted using the Huynh-Feldt epsilon. The Least Significant Difference was used to identify exactly where any main effects of time lay. When a significant trial x time interaction was revealed targeted

pairwise comparisons were used, specifically, comparisons within-trials with respect to the fasting measure and between trials at the same time point. Pearson's correlation coefficient was calculated in the control and in the exercise trial: for fasting FMD and plasma triacylglycerol concentration; for the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 3 h; for the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 6.5 h; for fasting FMD and basal diameter; for the change in FMD and basal diameter from 0 h to 3 h; and for the change in FMD and basal diameter from 0 h to 6.5 h (twelve correlations in total). Statistical significance was accepted at the $P<0.05$ level. All participant characteristics are presented as population marginal means (sd) and all experimental data are presented as population marginal means (sem).

6.3 Results

6.3.1 Responses to treadmill exercise

Mean oxygen uptake during the bouts of walking was 28.8 (sem 0.5) $mL \cdot kg^{-1} \cdot min^{-1}$, which represented 59.9 (sem 0.54)% of the participants $\dot{V}O_2$peak. Mean heart rate, ratings of perceived exertion and gross energy expenditure were 156 (sem 2) $beats \cdot min^{-1}$, 12 (sem 1) and 1918 (sem 142) kJ or 34.9 (sem 0.76) $kJ \cdot kg^{-1}$ body mass respectively.

6.3.2 Measures in the fasted state

No differences were observed between trials for fasting basal diameter (Table 6.2, P = 0.471), peak diameter (Table 6.2, P = 0.707), FMD (Figure 6.1(a), P = 0.449), normalised FMD (Figure 6.1(b), P = 0.379), plasma glucose concentration (Figure 6.2(b), P = 0.190) and plasma insulin concentration (Figure 6.2(c), P = 0.343). No differences were observed between trials for fasting HOMA-IR (Control vs. Exercise; 3.0 (sem 0.5) vs. 2.8 (sem 0.8), P = 0.971). Fasting plasma triacylglycerol concentration was 0.17 $mmol \cdot L^{-1}$ lower in the exercise trial (Figure 6.2(a); P = 0.001).

6.3.3 Measures in the postprandial state: Basal diameter, peak diameter, flow-mediated dilation and normalised flow-mediated dilation

Basal diameter and peak diameter increased following the ingestion of the high-fat test meals, but did not differ between trials (Table 6.2; basal diameter: main effect trial, $P = 0.239$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.253$; peak diameter: main effect trial, $P = 0.961$; main effect time, $P = 0.030$; interaction effect trial x time, $P = 0.190$). Postprandial FMD was lower in the control compared with the exercise trial both before (Figure 6.1(a); main effect trial, $P = 0.002$; main effect time, $P = 0.023$; interaction effect trial x time, $P = 0.088$) and after (Figure 6.1(b); main effect trial, $P = 0.003$; main effect time, $P = 0.002$; interaction effect trial x time, $P = 0.036$) normalisation for the post-occlusion shear rate. Pairwise comparisons identified that normalised FMD was significantly lower compared to the fasting measure at both 3 h ($P = 0.005$) and 6.5 h ($P = 0.013$) only in the control trial and that normalised FMD was significantly higher in the exercise trial, compared to the control trial, at 3 h ($P = 0.003$) and 6.5 h ($P = 0.011$).

Table 6.2. Basal diameter and peak diameter of the brachial artery during the measurement of flow-mediated dilation in the control and exercise trials*
(n 13)

	Control						Exercise					
	0 h		3 h		6.5 h		0 h		3 h		6.5 h	
	Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
Basal diameter (mm)†‡	3.54	0.15	3.64	0.15	3.70	0.15	3.51	0.15	3.60	0.14	3.61	0.15
Peak diameter (mm)‡	3.83	0.16	3.84	0.14	3.93	0.15	3.81	0.16	3.89	0.14	3.89	0.15

*Data was analysed using a mixed effects general linear model with two fixed factors (trial and time) and one random factor (participant): for both basal and peak diameter: main effect trial $P > 0.05$; main effect time $P < 0.05$; interaction effect trial x time, $P > 0.05$.
Least significant differences was used to identify where significant main effects lay.
† Main effect – time: Difference between 0 h and 3.5 h, $P < 0.05$
‡ Main effect – time: Difference between 0 h and 6.5 h, $P < 0.05$

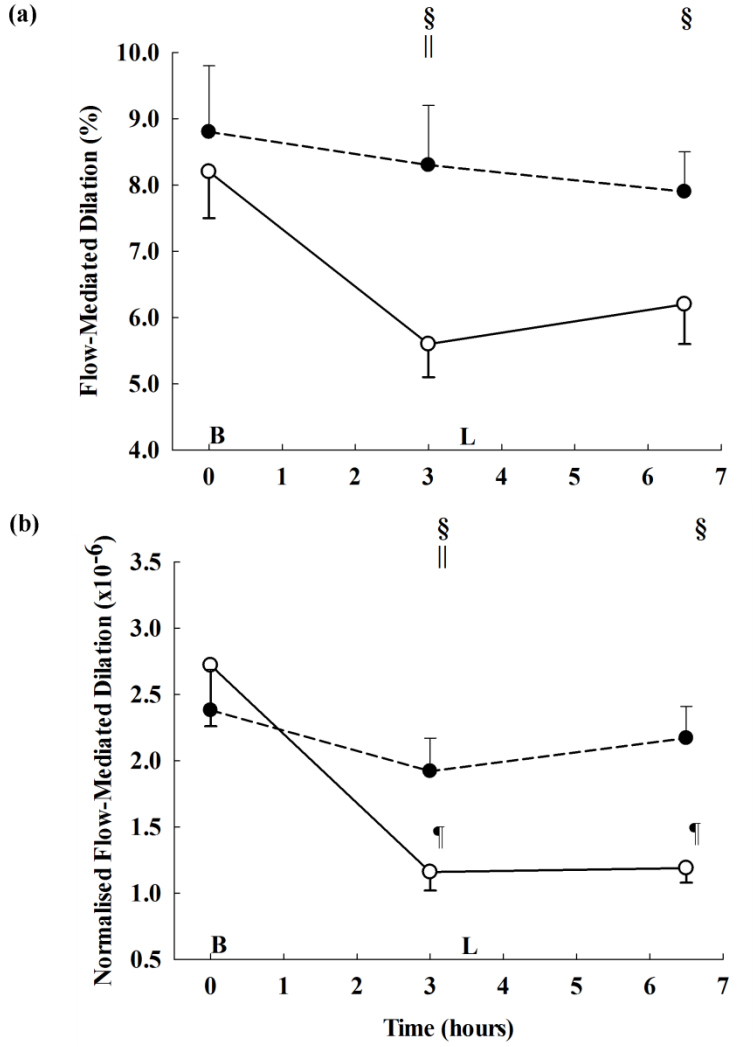


Figure 6.1. (a) Flow-mediated dilation and (b) normalised flow-mediated dilation. B = breakfast; L = lunch; values are mean (sem) (n 13); data were analysed using a mixed effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay and when a significant time x trial interaction was identified pre-specified planned comparisons were used; specifically, comparisons within-trial with respect to the fasting measure and between trials at the same time point. (a) Flow-mediated dilation: main effect trial, P = 0.002; main effect time, P = 0.023; interaction effect trial x time, P = 0.088. (b) Normalised flow-mediated dilation: main effect trial, P = 0.003, main effect time, P = 0.002, interaction effect trial x time, P = 0.036).

§ post-hoc analysis on main effect of time - different from 0 h, P < 0.05; || post-hoc analysis on main effect of time - different from previous time point, P < 0.05; ¶ post-hoc analysis on interaction effect of trial x time - different from 0 h in the same trial and different from exercise trial at same time point, P < 0.05.

Control Exercise

6.3.4 Measures in the postprandial state: Triacylglycerol, glucose and insulin concentrations

Postprandial plasma triacylglycerol concentration was lower in the exercise compared to the control trial (Figure 6.2(a); main effect trial, P = 0.009; main effect time, P < 0.001; interaction effect trial x time, P = 0.276). The total area under the plasma triacylglycerol concentration versus time curve was 22% lower in the exercise compared to the control trial (Control vs. Exercise; 12.68 (sem 1.37) vs. 9.84 (sem 0.75) $mmol \cdot L^{-1} \cdot 6.5h$, P = 0.018). The incremental area under the plasma triacylglycerol concentration versus time curve was 24% lower in the exercise compared to the control trial, however this did not reach statistical significance (Control vs. Exercise; 7.16 (sem 1.22) vs. 5.45 (sem 0.62) $mmol \cdot L^{-1} \cdot 6.5h$, P = 0.169).

Postprandial plasma glucose concentrations did not differ between trials (Figure 6.2(a); main effect trial, P = 0.877; main effect time, P < 0.001; interaction effect trial x time, P = 0.367). No differences were observed between the exercise and control trials for the total area under the plasma glucose concentration versus time curve (Control vs. Exercise; 42.91 (sem 0.66) vs. 42.62 (sem 0.83) $mmol \cdot L^{-1} \cdot 6.5h$, P = 0.429).

Postprandial plasma insulin concentrations did not differ between trials (Figure 6.2(c); main effect trial, P = 0.078; main effect time, P < 0.001; interaction effect trial x time, P = 0.558). However, the total area under the plasma insulin concentration versus time curve was lower in the exercise compared to the control trial (Control vs. Exercise; 1854.0 (sem 283.8) vs. 1713.6 (sem 276.0) $pmol \cdot L^{-1} \cdot 6.5h$, P = 0.047).

6.3.5 Correlations between flow-mediated dilation and basal diameter and triacylglycerol concentrations

The correlations between fasting FMD and plasma triacylglycerol concentrations were: Control, r = -0.106, P = 0.730; Exercise, r = 0.104, P = 0.736. The correlations between the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 3 h, were: Control, r = -0.138, P = 0.653; Exercise, r = 0.214, P = 0.482. The correlations between the change in FMD and the total area under the plasma

triacylglycerol concentration versus time curve from 0 h to 6.5 h, were: Control, r = 0.190, P = 0.534; Exercise, r = 0.223, P = 0.465.

The correlations between fasting FMD and basal diameter were: Control, r = -0.196, P = 0.521; Exercise, r = -0.310, P = 0.302. The correlations between the change in FMD and basal diameter from 0 h to 3 h, were: Control, r = -0.297, P = 0.325; Exercise, r = 0.246, P = 0.417. The correlations between the change in FMD and basal diameter from 0 h to 6.5 h, were: Control, r = -0.494, P = 0.086; Exercise, r = -0.42, P = 0.089.

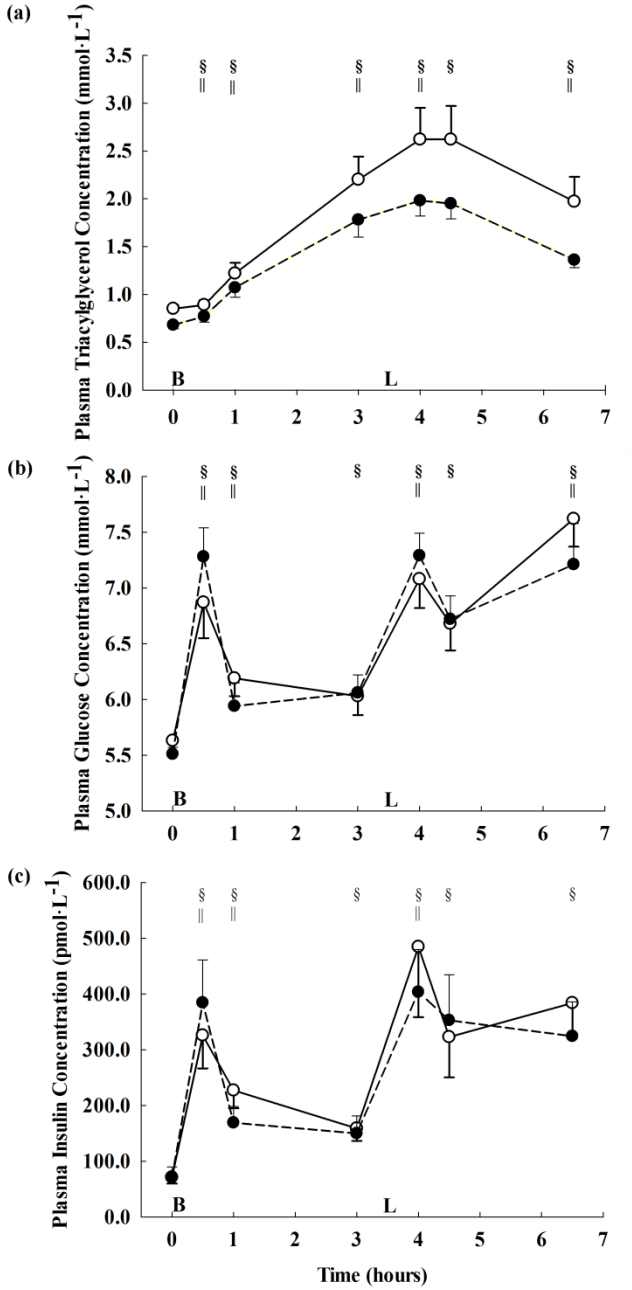


Figure 6.2. Plasma (a) triacylglycerol, (b) glucose and (c) insulin concentrations. B = breakfast; L = lunch; values are mean (sem) (n 13); data were analysed using a mixed effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay; triacylglycerol and insulin data were Ln transformed prior to the analysis. (a) Triacylglycerol: main effect trial, $P = 0.009$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.276$. (b) Glucose: main effect trial, $P = 0.877$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.361$. (c) Insulin: main effect trial, $P = 0.078$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.558$.
§ post-hoc analysis on main effect of time - different from 0 h, $P < 0.05$; || post-hoc analysis on main effect of time - different from previous time point, $P < 0.05$.

Control ○—— Exercise ●---

6.4 Discussion

The key finding of the present study was that, for the first time in adolescent boys, a bout of moderate-intensity exercise was shown to attenuate the decline in FMD observed following the ingestion of high-fat meals observed in a control trial. In fact, once FMD had been normalised for the post-occlusion shear rate, prior exercise prevented the postprandial decline in FMD entirely. No relationships were found between postprandial FMD and plasma triacylglycerol concentrations.

This is the first study to investigate endothelial function following the ingestion of high-fat meals in adolescent boys. The ingestion of a breakfast and lunch containing substantial quantities of fat (60% and 50% by energy content respectively) reduced FMD, compared to fasting, by 32% after breakfast and 24% after lunch. This observed endothelial dysfunction (as indicated by a decline in FMD) following the consumption of high-fat meals has been demonstrated repeatedly in adults. While this decline in FMD in asymptomatic adults can range between 17% (Nicholls et al., 2006) and 70% (Marchesi et al., 2000), the majority of studies report changes between 30-50% (Vogel et al., 1997; Bae et al., 2001; Gokce et al., 2001; Tushuizen et al., 2006; Tyldum et al., 2009). Importantly, the adolescent boys in the present study had none of the factors associated with CHD risk. Yet, the consumption of high-fat meals induced endothelial dysfunction to a similar extent to that previously observed in adults. Therefore, this study supports the assertion that ingestion of meals containing high quantities of fat place strain on the endothelium, and clearly demonstrates that this is the case even in healthy adolescents.

The ingestion of high-fat meals is not uncommon in adolescents, with 60% of teenagers in the United States of America reported to eat "fast food" (which is typically high in fat content) at least once a week (Taveras et al., 2005), and 30% reported to eat such foods every day (Schmidt et al., 2005). Therefore, a substantial number of adolescents may be exposed, on a regular basis, to the risks associated with the consumption of meals high in fat. While the observation period in the present study was very short, and the endothelial dysfunction following ingestion of a high fat meal has been shown to be transient, certainly in adults (Vogel et al., 1997; Marchesi et al., 2000), if the responses observed in this study were to be repeated on a regular basis over many years then the strain on the

vasculature could be substantial and have a lasting deleterious effect. Interventions that might reduce or prevent this strain on the vasculature could have important clinical implications. Research with adults suggests that moderate-intensity exercise undertaken before the ingestion of a high-fat meal reduces the subsequent postprandial endothelial dysfunction (Gill et al., 2004; Tyldum et al., 2009). Interestingly, if the exercise is of a high-intensity (85 to 95% of maximum heart rate) the dysfunction is prevented (Tyldum et al., 2009). In the present study we observed that after a bout of moderate-intensity exercise (performed 14 hours prior to the ingestion of the high-fat breakfast and lunch) the decline in FMD was attenuated. In fact, once FMD had been normalised for the post-occlusion shear rate, prior exercise prevented the postprandial decline in FMD entirely, indicating that prior exercise negated the deleterious effects of high-fat meals on the endothelium.

It has been argued that postprandial decreases in FMD do not signify endothelial dysfunction but are purely a consequence of an increase in basal diameter (Raitakari et al., 2000; Gokce et al., 2001). Arteries with large diameters have been shown to exhibit lower FMD than arteries with small diameters (Herrington et al., 2001; Silber et al., 2001; Pyke et al., 2004; Thijssen et al., 2008) as the post occlusion shear stress stimulus (the stimulus for post-occlusion dilation) is inversely related to vessel diameter (Herrington et al., 2001; Pyke et al., 2004). As an increase in mean basal diameter from 0 h to 3 h was observed in both the control and exercise trial, in the present study, which could influence the FMD responses, an attempt was made to account for this increase in basal diameter by normalising FMD to the area under the shear rate versus time curve. Normalised FMD is not associated with basal diameter (Pyke et al., 2004; Thijssen et al., 2008). As both FMD and normalised FMD responses to the meals and exercise were similar, despite the similar changes in basal diameter in the control and exercise trials, it is therefore likely that the effect of the high-fat mixed meals and continuous moderate-intensity exercise on FMD are not solely a consequence of changes in basal diameter. This is further supported by the lack of any significant correlation between the postprandial changes in basal artery diameter and FMD.

In the present study as well as observing lower postprandial triacylglycerol concentrations following exercise we also noted a reduction in fasting triacylglycerol concentration. The

reduction in fasting triacylglycerol concentration seen as a result of exercise in this study has also been observed frequently in previous studies (Barrett et al., 2007; Miyashita et al., 2006; Al-Shayji et al., 2012). Given that the correlations between fasting triacylglycerol concentration and FMD in the control and exercise trials in the present study were low and not statistically significant, it is unlikely that the differences in fasting triacylglycerol concentration in the control and exercise trials would have confounded the main results of the study.

In previous research a relationship between triacylglycerol concentrations and FMD has been demonstrated postprandially, but no relationship between these variables was evident in the fasted state (Plotnick et al., 1997; Vogel et al., 1997; Anderson et al., 2006). In previous studies where a significant relationship has been found between postprandial triacylglycerol concentrations and change in endothelial function, Pearson correlations have ranged from -0.31 (Bae et al., 2001) to -0.70 (Marchesi et al., 2000). This has led to the hypothesis that prior exercise can prevent the postprandial endothelial dysfunction by reducing postprandial triacylglycerol concentrations (Chandrruangphen & Collins, 2002; Gill et al., 2004). However, not all studies have observed this association (chapter 5 and Williams et al., 1999; Steer et al., 2003; Tushuizen et al., 2006; Rudolph et al., 2007; Wallace et al., 2010; Johnson et al., 2011). In the present study postprandial triacylglycerol concentrations were lower and FMD higher in the exercise compared with the control trial; but, we found no evidence of a direct association between these variables. A lack of association between the exercise induced changes in triacylglycerol concentration and endothelial function has also been noted by Tyldum and colleagues (2009) who found that exercise reduced postprandial endothelial dysfunction but did not affect postprandial triacylglycerol concentration. Gill and colleagues (2003a) also observed that postprandial endothelial function was similar before and after a period of detraining, despite postprandial triacylglycerol concentration being higher following detraining. There are a number of possible factors which may contribute to the inconsistent associations observed across studies between postprandial triacylglycerol and endothelial function including differences in the methods used to quantify variables, variability in the timing of measurements and in the timing of postprandial events in vivo, and differences in the lipid load and in the number of study participants (Wallace et al., 2010; Johnson et al., 2011).

The lack of association between triacylglycerol concentration and endothelial function in the present study may suggest that while postprandial changes in triacylglycerol concentrations and FMD may coincide, they are not dependent upon each other; however, due to the small sample size, such conclusions need to be drawn cautiously. Nonetheless, elevated postprandial triacylglycerol concentrations are an independent risk factor for CHD (Bansal et al., 2007) and, consistent with other reports from the literature which have used a single meal (Barrett et al., 2007; Tolfrey et al., 2008; MacEneaney et al., 2009; Tolfrey et al., 2012a), the results of the present study demonstrate that exercise reduces postprandial plasma triacylglycerol concentrations following the ingestion of two high-fat meals in adolescent boys.

In the exercise trial, despite no change in HOMA-IR, the total area under the plasma insulin concentration versus time curve was lower than in the control trial, while glucose concentrations were not different; suggesting that the insulin sensitivity of the participants was improved after exercise. The only other study that has measured both postprandial glucose and insulin concentrations in adolescents following high-fat mixed meals found no effect of exercise (MacEneaney et al., 2009). When HOMA-IR values were compared between the present study and the study by MacEneaney and colleagues (2009) the participants in the present study were found to be more insulin resistant, this may explain the difference in findings between the two studies. It is well established that during puberty a transient state of insulin resistance is experienced with the greatest insulin resistance occurring mid-puberty (Tanner stage 3) (Amiel et al., 1986; Moran et al., 1999). MacEneaney and colleagues (2009) did not state the pubertal status of the boys in their study however the boys were older than those in the present study (15.9 (sd 0.4) versus 13.6 (sd 0.6) years respectively) and therefore would be expected to be more mature and therefore less insulin resistant. Previous studies have identified greater insulin sensitivity in adolescents who are more physically active (Andersen et al., 2006; Rizzo et al., 2008; Mitchell et al., 2010) or have undergone a period of exercise training (Nassis et al., 2005; van der Heijden et al., 2009). The present study suggests that an acute bout of exercise also has the ability to improve insulin sensitivity. Clearly, this finding warrants further investigation.

The present study did not directly investigate the mechanisms by which exercise attenuates endothelial dysfunction but a number of possibilities have been proposed. Measures of endothelial function, including FMD, are essentially markers of nitric oxide bioavailability (Pyke & Tschakovsky, 2005), and a decrease in nitric oxide production and/or an increase in nitric oxide inactivation can result in a decline in FMD. It has been suggested that prior exercise may reduce the postprandial rise in oxidative stress by increasing the body's antioxidant capacity, consequently reducing the inactivation of nitric oxide, and so attenuating the decline in FMD (Tyldum et al., 2009). Exercise may also increase endothelial nitric oxide synthase mRNA expression, and induce phosphorylation of endothelial nitric oxide synthase (Hambrecht et al., 2003), both of which will increase the production of nitric oxide which would attenuate the decline in FMD. Therefore, there are several mechanisms which may explain why exercise reduces the strain experienced by the endothelium following ingestion of high-fat meals.

Due to the study design it is not possible to determine the mechanisms for the observed exercise induced reduction in triacylglycerol concentration but, based on other research literature, it appears that exercise reduces triacylglycerol concentration through two complementary pathways: an increased clearance of triacylglycerol rich lipoproteins and/or a reduced hepatic secretion of VLDL triacylglycerol (Gill & Hardman, 2003). Recent data from kinetic studies suggest the predominant cause of exercise induced reductions in triacylglycerol concentration is the increased clearance of triacylglycerol rich lipoproteins, with this increased clearance being mediated by the secretion of fewer but more triacylglycerol-rich VLDL particles by the liver following exercise (Magkos et al., 2006; Tsekouras et al., 2007; Al Shayji et al., 2012). These triacylglycerol rich VLDL particles are hydrolysed faster by lipoprotein lipase and removed faster from the circulation (Streja, 1979; Fisher et al., 1995). In addition, triacylglycerol clearance may be enhanced by increases in skeletal muscle lipoprotein lipase activity after exercise (Kiens & Lithell, 1989; Malkova et al., 2000).

6.5 Conclusions

In conclusion, 60 min of moderate-intensity exercise, performed 14 h before the ingestion of a high-fat breakfast and lunch, attenuated the postprandial endothelial dysfunction that

was observed in a control trial where no exercise was performed. These results demonstrate for the first time that meals high in fat place a strain on the endothelium of healthy adolescent boys, but also that exercise has prophylactic properties which negate at least some of the deleterious effects associated with the consumption of these meals.

Chapter 7: Effect of repeated short sprints on postprandial endothelial function and triacylglycerol concentrations in adolescent boys

7.1 Introduction

Acute exercise interventions have been effective at attenuating endothelial dysfunction or the rise in triacylglycerol concentrations evident after ingestion of meals high in fat. A single 30 to 180 min session of moderate-intensity exercise completed 12 to 18 h prior to the ingestion of a high-fat meal has consistently been shown to lower postprandial triacylglycerol concentrations (see chapter 6 and Gill & Hardman, 2003; Petitt & Cureton, 2003, Gill et al., 2004; Barrett et al., 2007; Tolfrey et al., 2008; MacEneaney et al., 2009; Tolfrey et al., 2012a) and more recently to attenuate postprandial endothelial dysfunction in both adults and adolescents (see chapter 6 and Gill et al., 2004; Tyldum et al., 2009). Repeated sprints of relatively long duration (30 s), interspersed with periods of active low-intensity recovery, have also been shown to elicit health benefits in adults (Gibala et al., 2012) including reductions in postprandial triacylglycerol concentrations (Freese et al., 2011; Gabriel et al., 2012). However, continuous moderate-intensity exercise or long duration sprinting is not typical of young people's activity patterns, which are usually intermittent in nature and characterised by multiple short-duration bouts of vigorous activity typically lasting less than 10 s (median duration 4 s) (Baquet et al., 2007; Stone et al., 2009a). Since the young appear to undertake very short bouts of vigorous activity, repeated short-duration (<10 s) sprints may offer a better and more attractive exercise model for the young. Barrett and colleagues (2006; 2007) did find that 90 minutes of games type activity (15 m sprints interspersed with walking, jogging and faster running) reduced postprandial triacylglycerol concentrations in adolescent boys and men, but it is not possible from these studies to establish what the effect of the short sprints alone was on the postprandial triacylglycerol concentrations. As well as being closer to the typical activity pattern of the young, repeated very short sprints may be more practical and better tolerated than the repeated 30 s sprints shown to induce health benefits in adults (Gibala et al., 2012), especially as the repeated longer sprints require high levels of motivation to complete and are often associated with feelings of nausea and light-headedness (Richards et al., 2010).

The effect of sprint exercise on postprandial endothelial function has not previously been investigated in adults or the young. However, repeated sprints (30 s), completed the evening prior to the ingestion of a high-fat meal have been reported to reduce postprandial triacylglycerol concentrations (Freese et al., 2011; Gabriel et al., 2012), and oxidative stress (Gabriel et al., 2012). As previous research suggests that the high-fat meal induced endothelial dusfunction is a consequence of high postprandial triacylglycerol concentrations and oxidative stress (Wallace et al., 2010), repeated sprint exercise could also have the ability to attenuate postprandial endothelial dysfunction.

Therefore, the purpose of the present study was to investigate the effect of a session of repeated very short duration (6 s) maximal sprints on postprandial endothelial function and triacylglycerol concentrations in apparently healthy adolescent boys. It was hypothesised that the repeated very short duration maximal sprints would attenuate postprandial endothelial dysfunction and lower postprandial triacylglycerol concentrations.

7.2 Experimental methods

7.2.1 Participants

Fifteen adolescent boys volunteered to participate in the study, with nine boys completing the study (aged 12.5 to 14.1 years, Tanner Stage 1 to 4 (median 3)). Non-completion of the study was due to a failure to tolerate the exercise (n = 5) or a dislike of the blood sampling procedures (n = 1). The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by Loughborough University Ethical Advisory Committee. Prior to testing the procedures and risks of participating in the study were explained to the boys. Following this written consent from a parent and written assent from each participant was obtained. Participants were asked to complete a health history questionnaire which indicated that they were apparently healthy and not taking any drugs known to affect lipid or carbohydrate metabolism. Participant characteristics are presented in Table 7.1.

Table 7.1. Physical characteristics of participants
(n 9)

	Mean	sd
Age (years)	13.1	0.6
Height (m)	1.59	0.08
Body mass (kg)	49.7	9.8
BMI ($kg \cdot m^{-2}$)	19.4	2.3
Sum of four skin folds (mm)[a]	45.2	12.9
Waist circumference (cm)	70.9	7.6
Hip circumference (cm)	86.4	8.2
Tanner Stage: Genital[b]	3	2 - 5
Tanner Stage: Pubic Hair[b]	3	1 - 5
Age from PHV (years)	-0.7	0.9

BMI, Body mass index; PHV, Peak height velocity.
[a] Four skinfold sites were biceps, triceps, subscapular and suprailiac.
[b] Values are median (range).

7.2.2 Study design

Following preliminary tests participants undertook two, 2-day main trials which were separated by seven days (control trial and exercise trial) in a 2 x 2 replicated Latin square design.

7.2.3 Preliminary tests

Height, sitting height, body mass, skinfold thicknesses (biceps, triceps, subscapular and suprailiac), waist circumference and hip circumference were measured (see section 3.4). Age from peak height velocity was also estimated using equations developed by Mirwald and colleagues (2002). Each participant undertook a self-assessment of maturity using a five-point scale described by Tanner (1989) (see section 3.4.4). Finally each participant was familiarised with the repeated sprint exercise.

7.2.4 Exercise familiarisation

Participants were asked to warm-up, against an air resistance setting of 1, on a cycle ergometer (Wattbike, Nottingham, UK). The warm-up consisted of 5 min cycling at 60 rev·min^{-1}, 5 min stretching, 3 x 30 s cycling at 80 rev·min^{-1} and 2 x 30 s cycling at 100 rev·min^{-1} with 30 s recovery between sprints. When the warm-up was finished participants were asked to complete 3 x 6 s maximal sprints with 90 s recovery between sprints. Following a 5 min rest participants completed one block of the sprint exercise: 10 x 6 s maximal sprints with 90 s recovery between sprints.

7.2.5 Main trials

Participants were asked not to consume alcohol or supplements in the seven days prior to the main trials and to refrain from undertaking exercise and to be as physically inactive as possible for one day before each main trial. In order to standardise their diet participants were asked to complete a food diary for one day before and on day 1 of the first main trial and were then asked to replicate this diet for the subsequent trial.

On day 1 of the control trial participants were asked to be physically inactive; whereas in day 1 of the exercise trial they were asked to be physically inactive until 15:30 when they reported to the laboratory to complete a warm up and four blocks of 10 x 6 s sprints (as described above in section 7.2.4). Each block was separated by 3 min of rest. During the last sprint and the 90 s recovery period following each block; heart rate was recorded, ratings of perceived exertion assessed (Borg, 1973) and oxygen uptake was determined using standard Douglas bag techniques (Williams & Nute, 1983). Energy expenditure was estimated using the mechanical work done, assuming a mechanical efficiency of 18.5 % (Smith & Hill, 1991) and assuming 1 L of oxygen uptake equates to 20.3 kJ (Frayn & Macdonald, 1997).

On day 2 of each main trial following an overnight fast participants reported to the laboratory at 08:00, where FMD was assessed (see section 3.7) and a capillary blood sample collected (see section 3.8). Participants were then given breakfast (see section 3.6.1) and a clock was started when participants took their first mouthful of the meal.

Having eaten breakfast participants rested for 6.5 h. Participants were provided with lunch (see section 3.6.2) at 3.5 h. Further measures of FMD were undertaken at 3 and 6.5 h and additional blood samples were collected at 0.5, 1, 3, 4, 4.5 and 6.5 h.

Collected capillary blood was centrifuged (see sections 3.8) and the resulting plasma was later analysed to determine plasma triacylglycerol, glucose and insulin concentrations (see section 3.9). Concentrations were not adjusted for plasma volume because plasma volumes were similar between trials and across time (main effect trial, $P = 0.423$; main effect time, $P = 0.430$; interaction effect trial x time, $P = 0.928$).

7.2.6 Statistical analysis

Based on previously published data and pilot research conducted in our laboratory the size of the effect of the ingestion of exercise on postprandial FMD and triacylglycerol concentrations was expected to be large (Cohen's d, ~0.9). Consequently, a priori power calculations (ES = 0.9; $\alpha = 0.05$, $\beta = 0.8$, two tailed) suggested that a sample size of 12 would be sufficient to discern differences in FMD when the fat content of the meals or the amount of exercise performed was manipulated. As a 20% drop out was deemed possible 15 participants were recruited to the study.

The total and incremental (after correcting for fasting concentrations) area under the plasma concentration versus time curves for triacylglycerol and the total area under the plasma concentration versus time curves for glucose and insulin were calculated using the trapezium rule. Insulin resistance was estimated using the HOMA-IR (Matthews et al., 1985). Data was analysed using the PASW statistics software version 18.0 for Windows (SPSS Inc, Chicago, IL). Normality of the residual errors were tested using the Shapiro–Wilk test and where necessary data was logarithmically transformed prior to statistical analysis. Paired Student's t-test were used to compare differences between trials for; fasting basal diameter, peak diameter, FMD, normalised FMD and plasma triacylglycerol, glucose and insulin concentration, areas under the plasma concentration versus time curves and HOMA-IR. A mixed effects general linear model with two fixed factors (trial and time) and one random factor (participant) was used to compare differences between trials over time, and any possible interactions between trial and time, for basal diameter, peak

diameter, FMD, normalised FMD and plasma triacylglycerol, glucose and insulin concentration. For further information on this design and analysis see Winer (1971). Where there was a violation of compound symmetry, based on Mauchly's test, the degrees of freedom were adjusted using the Huynh-Feldt epsilon. The Least Significant Difference was used to identify exactly where any main effects of time lay. When a significant trial x time interaction was revealed targeted pairwise comparisons were used, specifically, comparisons within-trials with respect to the fasting measure and between trials at the same time point. Pearson's correlation coefficient was calculated in the control and in the exercise trial: for fasting FMD and plasma triacylglycerol concentration; for the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 3 h; for the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 6.5 h; for fasting FMD and basal diameter; for the change in FMD and basal diameter from 0 h to 3 h; and for the change in FMD and basal diameter from 0 h to 6.5 h (twelve correlations in total). Statistical significance was accepted at the $P<0.05$ level. All participant characteristics are presented as population marginal means (sd) and all experimental data are presented as population marginal means (sem).

7.3 Results

7.3.1 Responses to sprint exercise

Peak power, mean power, peak cadence and mean cadence during the 6 s sprints were 472 (sem 41) W, 417 (sem 33) W, 113 (sem 3) $rev \cdot min^{-1}$ and 104 (sem 3) $rev \cdot min^{-1}$ respectively. Peak heart rate, mean heart rate and ratings of perceived exertion were 156 (sem 6) $beats \cdot min^{-1}$, 133 (sem 5) $beats \cdot min^{-1}$ and 12 (sem 1) respectively. The total mechanical work done during each 6 s sprint was 2.5 (sem 0.6) kJ resulting in a total mechanical work done over the 40 sprints of 98.9 (sem 7.9) kJ. Energy expenditure over the 40 sprints was estimated to be between: 533 (sem 43) kJ (if a mechanical efficiency of 18.5% was assumed) and 1077 (sem 52) kJ (assuming that 1 L oxygen uptake is equivalent to 20.3 kJ).

7.3.2 Measures in the fasted state

No differences were observed between trials for fasting basal diameter (Table 7.2, P = 0.235), peak diameter (Table 7.2, P = 0.186), FMD (Fig. 7.1(a), P = 0.479), normalised FMD (Fig. 7.1(b), P = 0.231), plasma triacylglycerol concentration (Fig 7.2 (a), P = 0.141), plasma glucose concentration (Fig. 7.2(b), P = 0.780) and plasma insulin concentration (Fig. 7.2(c), P = 0.070). No differences were observed between trials for fasting HOMA-IR (Control vs. Exercise; 2.1 (sem 0.3) vs. 1.6 (sem 0.2), P = 0.971).

7.3.3 Measures in the postprandial state: Basal diameter, peak diameter, flow-mediated dilation and normalised flow-mediated dilation.

Basal diameter and peak diameter increased following the ingestion of the high-fat test meals, but did not differ between trials (Table 7.2; basal diameter: main effect trial, P = 0.209; main effect time, P < 0.001; interaction effect trial x time, P = 0.131; peak diameter: main effect trial, P = 0.614; main effect time, P = 0.002; interaction effect trial x time, P = 0.052).

Postprandial FMD was lower in the control compared with the exercise trial (Fig 7.1(a); main effect trial, P = 0.012; main effect time, P = 0.004; interaction effect trial x time, P = 0.003). Pairwise comparisons identified that FMD was lower compared to the fasting measure at both 3 h (P = 0.003) and 6.5 h (P = 0.003) in the control trial only and that FMD was significantly higher in the exercise trial, compared to the control trial, at 3 h (P = 0.002) and 6.5 h (P = 0.002). Normalised FMD decreased following the ingestion of the high-fat meals, but did not differ between trial (Fig. 7.1(b); main effect trial, P = 0.968; main effect time, P = 0.001; interaction effect trial x time, P = 0.077).

Table 7.2. Basal diameter and peak diameter of the brachial artery during the measurement of flow-mediated dilation in the control and exercise trials*
(n 9)

	Control						Exercise					
	0 h		3 h		6.5 h		0 h		3 h		6.5 h	
	Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
Basal diameter (mm) †‡	3.33	0.11	3.47	0.10	3.45	0.11	3.28	0.11	3.39	0.10	3.44	0.11
Peak diameter (mm) †‡	3.60	0.13	3.70	0.13	3.66	0.13	3.54	0.14	3.66	0.13	3.70	0.13

*Data was analysed using a mixed effects general linear model with two fixed factors (trial and time) and one random factor (participant): for both basal and peak diameter: main effect trial $P > 0.05$; main effect time $P < 0.05$; interaction effect trial x time, $P > 0.05$.
Least significant differences was used to identify where significant main effects lay.
† Main effect – time: Difference between 0 h and 3.5 h, $P < 0.05$
‡ Main effect – time: Difference between 0 h and 6.5 h, $P < 0.05$

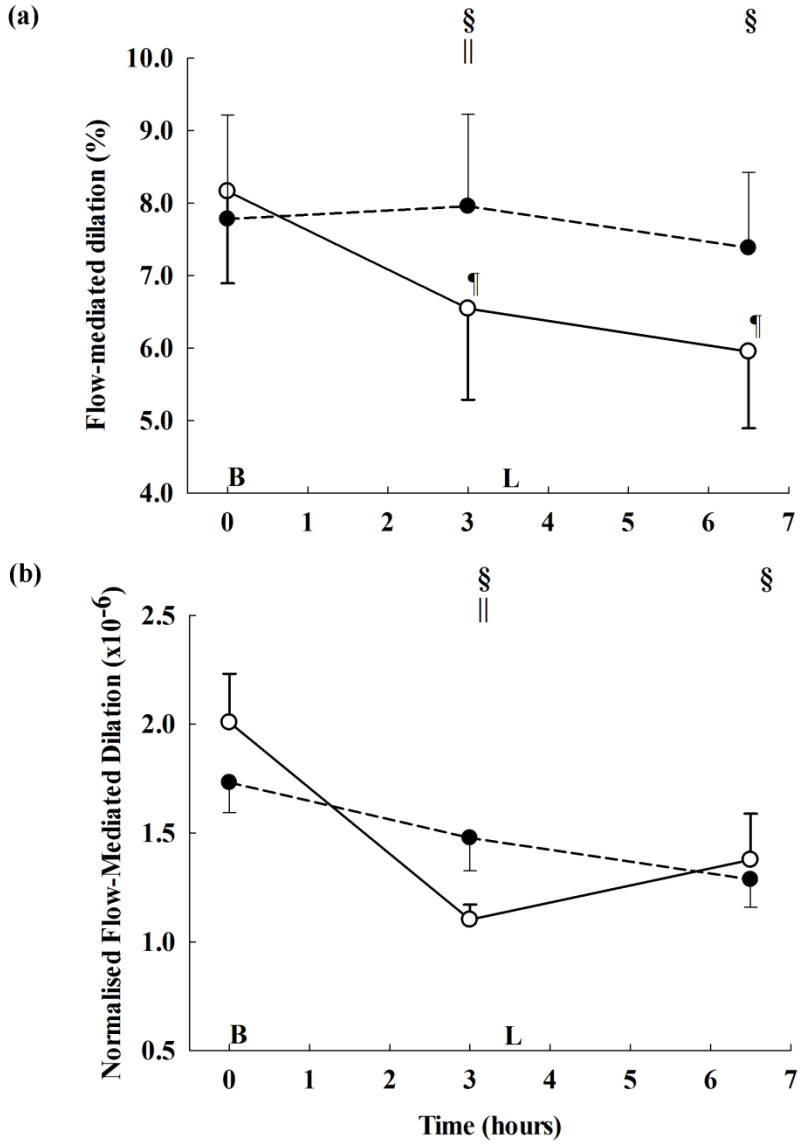


Figure 7.1. (a) Flow-mediated dilation and (b) normalised flow-mediated dilation. B, breakfast; L, lunch. Values are means (sem) (n 9). Data were analysed using a mixed-effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay and when a significant time x trial interaction was identified, pre-specified planned comparisons were used, specifically, comparisons within trial with respect to the fasting measure and between trials at the same time point. (a) Flow-mediated dilation: main effect trial, P = 0.012; main effect time, P = 0.004; interaction effect trial x time, P = 0.003. (b) Normalised flow-mediated dilation: main effect trial, P = 0.968; main effect time, P = 0.001; interaction effect trial x time, P = 0.077).

§ post-hoc analysis on main effect of time - different from 0 h, $P < 0.05$; || post-hoc analysis on main effect of time - different from previous time point, $P < 0.05$; ¶ post-hoc analysis on interaction effect of trial x time - different from 0 h in the same trial and different from exercise trial at same time point, $P < 0.05$.

Control Exercise

7.3.4 Measures in the postprandial state: Triacylglycerol, glucose and insulin concentrations

Postprandial plasma triacylglycerol concentration was lower in the exercise compared to the control trial (Fig. 7.2(a); main effect trial, P = 0.019; main effect time, P < 0.001; interaction effect trial x time, P = 0.071). The total area under the plasma triacylglycerol concentration versus time curve was 13% lower following exercise (Exercise vs. Control: 8.65 (sem 0.97) vs. 9.92 (sem 1.16) $mmol \cdot L^{-1} \cdot 6.5h$, P = 0.023). The incremental area under the plasma triacylglycerol concentration versus time curve was 15% lower following exercise (Exercise vs. Control: 4.52 (sem 0.61) vs. 5.32 (sem 0.75) $mmol \cdot L^{-1} \cdot 6.5h$, P = 0.036).

Postprandial plasma glucose concentrations did not differ between trials (Fig. 7.2(b); main effect trial, P = 0.120; main effect time, P < 0.001; interaction effect trial x time, P = 0.389). The total area under the plasma glucose concentration versus time curve did not differ between trials (Exercise vs. Control: 42.22 (sem 1.40) vs. 41.13 (sem 0.89) $mmol \cdot L^{-1} \cdot 6.5h$, P = 0.223).

Postprandial plasma insulin concentrations did not differ between trials (Fig 7.2(c); main effect trial, P = 0.266; main effect time, P = 0.001; interaction effect trial x time, P = 0.491). The total area under the plasma insulin concentration versus time curve did not differ between trials (Exercise vs. Control: 1829.4 (sem 273.0) vs. 1741.8 (sem 224.4) $pmol \cdot L^{-1} \cdot 6.5h$, P = 0.379).

7.3.5 Correlations between flow-mediated dilation and basal diameter and triacylglycerol concentrations

The correlations between fasting FMD and plasma triacylglycerol concentrations were: Control, r = -0.604, P = 0.085; Exercise, r = -0.394, P = 0.294. The correlations between the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 3 h, were: Control, r = 0.289, P = 0.451; Exercise, r = -0.059, P = 0.880. The correlations between the change in FMD and the total area under the plasma

triacylglycerol concentration versus time curve from 0 h to 6.5 h, were: Control, r = 0.514, P = 0.157; Exercise, r = 0.210, P = 0.587.

The correlations between fasting FMD and basal diameter were: Control, r = 0.164, P = 0.672; Exercise, r = 0.371, P = 0.325. The correlations between the change in FMD and basal diameter from 0 h to 3 h, were: Control, r = -0.417, P = 0.265; Exercise, r = 0.546, P = 0.128. The correlations between the change in FMD and basal diameter from 0 h to 6.5 h, were: Control, r = -0.539, P = 0.134; Exercise, r = 0.417, P = 0.264.

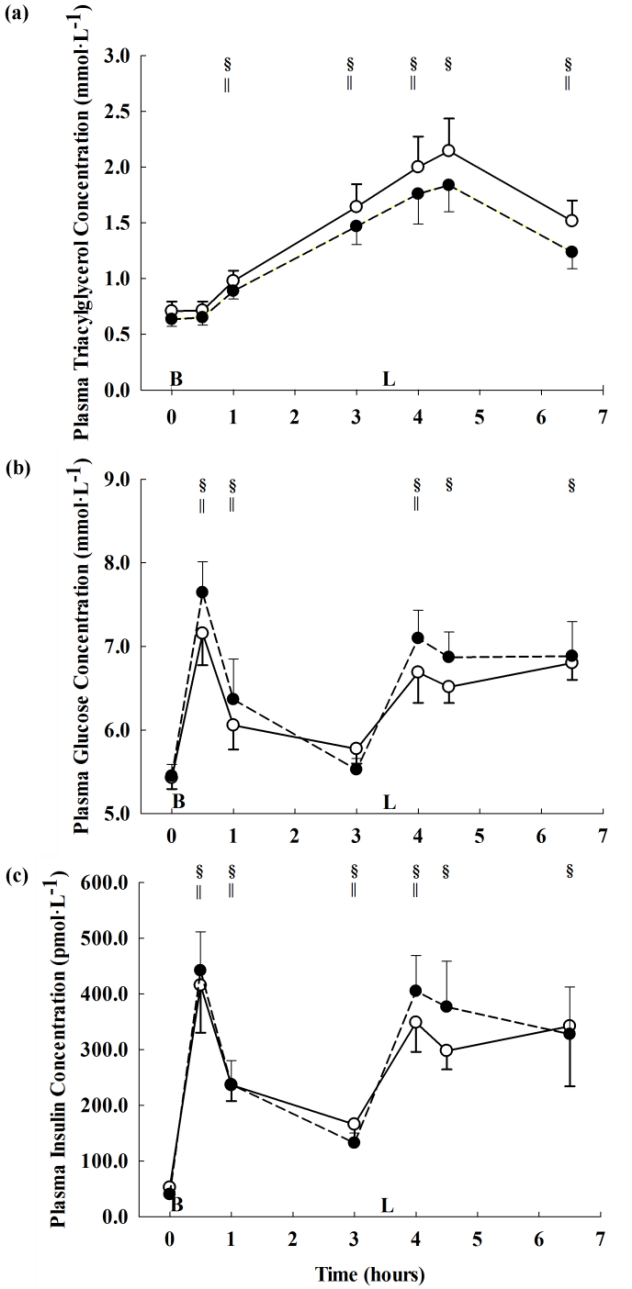


Figure 7.2. Plasma (a) triacylglycerol (b) glucose (c) insulin concentrations. B, breakfast; L, lunch. Values are means (sem) (n 9). Data were analysed using a mixed-effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay; triacylglycerol data were Ln transformed prior to the analysis. (a) Triacylglycerol: main effect trial, P = 0.019; main effect time, P < 0.001; interaction effect trial x time, P = 0.071. (b) Glucose: main effect trial, P = 0.120; main effect time, P < 0.001; interaction effect trial x time, P = 0.389. (c) Insulin: main effect trial, P = 0.266; main effect time, P = 0.001; interaction effect trial x time, P = 0.491.

§ post-hoc analysis on main effect of time - different from 0 h, P < 0.05; || post-hoc analysis on main effect of time - different from previous time point, P < 0.05.

Control ○—— Exercise ●---

7.4 Discussion

This is the first study to investigate the effect of a session of repeated sprints on postprandial endothelial dysfunction and triacylglycerol concentrations in adolescents. The main finding of the present study was that 40 maximal 6 s sprints, completed 14 hours prior to the consumption of a high-fat breakfast and high-fat lunch, attenuated the decline in FMD evident in a control trial in healthy adolescent boys. The prior session of 6 s sprints also lowered postprandial triacylglycerol concentrations, reducing the total and incremental area under the triacylglycerol concentration versus time curves by 13% and 15% respectively.

In the present study postprandial endothelial dysfunction was evident in the control trial following breakfast and lunch with FMD being reduced, compared to fasting, by 20 and 27% respectively. This state of endothelial dysfunction following consumption of high-fat meals has been shown previously in adults (see chapter 5 and Vogel et al, 1997; Marchesi et al., 2000; Gill et al., 2003a; Gill et al., 2004; Tyldum et al., 2009) and in adolescents (see chapter 6). In contrast when the adolescent boys in the present study completed the 40 maximal 6 s sprints 14 hours prior to the high-fat breakfast, postprandial endothelial dysfunction was not apparent, as there was no change in FMD compared to fasting. This finding is consistent with a previous study in adults that used high-intensity intermittent exercise (4 x 4 min cycle at 85 to 95% of maximum heart rate with each interspersed with 3 min cycle at 50 to 60% of maximum heart rate) which also found no evidence of endothelial dysfunction when exercise preceded consumption of a high fat meal (Tyldum et al., 2009). While exercise of a moderate intensity has also been shown to influence postprandial endothelial function in adults (Gill et al., 2004; Tyldum et al., 2009) and adolescents (see chapter 6) the beneficial effects may be more muted when the exercise intensity is moderate as these studies tended to only find an attenuation in the extent of postprandial endothelial dysfunction. While there may be some uncertainty as to whether high-intensity or repeated very short sprint exercise has a greater effect on postprandial endothelial function than moderate-intensity exercise, clearly the exercise model used in the present study was effective based on the FMD responses.

It has been recommended that FMD should be normalised to account for the post-occlusion shear stress stimulus (Pyke & Tschakovsky, 2005) as this removes any association with basal diameter (Pyke et al., 2004; Thijssen et al., 2008). In the present study normalised FMD was observed to decrease following the ingestion of the high-fat meals but, in contrast with the non-normalised findings, this decrease was not attenuated by the sprint exercise. Previous studies investigating the effect of continuous moderate-intensity and high-intensity intermittent exercise on postprandial FMD have reported attenuation in the postprandial decline in both FMD and normalised FMD (see chapter 6 and Tyldum et al., 2009), so the findings in the present study are perhaps surprising. However, any conclusions based on the results of normalised FMD may need to be made cautiously as the methods used to normalise FMD are currently under debate (Atkinson et al., 2009; McCully, 2012; Stoner et al., 2013).

The reduction in triacylglycerol concentrations evident in the present study following the session of 40 maximal 6 s sprints is consistent with previous research in adults which has investigated the effect of sprint exercise on postprandial triacylglycerol concentrations. Four to five, 30 s maximal effort sprints, completed 14 to 18 hours prior to the consumption of a high-fat meal, were found to lower subsequent postprandial triacylglycerol concentrations in adults by 18 to 21% (Freese et al., 2011; Gabriel et al., 2012). Maximum effort cycle sprints also seemed more effective at reducing postprandial triacylglycerol concentrations than moderate-intensity exercise (30 min brisk treadmill walking) (Gabriel et al., 2012). In previous studies in adolescents, moderate-intensity exercise has been reported to reduce the total and incremental area under the plasma triacylglycerol concentrations versus time curve by 14 to 22% and 5 to 38% respectively (see chapter 6 and Barrett et al., 2007; Tolfrey et al., 2008; MacEneaney et al., 2009; Tolfrey et al., 2012a). In the present study the reductions were 13 and 15% respectively, demonstrating that repeated bouts of very short maximal sprints are effective at reducing postprandial triacylglycerol concentrations. These findings, and the FMD finding described above, support the assertion that repeated sprints of very short duration offer an alternative mode of exercise to moderate-intensity exercise to reduce postprandial triacylglycerol concentrations in adolescents, and that the typical activity pattern undertaken by children and adolescents during games and free play, involving intermittent bursts of vigorous activity, is probably sufficient to gain health benefits.

In adults it appears that a dose-response relationship exists between energy expenditure induced by exercise and the subsequent reductions seen in postprandial triacylglycerol concentrations (Petitt & Cureton, 2003), although this relationship may not exist in adolescent boys (Tolfrey et al., 2008; Tolfrey et al., 2012a). Research also suggests that a threshold energy expenditure of 2000 kJ is required to lower postprandial triacylglycerol concentrations (Maraki & Sidossis, 2010). It is therefore perhaps surprising that repeated very short duration sprinting lowered postprandial triacylglycerol concentrations in the present study as the energy expenditure associated with the exercise was certainly much lower than the 2000 kJ threshold. This 2000 kJ threshold must be viewed sceptically as a number of studies have observed an effect of exercise on postprandial triacylglycerol concentrations when the exercise induced an energy exercise of less than 2000 kJ (Miyashita, 2008; Miyashita & Tokuyama, 2008; Miyashita et al., 2008; Peddie et al., 2012). While it must be noted that the accurate measurement of energy expenditure during sprinting is problematic, the findings of the present and previous studies (Freese et al., 2011; Gabriel et al., 2012) suggest that the effect of sprint exercise on postprandial triacylglycerol concentrations may not be dependent on energy expenditure, or that, when elicited by sprint exercise, much lower energy expenditures are sufficient to lower postprandial triacylglycerol concentrations.

It has been hypothesised that prior exercise protects against postprandial endothelial dysfunction by reducing postprandial triacylglycerol concentrations (Chandrruangphen & Collins, 2002; Gill et al., 2004) as a number of studies have reported significant correlations between postprandial triacylglycerol concentrations and endothelial dysfunction (Wallace et al., 2010). However, as in the present study, not all studies have observed such correlations (see chapter 5 and 6 and Williams et al., 1999; Steer et al., 2003; Tushuizen et al., 2006; Rudolph et al., 2007; Johnson et al., 2011). Discrepancies in these findings may be a consequence of the large range of methods used to quantify the postprandial triacylglycerol concentrations and endothelial dysfunction across studies (Wallace et al., 2010). Although we did observe parallel alterations in postprandial triacylglycerol concentrations and FMD in the present study, it appears that exercise-induced alterations in postprandial endothelial function may not be dependent on alterations in postprandial triacylglycerol concentrations (Gill et al., 2003a; Tyldum et al.,

2009). Further research must be conducted to identify if the effects of exercise on postprandial triacylglycerol concentrations and endothelial dysfunction are linked.

Although this study did not directly measure the mechanisms through which sprint exercise may protect against postprandial endothelial dysfunction, it is known that measures of endothelial function, including FMD, are markers of nitric oxide bioavailability (Doshi et al., 2001; Corretti et al., 2002), and a decrease in nitric oxide production and/or an increase in nitric oxide inactivation can result in a decline in FMD. It is believed that a transient rise in oxidative stress parallel to a rise in triacylglycerol concentrations following a high-fat meal induces postprandial endothelial dysfunction (Anderson et al., 2001; Tsai et al., 2004; Wallace et al., 2010) through the ability of free radicals to inactivate nitric oxide (Beckman & Koppenol, 1996). Prior exercise may reduce the postprandial rise in oxidative stress by increasing the body's antioxidant capacity, consequently reducing the inactivation of nitric oxide, and so attenuating the decline in FMD (Tyldum et al., 2009). Such a mechanism could explain why high-intensity exercise may be more effective than moderate-intensity exercise at attenuating postprandial endothelial dysfunction as it has been shown, at least in adults, to ellicit a greater total antioxidant capacity (Tyldum et al., 2009) and to attenuate the postprandial rise in oxidative stress (Gabriel et al., 2012). Exercise may also increase endothelial nitric oxide synthase mRNA expression, and induce phosphorylation of endothelial nitric oxide synthase (Hambrecht et al., 2003), both of which will increase the production of nitric oxide which would attenuate the decline in FMD.

Exercise involving repeated very short duration sprinting may lower postprandial triacylglycerol concentrations through a combination of increased triacylglycerol-rich lipoprotein clearance from the circulation and a decreased hepatic secretion of VLDL-triacylglycerol (Gill & Hardman, 2003). Prior exercise has been shown to increase skeletal muscle lipoprotein lipase activity (Kiens & Lithell, 1989; Malkova et al., 2000), localised to the contracting muscles (Hamilton et al., 1998), resulting in an increased hydrolysis and clearance of triacylglycerol from the bloodstream. The vigorous nature of contractions and the larger recruitment of muscle mass seen during sprint exercise could conceivably increase skeletal muscle lipoprotein lipase activity to a greater extent than moderate-intensity exercise and may explain why various sprinting regimens reduce postprandial

triacylglycerol concentrations, despite the low energy expenditure. Sprint exercise is also associated with large increases in the concentrations of catecholamines and growth hormone (Ekbolm et al., 1971; Kindermann et al., 1982; Weltman et al., 1994; Pullinen et al., 1998; Stokes, 2003) which in turn are associated with increases in skeletal muscle lipoprotein lipase activity (Eckel et al., 1996; Oscarsson et al., 1999; Pedersen et al., 1999). More recent evidence has also identified that exercise can reduce hepatic secretion of VLDL-triacylglycerol and alter the composition of VLDL particles, resulting in larger, more triacylglycerol-enriched VLDL particles (Magkos et al., 2006; Tsekouras et al., 2007; Al-Shayji et al., 2012). This triacylglycerol enrichment of VLDL particles increases their affinity with lipoprotein lipase ultimately increasing their hydrolysis and triacylglycerol clearance from the circulation (Streja, 1979; Fisher et al., 1995).

Longer duration (e.g. 30 s) repeated sprint protocols require high levels of motivation to complete and are often associated with feelings of nausea and light-headedness (Richards et al., 2010). It was thought that the repeated 6 s sprints in the present study would be less likely to elicit these type of responses, and that the short intense bursts of activity associated with repeated short sprints might be more enjoyable and more appropriate for the young given their typical activity patterns, certainly when compared with moderate-intensity exercise, (Bartlett et al., 2011; Crisp et al., 2012). However, of the 15 recreationally active individuals who participated in the present study, 5 proved unable to tolerate the exercise pattern even though the sprints were only of 6 s duration. Typically these participants stopped exercising because they felt light-headed and nauseous; in 2 cases participants vomited. These responses occurred after only 7 (sd 2) sprints which suggests that some individuals cannot easily tolerate sprinting per se. It should be acknowledged that a low-intensity active recovery between the repeated sprints may be one method to increase tolerance to this type of sprint exercise, as it may reduce venous pooling in the legs and reduce feelings of light-headiness and nausea (Rakobowchuk et al., 2008), but this was not done in the present study in order to investigate the physiological effects of repeated short sprints alone. Future studies might also examine the effect of intense, although not maximal, sprints which may be better tolerated.

While the potential health benefits associated with repeated sprint exercise seem clear (Gibala et al., 2012) it has also been argued that repeated sprint protocols have the added

benefit of being time efficient (Burgomaster et al., 2008). As lack of time is often cited as an obstacle to involvement in an exercise programme the apparent shorter duration of sprint exercise based sessions may be attractive to the larger population (Tappe et al., 1989). While the duration of sprint exercise in the present study was only 4 min (11 min of exercise in total if the warm-up was included) the total duration of the repeated sprint protocol was 90 min (the length of an association football game), which is 50% greater in duration than the 60 min of moderate-intensity exercise currently recommended in the physical activity guidelines for adolescents (Department of Health, 2011). So while the duration of actual exercise was short the total exercise session would represent a substantial time commitment.

7.5 Conclusions

In conclusion the findings of the present study demonstrate that a session of 40 maximal effort 6 s sprints, completed 14 hours prior to the consumption of a high-fat breakfast and lunch, attenuated postprandial endothelial dysfunction and reduced postprandial triacylglycerol concentrations. This study is the first to demonstrate that exercise based on repeated very short duration sprints can elicit health benefits in adolescents; however, among some individuals, tolerance for this specific type of exercise may be poor. It is important to recognise that sprint exercise may be unsuitable for many individuals (for example those with pre-existing medical conditions or suseptiblity to injury) and should not be recommended for all populations. However, if short sprints could be incorporated into regularly undertaken activity/training programmes it is possible that health benefits could be gained

Chapter 8: The accumulation of exercise and postprandial endothelial function and triacylglycerol concentrations in adolescent boys

8.1 Introduction

Current physical activity guidelines (60 min of moderate to vigorous-intensity physical activity daily for children and adolescents) indicate that the recommended volume of physical activity does not need to be undertaken in a single, continuous bout but can be accumulated, over the day, in multiple bouts of shorter duration (World Health Organization, 2010; Department of Health, 2011). Despite this, experimental research supporting this concept of accumulating activity in the young is, at best, sparse. However, such an activity pattern may be particularly suitable for the young, as young people's activity bouts are typically spontaneous and of a short duration (Stone et al., 2009a; Stone et al., 2009b) and the young are also known to perceive prolonged exercise to be more demanding than adults (Timmons & Bar-Or, 2003). In addition, if the duration of exercise bouts are short it may be possible to undertake exercise at a higher intensity, ultimately resulting in a larger volume of exercise being completed throughtout the day. Consequently, accumulating physical activity in multiple short-duration bouts over a day may not just be suitable, but also an appealing activity pattern for the young.

Previous research in adults has observed that a single session of continuous moderate-intensity walking or running, completed 14 to 16 hours before the ingestion of a high-fat meal, can attenuate postprandial endothelial dysfunction (Gill et al., 2004; Tyldum et al., 2009) and lower postprandial triacylglycerol concentrations (Gill & Hardman, 2003). In chapter 6 of this thesis prior continuous moderate-intensity walking was shown to attenuate postprandial endothelial dysfunction and lower postprandial triacylglycerol concentrations in adolescent boys. Research in adults has observed that similar reductions in postprandial triacylglycerol concentrations are evident when an equal volume of exercise is completed in a single session or in multiple, short-duration sessions spread throughout the day (Gill et al., 1998; Miyashita et al., 2006; Miyashita, 2008; Miyashita et al., 2008). The effect of accumulating exercise on postprandial endothelial dysfunction and triacylglycerol concentrations has yet to be investigated in children or adolescents.

Therefore the purpose of this study was to investigate if accumulating 60 min of running could attenuate postprandial endothelial dysfunction and lower postprandial triacylglycerol concentrations in adolescent boys. It was hypothesised that accumulating a total of 60 min of moderate-intensity running would attenuate postprandial endothelial dysfunction and lower postprandial triacylglycerol concentrations.

8.2 Experimental methods

8.2.1 Participants

Seventeen adolescent boys volunteered to participate in the study, with fourteen boys completing the study (aged 12.1 to 14.4 years). Non-completion of the study was due to signs and symptoms of presyncope during blood sampling (n = 3). The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by Loughborough University Ethical Advisory Committee. Before any testing took place all participants had the procedures and risks associated with involvement in the study explained to them. Written consent from a parent and written assent from each participant was then obtained. All participants were apparently healthy and not taking any drugs known to affect lipid or carbohydrate metabolism. The physical characteristics of those who completed the study are shown in Table 8.1.

Table 8.1. Physical characteristics of participants
(n 14)

	Mean	sd
Age (years)	12.9	0.7
Height (m)	1.54	0.08
Body mass (kg)	48.4	10.0
BMI ($kg \cdot m^{-2}$)	20.3	3.5
Sum of four skin folds (mm)[a]	53.5	26.3
Waist circumference (cm)	70.3	8.3
Hip circumference (cm)	89.5	11.2
Waist : Hip ratio	0.82	0.04
Age from PHV (years)	-1.0	0.6
Tanner Stage: Genital[b]	3	2 – 4
Tanner Stage: Pubic Hair[b]	3	1 – 4
$\dot{V}O_2$peak ($mL \cdot kg^{-1} \cdot min^{-1}$)	45.4	7.9

BMI, Body mass index, PHV, Peak height velocity, $\dot{V}O_2$peak, Peak oxygen uptake.
[a] Four skinfold sites were biceps, triceps, subscapular and suprailiac.
[b] Values are median (range)**.**

8.2.2 Study design

Participants completed preliminary tests and then undertook two, 2-day main trials which were separated by seven days (a control trial and an exercise trial) in a 2 x 2 replicated Latin square design.

8.2.3 Preliminary tests

Height, sitting height, body mass, skinfold thicknesses (biceps, triceps, subscapular and suprailiac), waist circumference and hip circumference were measured (see section 3.4). A self-assessment of maturity, based on secondary sexual characteristics, was completed (Tanner, 1989) (see section 3.4.4). Age from peak height velocity was also estimated (Mirwald et al., 2002) (see section 3.4.1).

Following anthropometric measures a sub-maximal treadmill test (see section 3.5.3.1) was completed in order to determine the relationship between running speed and oxygen uptake which was followed by an incremental uphill treadmill test until volitional fatigue to determine $\dot{V}O_2$peak (see section 3.5.3.2).

8.2.4 Main trials

Participants were instructed not to consume alcohol or supplements in the seven days before and throughout each main trial. Participants recorded all food and drinks consumed during the day before (day 0) and day 1 of the first main trial and were asked to replicate this diet prior to the subsequent trial. Participants were also instructed to refrain from any physical activity, except that prescribed by the investigators, on day 0 and throughout each main trial. During this time participants wore an ActiGraph GT1M accelerometer during waking hours to monitor physical activity. The ActiGraph is a uniaxial electromechanical device that records movement in the vertical plane. The sampling epoch was 5 seconds. During data processing 60 minutes of consecutive zeroes was considered indicative of non-wearing and these data were excluded. Minimum day length was set at 9 hours and time spent in low- and moderate- and vigorous-intensity physical activity was calculated using age specific cutpoints (Freedson et al., 1997) and daily energy expenditure estimated using previously published equations (Trost et al., 1998).

On day 1 of the control trial participants were instructed to be as physically inactive as possible and to refrain from any form of exercise whilst in their free-living environment, whereas in the exercise trial they reported to the laboratory at 09:00 and completed 6 x 10 min runs on a treadmill at 70% of $\dot{V}O_2$peak. Each run was followed by 50 min of rest. The first run began at 09:30 resulting in the final run ceasing at 14:40. Heart rate, ratings of perceived exertion and expired air samples were collected in the last minute of each run. Energy expenditure was estimated using indirect calorimetry (Frayn, 1983). On leaving the laboratory after the last run the participants were reminded to be as physically inactive as possible and to refrain from any form of exercise whilst in their free-living environment.

On day 2 of each main trial participants reported to the laboratory at 08:00, following an overnight fast, where FMD was assessed (see section 3.7) and a capillary blood sample

collected (see section 3.8). Participants then ate breakfast (see section 3.6.1) at 08:40 and a clock was started on commencement of the meal. After breakfast participants rested for 6.5 h in the laboratory with lunch (see section 3.6.1) provided at 3.5 h. Flow-mediated dilation was assessed again at 3 and 6.5 h and additional blood samples collected at 0.5, 1, 3, 4, 4.5 and 6.5 h.

Collected capillary blood was centrifuged (see sections 3.8) and the resulting plasma was later analysed to determine plasma triacylglycerol, glucose and insulin concentrations (see section 3.9). Concentrations were not adjusted for plasma volume because plasma volumes were similar between trials and across time (main effect trial, $P = 0.615$; main effect time, $P = 0.113$; interaction effect trial x time, $P = 0.533$).

8.2.5 Statistical analysis

Based on previously published data and pilot research conducted in our laboratory the size of the effect of the ingestion of exercise on postprandial FMD and triacylglycerol concentrations was expected to be large (Cohen's d, ~0.9). Consequently, a priori power calculations (ES = 0.9; $\alpha = 0.05$, $\beta = 0.8$, two tailed) suggested that a sample size of 12 would be sufficient to discern differences in FMD when the fat content of the meals or the amount of exercise performed was manipulated. With the additional time requirements in this study a 30% drop out was deemed possible, therefore 17 participants were recruited to the study.

Data was analysed using the PASW statistics software version 18.0 for Windows (SPSS Inc, Chicago, IL). The total and incremental (after correcting for fasting concentrations) areas under the plasma concentration versus time curves for triacylglycerol and total area under the plasma concentration versus time curves for glucose and insulin were calculated using the trapezium rule. Insulin resistance was evaluated according to HOMA-IR (Matthews et al., 1985). Residual errors were tested for normality using the Shapiro–Wilk test, where necessary data was logarithmically transformed prior to statistical analysis. Time spent in low and moderate-to-vigorous intensity physical activity during day 0 and day 1 of the main trials were compared between trials using paired Student's t-test. Fasting basal diameter, peak diameter, FMD, normalised FMD and plasma triacylglycerol,

glucose and insulin concentration and the areas under the plasma concentration versus time curves were compared between trials using paired Student's *t*-test. A mixed effects general linear model with two fixed factors (trial and time) and one random factor (participant) was used to compare differences between trials over time, and any possible interactions between trial and time, for basal diameter, peak diameter, FMD, normalised FMD and plasma triacylglycerol, glucose and insulin concentration. For further information on this design and analysis see Winer (1971). Least significant difference post-hoc analysis was used to identify where any significant effects of time lay. When a significant trial x time interaction was revealed targeted pair wise comparisons were used, specifically, comparisons within-trial with respect to the fasting measure and between-trials at the same time point. Pearson's correlation coefficient was calculated in the control and in the exercise trial: for fasting FMD and plasma triacylglycerol concentration; for the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 3 h; for the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 6.5 h; for fasting FMD and basal diameter; for the change in FMD and basal diameter from 0 h to 3 h; and for the change in FMD and basal diameter from 0 h to 6.5 h (twelve correlations in total). Statistical significance was accepted as $P < 0.05$. All participant characteristics are presented as population marginal means and standard deviations (sd) and experimental data are presented as population marginal means and standard error of the mean (sem).

8.3 Results

8.3.1 Physical activity

Complete accelerometer data was provided by ten boys. One boy and three boys did not provide data for day 0 in the control and exercise trial, respectively. During day 0, no differences were observed between trials in the time spent in low-, moderate- and vigorous-intensity physical activity (Table 8.2; $P = 0.605$, $P = 0.516$ and $P = 0.848$, respectively). During day 1 less time was spent in low- and moderate-intensity and more in vigorous-intensity physical activity in the exercise trial compared to the control trial (Table 8.2; all $P < 0.001$). During day 0 energy expenditure was not different between trials, but during day 1 energy expenditure was 1129 (sem 205) kJ greater during the

exercise trial compared to the control trial (Table 8.2; P = 0.294 and P < 0.001, respectively).

Table 8.2. Time spent in low-, moderate- and vigorous-intensity physical activity and estimated energy expenditure during the day before the main trials (day 0) and day 1 of the main trials
(n 10 for day 0 and n 14 for day 1)

		Control		Exercise		
		Mean	sem	Mean	sem	P^a
Low-intensity (min)	Day 0	136	13	130	13	0.606
	Day 1	138	8	93	8	<0.001
Moderate-intensity (min)	Day 0	69	10	64	10	0.652
	Day 1	67	6	41	5	<0.001
Vigorous-intensity (min)	Day 0	12	3	15	3	0.516
	Day 1	14	2	68	3	<0.001
Energy expenditure (kJ)[b]	Day 0	7084	1109	6900	1143	0.294
	Day 1	6417	747	7546	720	<0.001

[a] Paired Student's t-test.
[b]Energy expenditure estimated as $4.18 * [(-2.23 + (0.0008 * \overline{counts \cdot min}^{-1}) + (0.08 * body\ mass\ (kg)) * wear\ time$ (min)](Trost et al., 1998)

8.3.2 Responses to treadmill exercise

The six, 10 min bouts of running elicited a mean heart rate, ratings of perceived exertion and oxygen uptake of 169 (sem 4) beats·min^{-1}, 13 (sem 1) and 32.6 (sem 1.5) ml·kg^{-1}·min^{-1} (72 (sem 1)% of participants $\dot{V}O_2$peak) respectively. The gross energy expenditure of the total exercise protocol (6 x 10 min) was 1900 (sem 79) kJ or 39.2 (sem 1.7) kJ·kg^{-1} body mass.

8.3.3 Measures in the fasted state

No differences were observed between trials for fasting basal diameter (Table 8.3, P = 0.477), peak diameter (Table 8.3, P = 0.650), FMD (Fig 8.1(a), P = 0.664), normalised FMD (Fig 8.1(b), P = 0.329), plasma triacylglycerol concentration (Fig 8.2(a), P = 0.706), plasma glucose concentration (Fig 8.2(b), P = 0.471) and plasma insulin concentration (Fig

8.2(c), P = 0.656). No differences were observed between trials for fasting HOMA-IR (Control vs. Exercise: 1.6 (sem 0.3) vs. 1.5 (sem 0.2), P = 0.625).

8.3.4 Measures in the postprandial state: Basal diameter, peak diameter, flow-mediated dilation and normalised flow-mediated dilation

Basal and peak diameter increased following the ingestion of the high-fat meals, but no differences were evident between trials (Table 8.3; basal diameter: main effect trial, P = 0.178; main effect time, P < 0.001; interaction effect trial x time, P = 0.088; peak diameter: main effect trial, P = 0.524; main effect time, P = 0.004; interaction effect trial x time, P = 0.972).

Postprandial FMD was lower in the control compared with the exercise trial both before (Fig 8.1(a); main effect trial, P = 0.004; main effect time, P < 0.001; interaction effect trial x time, P < 0.001) and after normalisation for the post-occlusion shear rate (Fig 8.1(b); main effect trial, P = 0.020; main effect time, P < 0.001; interaction effect trial x time, P = 0.014). Pairwise comparisons revealed that FMD, both before and after normalisation for the post-occlusion shear rate, was significantly lower than the fasting measure at both 3 h (P < 0.001) and 6.5 h (P < 0.001) in the control trial only. Pairwise comparisons also revealed that FMD, both before and after normalisation, was significantly higher in the exercise trial compared to the control trial at 3 h (P < 0.001 and P = 0.048) and 6.5 h (P < 0.001 and P = 0.002).

Table 8.3. Basal diameter and peak diameter of the brachial artery during the measurement of flow-mediated dilation in the control and exercise trials*
(n 13)

	Control						Exercise					
	0 h		3 h		6.5 h		0 h		3 h		6.5 h	
	Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
Basal diameter (mm) †‡	3.12	0.08	3.24	0.08	3.27	0.08	3.14	0.09	3.20	0.10	3.23	0.09
Peak diameter (mm) ‡	3.38	0.08	3.43	0.08	3.46	0.08	3.40	0.09	3.46	0.10	3.49	0.09

*Data was analysed using a mixed effects general linear model with two fixed factors (trial and time) and one random factor (participant): for both basal and peak diameter: main effect trial $P > 0.05$; main effect time $P < 0.05$; interaction effect trial x time, $P > 0.05$.
Least significant differences was used to identify where significant main effects lay.
† Main effect – time: Difference between 0 h and 3.5 h, $P < 0.05$
‡ Main effect – time: Difference between 0 h and 6.5 h, $P < 0.05$

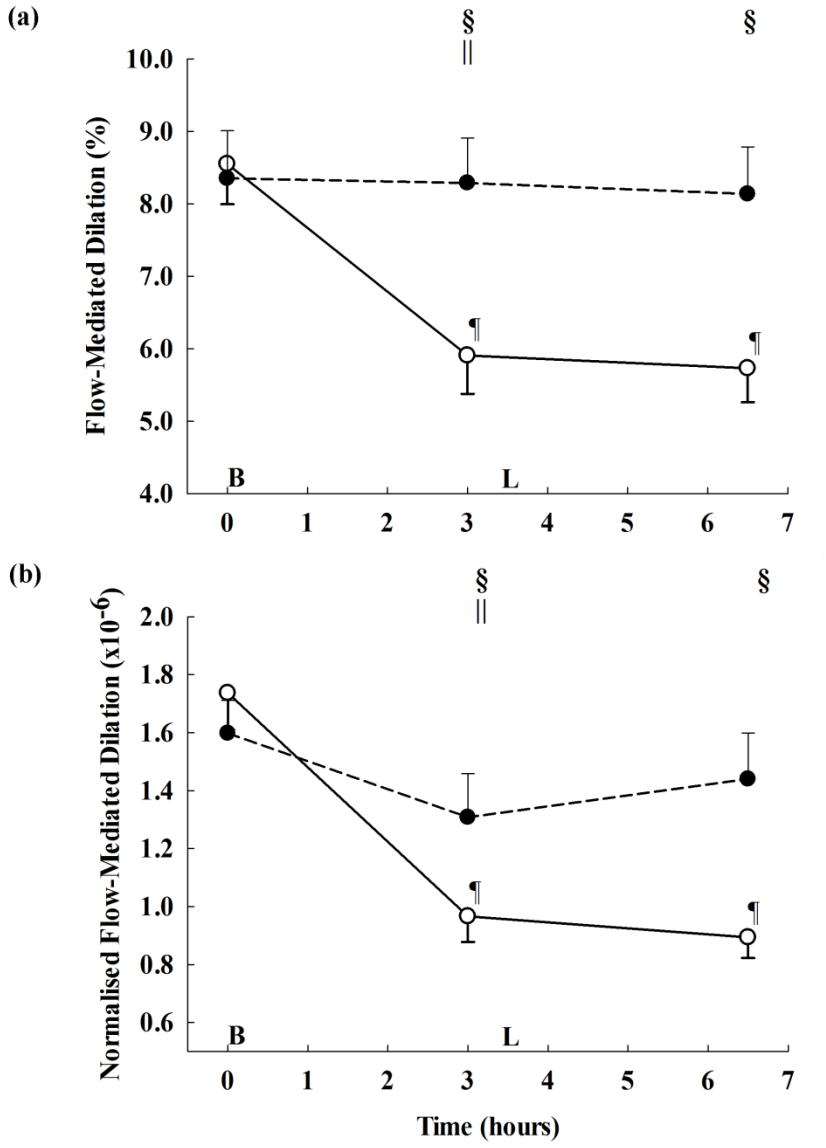


Figure 8.1. (a) Flow-mediated dilation and (b) normalised flow-mediated dilation. B, breakfast; L, lunch. Values are means (sem) (n 13). Data were analysed using a mixed-effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay and when a significant time x trial interaction was identified, pre-specified planned comparisons were used, specifically, comparisons within trial with respect to the fasting measure and between trials at the same time point. (a) Flow-mediated dilation: main effect trial, $P = 0.004$; main effect time, $P < 0.001$; interaction effect trial x time, $P < 0.001$. (b) Normalised flow-mediated dilation: main effect trial, $P = 0.020$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.014$.

§ post-hoc analysis on main effect of time - different from 0 h, $P < 0.05$; || post-hoc analysis on main effect of time - different from previous time point, $P < 0.05$; ¶ post-hoc analysis on interaction effect of trial x time - different from 0 h in the same trial and different from exercise trial at same time point, $P < 0.05$.

Control ○—— Exercise ●- - -

8.3.5 Measures in the postprandial state: Triacylglycerol, glucose and insulin concentrations

Postprandial plasma triacylglycerol concentrations did not differ between trials (Fig 8.2(a); main effect trial, P = 0.385; main effect time, P < 0.001; interaction effect trial x time, P = 0.091). The total area under the plasma triacylglycerol concentration versus time curve was 11% lower on average in the exercise compared to the control trial; however, this did not reach statistical significance (Control vs. Exercise: 10.71 (sem 0.94) vs. 9.56 (sem 0.67) $mmol \cdot L^{-1} \cdot 6.5h$, respectively, P = 0.183). The incremental area under the plasma triacylglycerol concentration versus time curve was 16% lower in the exercise compared to the control trial, however this did not reach statistical significance (Control vs. Exercise: 6.13 (sem 0.63) vs. 5.14 (sem 0.41) $mmol \cdot L^{-1} \cdot 6.5h$, respectively, P = 0.088).

Postprandial plasma glucose concentrations did not differ between trials (Fig 8.2(b); main effect trial, P = 0.916; main effect time, P < 0.001; interaction effect trial x time, P = 0.066). No difference was observed between the control and exercise trial for the total area under the plasma glucose concentration versus time curve (Control vs. Exercise: 41.17 (sem 0.79) vs. 41.32 (sem 0.84) $mmol \cdot L^{-1} \cdot 6.5h$, P = 0.741).

Postprandial plasma insulin concentrations did not differ between trials (Fig 8.2(c); main effect trial, P = 0.435; main effect time, P < 0.001; interaction effect trial x time, P = 0.515). No difference was observed between the control and exercise trial for the total area under the plasma insulin concentration versus time curve (Control vs. Exercise: 1685.4 (sem 207.6) vs. 1642.2 (sem 255.0) $pmol \cdot L^{-1} \cdot 6.5h$, P = 0.271).

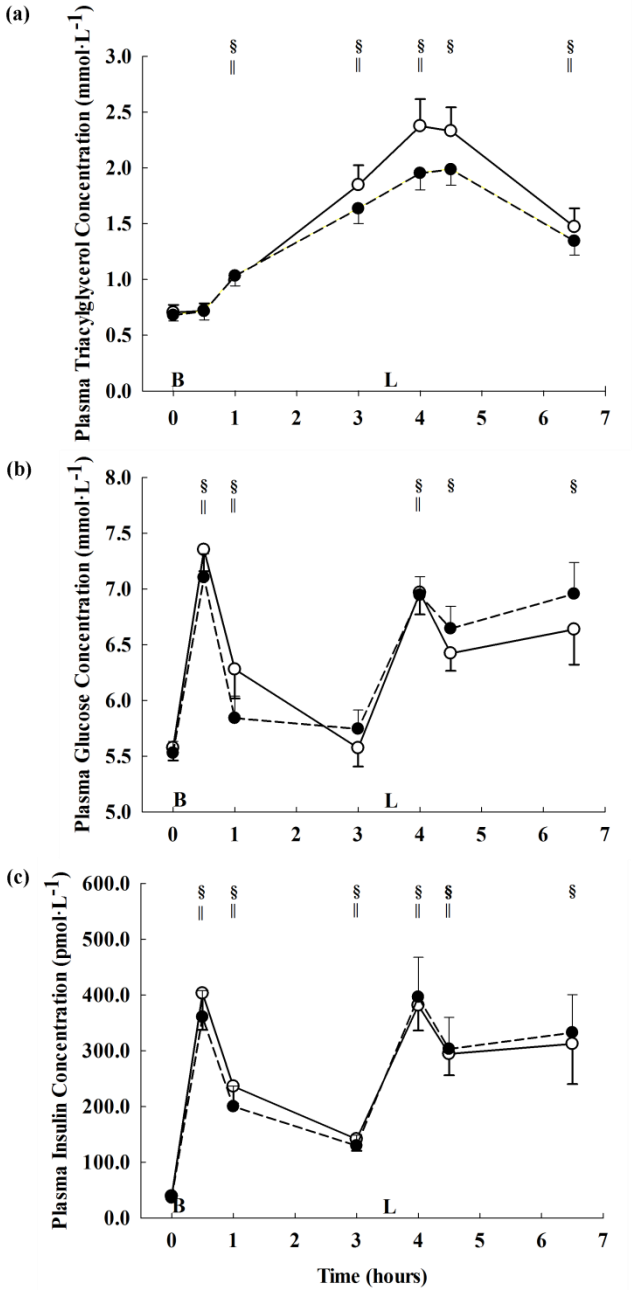


Figure 8.2. Plasma (a) triacylglycerol (b) glucose (c) insulin concentrations. B, breakfast; L, lunch. Values are means (sem) (n 14). Data were analysed using a mixed-effects general linear model with two fixed factors (trial and time) and one random factor (participant). Least significant differences post-hoc analysis was used to identify where significant main effects lay; triacylglycerol data were Ln transformed prior to the analysis. (a) Triacylglycerol: main effect trial, $P = 0.385$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.091$. (b) Glucose: main effect trial, $P = 0.916$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.066$. (c) Insulin: main effect trial, $P = 0.435$; main effect time, $P < 0.001$; interaction effect trial x time, $P = 0.515$.

§ post-hoc analysis on main effect of time - different from 0 h, $P < 0.05$; || post-hoc analysis on main effect of time - different from previous time point, $P < 0.05$.

Control ○—○ Exercise ●---●

8.3.6 Correlations between flow-mediated dilation and basal diameter and triacylglycerol concentrations.

The correlations between fasting FMD and fasting plasma triacylglycerol concentrations were: Control, r = 0.062, P = 0.841; Exercise, r = -0.347, P = 0.245. The correlations between the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 3 h, were: Control, r = -0.073, P = 0.812; Exercise, r = -0.133, P = 0.666. The correlations between the change in FMD and the total area under the plasma triacylglycerol concentration versus time curve from 0 h to 6.5 h, were: Control, r = -0.295, P = 0.328; Exercise, r = -0.342, P = 0.253.

The correlations between fasting FMD and fasting basal diameter were: Control, r = -0.653, P = 0.016; Exercise, r = -0.554, P = 0.049. The correlations between the change in FMD and basal diameter from 0 h to 3 h, were: Control, r = -0.244, P = 0.422; Exercise, r = 0.396, P = 0.181. The correlations between the change in FMD and basal diameter from 0 h to 6.5 h, were: Control, r = -0.218, P = 0.473; Exercise, r = -0.243, P = 0.424.

8.4 Discussion

The main finding of the present study was that, for adolescent boys, accumulating 60 min of exercise in short bouts throughout a day, before the consumption of a high-fat breakfast and lunch, attenuated the postprandial decline in FMD seen in the control trial. The total and incremental areas under the triacylglycerol concentration versus time curve were reduced by 11% and 16% respectively when the accumulated exercise trial was compared with the control, although these differences were not statistically significant.

In the present study postprandial endothelial dysfunction was evident in the control trial following breakfast and lunch with FMD being reduced, compared to fasting, by 30 and 33% respectively (both $P < 0.05$). This postprandial endothelial dysfunction following food ingestion has been seen previously in both adults (see chapter 5 and Vogel et al., 1997; Marchesi et al., 2000; Gill et al., 2003a; Gill et al., 2004; Wallace et al., 2010) and adolescents (see chapter 6 and 7). When the adolescent boys in the present study accumulated 60 min of exercise in short bouts throughout a day (6 x 10 min of treadmill

running at 70% $\dot{V}O_2$peak), the postprandial endothelial dysfunction seen in the control trial was not apparent as fasting FMD and postprandial FMD in the exercise trial were similar. This finding is consistent with previous studies that used a longer continuous bout of exercise rather than the shorter accumulated bouts used in the present study (see chapter 6 and Gill et al., 2004; Tyldum et al., 2009). When FMD was normalised, for the area under the shear rate vs. time curve, the postprandial decline in FMD was still evident in the control trial and again the accumulated exercise attenuated this decline. Therefore, the results from this study demonstrate for the first time, that accumulating 60 min of exercise in short bouts throughout a day is effective at attenuating postprandial endothelial dysfunction, at least in adolescent boys.

The six short bouts of exercise accumulated over a day also attenuated postprandial triacylglycerol responses, with the total and incremental areas under the triacylglycerol concentration versus time curve reduced by 11% and 16% respectively when the exercise trial was compared with the control, although these differences were not statistically significant ($P < 0.183$ and $P < 0.088$ respectively). In previous studies involving adolescents' continuous moderate-intensity exercise reduced total and incremental area by 14-22% and 5-38% respectively (see chapter 6 and Barrett et al., 2007; Tolfrey et al., 2008; MacEneaney et al., 2009; Tolfrey et al., 2012a), so the findings from the present study seem consistent with those previously seen. In adults 30 min of moderate-intensity exercise, undertaken either in a single bout or accumulated over short bouts throughout a day, proved equally effective at significantly lowering postprandial triacylglycerol concentrations (Miyashita et al., 2006; Miyashita, 2008; Miyashita et al., 2008). In the present study, despite the changes in postprandial triacylglycerol concentrations induced by the accumulated exercise not reaching statistical significance, the reduction in the total and incremental area under the triacylglycerol concentration versus time curve is in line with previous research in adolescents, and may still be meaningful and have physiological importance.

Analysis of the accelerometer data collected in the two days preceding the day when the high-fat meals were consumed, undertaken to confirm the physical activity levels of the study's participants, demonstrated that the time spent in low-, moderate- and vigorous-intensity physical activity was not different during day 0 of the control and exercise trials.

During day 1 of the exercise trial the boys did spend more time in vigorous-intensity physical activity, as would have been expected, but they actually spent less time in low- and moderate-intensity physical activity than in the control trial (see Table 8.2). Consequently, although the boys expended 1900 (sem 79) kJ during the prescribed exercise, the estimated difference in total daily energy expenditure between the exercise and control trials was substantially less than this (estimated to be 1128 (sem 205) kJ greater on day 1 of the exercise trial compared with day 1 of the control trial). Therefore, although the participants were instructed to be as physically inactive as possible and to refrain from any form of exercise whilst in their free-living environment, their adherence to these instructions, perhaps not unexpectedly, was poorer under free-living conditions compared with that achieved in the more controlled laboratory conditions where physical inactivity was ensured. The estimated energy expenditures were calculated using a regression equation reported by Trost and colleagues (1998) between accelerometer counts and energy expenditure, determined through indirect calorimetry, during treadmill walking. In a cross validation they reported that this regression equation predicted energy expenditure to 0.4 (standard error of the estimate 3.9) $kJ \cdot min^{-1}$ which if extrapolated to a nine hour wear time result in the daily energy expenditure lying within 4114 kJ of the estimate (95% confidence). Clearly, there are large errors associated with estimating energy expenditure using accelerometry, but the estimates obtained in this study do provide a picture of what activity (and the associated energy expenditure) the boys undertook.

The smaller than expected net increase in energy expenditure may have influenced the participant's triacylglycerol responses in the present study. Previous research in adults suggests that the effect of prior exercise on postprandial triacylglycerol concentrations is dependent on the energy expended (Petitt and Cureton, 2003). Maraki and Sidossis have argued that an expenditure of at least 2000 kJ is required to observe any reduction in postprandial triacylglycerol concentrations (Maraki and Sidossis, 2010), although prior moderate-intensity exercise has been shown to lower postprandial triacylglycerol concentrations when the energy expended has been below 2000 kJ (Miyashita, 2008; Miyashita & Tokuyama, 2008; Miyashita et al., 2008; Peddie et al., 2012).. Recent work in adolescents has failed to establish that energy expenditure per se is the primary determinant of attenuated postprandial triacylglycerol concentrations. Tolfrey and

colleagues (2008, 2012) reported similar reductions in postprandial triacylglycerol concentrations in adolescent boys when 60 min of running at 53% or 75% $\dot{V}O_2$peak (Tolfrey et al., 2008) and 30 min or 60 min of running at 55% $\dot{V}O_2$peak (Tolfrey et al., 2012) was completed 15 h before the ingestion of a high-fat milkshake. Further research is required to establish the influence of energy expenditure or the presence of an energy expenditure threshold in adolescent boys. Postprandial endothelial function appears to be more sensitive to vigorous-intensity exercise (Tyldum et al., 2009). Therefore, the substantial increase in vigorous-intensity exercise but smaller increase in total energy expenditure could explain why endothelial dysfunction was attenuated, but a smaller than expected and non-significant effect on postprandial triacylglycerol concentrations was evident, following the accumulated exercise.

It has previously been hypothesised that prior exercise protects against postprandial endothelial dysfunction by lowering postprandial triacylglycerol concentrations (Chandrruangphen & Collins, 2002; Gill et al., 2004), as a number of studies have reported significant correlations between some quantification of postprandial triacylglycerol concentrations and endothelial dysfunction (Wallace et al., 2010). However, not all studies have reported significant correlations (see chapter 5, chapter 6 and chapter 7 and Williams et al., 1999; Steer et al., 2003; Tushuizen et al., 2006; Rudolph et al., 2007; Wallace et al., 2010; Johnson et al., 2011) and this was also the case in the present study. Importantly, the present study observed a significant attenuation of postprandial endothelial dysfunction, despite no significant reduction in postprandial triacylglycerol concentrations following the accumulated exercise. This observation provides additional support for the suggestion that exercise-induced alterations in postprandial endothelial function may be independent of changes in postprandial triacylglycerol concentrations (Gill et al., 2003a; Tyldum et al., 2009).

This study did not investigate the mechanisms through which accumulated exercise attenuated the postprandial endothelial dysfunction evident in the control trial but there are a number of potential explanations. FMD is a marker of nitric oxide bioavailability (Joannides et al., 1995; Doshi et al., 2001; Corretti et al., 2002), with an increase in the inactivation of nitric oxide and/or a decrease in the production of nitric oxide likely to reduce FMD. It is currently believed that a transient rise in oxidative stress, following a

high-fat meal, induces a postprandial endothelial dysfunction (Plotnick et al., 1997; Bae et al., 2001; Tsai et al., 2004; Wallace et al., 2010) through free radicals ability to inactivate nitric oxide (Beckman and Koppenol, 1996). Prior exercise may reduce the postprandial rise in oxidative stress by increasing the body's antioxidant capacity, consequently reducing the inactivation of nitric oxide, and so attenuating the decline in FMD (Tyldum et al., 2009).

Postprandial endothelial function and triacylglycerol concentrations were not evaluated on day 1 of the main trials. Previous research in adults has reported that exercise undertaken 2 h following the consumption of a high-fat meal reduced postprandial endothelial dysfunction (Padilla et al., 2006) and that postprandial triacylglycerol concentrations are lower throughout the day on which exercise is accumulated (Miyashita et al., 2009). Therefore, the full effects of the accumulated exercise on postprandial endothelial function and triacylglycerol concentrations may not have been revealed in the present study. Additional research should investigate if an effect of exericise on endothelial function or triacylglycerol concentrations are evident on the day that the exercise is accumulated in young people.

Endothelial dysfunction has been implicated in the early stages of atherosclerosis (Ross, 1999) and there is evidence that atherosclerotic development and progression is dependent on the presence of endothelial dysfunction (Juonala et al., 2004). Therefore, any intervention that alters endothelial function could have clinical relevance. The findings of the present study provide experimental support for the concept of accumulating physical activity for good health in young people (World Health Organization, 2010; Department of Health, 2011). Previous research has observed that physical activity completed either in prolonged sessions or accumulated in short bouts have similar positive effects on a child's coronary heart disease risk profile (Stone et al., 2009b). Accumulated exercise may be a particularly attractive activity pattern for young people who often perceive prolonged activity to be more exerting than adults (Timmons & Bar-Or, 2003); it may also have considerable practical utility as short bouts of exercise may be relatively easy to incorporate into a school day.

8.5 Conclusions

In conclusion accumulating a total of 60 min of exercise through a series of 10 min exercise bouts spread throughout a day, prior to the consumption of a high-fat breakfast and lunch, attenuated postprandial endothelial dysfunction in adolescent boys. While the total and incremental areas under the triacylglycerol concentration versus time curve were reduced by 11% and 16% respectively by the accumulated exercise, these differences were not statistically significant.

Chapter 9: General discussion

9.1 Key findings

The research presented in chapter 4 established the validity of measuring lipid and lipoprotein concentrations from a capillary blood sample and chapter 5 established the reproducibility of fasting and postprandial endothelial function and triacylglycerol concentrations. The research presented in chapters 6, 7 and 8 investigated the effect of different modes of exercise on postprandial endothelial function and triacylglycerol concentrations in adolescent boys. The main findings of these studies are summarised below.

1. The ingestion of a high-fat breakfast was shown to induce a state of endothelial dysfunction in both adult (chapter 5) and adolescent males (chapters 6, 7 and 8) as evidenced by a decline in FMD three hours following breakfast compared to fasting. The ingestion of a second high-fat meal later in the day, lunch, maintained this endothelial dysfunction as evidenced by no further change in FMD between 3 h and 6.5 h (chapters 6, 7 and 8) or 7 h (chapter 5). These postprandial changes in FMD were also evident once FMD had been normalised to account for the post-occlusion shear rate.

2. All three modes of exercise investigated in the studies described in this thesis (continuous moderate-intensity exercise, repeated very short duration sprints and accumulated moderate-intensity exercise) were shown to attenuate the postprandial endothelial dysfunction evident in the control trial where no exercise was performed.

3. Continuous moderate-intensity exercise and repeated very short duration sprints, completed 14 h before the ingestion of the high-fat breakfast, significantly lowered postprandial triacylglycerol concentrations. The continuous moderate-intensity exercise resulted in the total and incremental area under the triacylglycerol concentrations versus time curve being reduced by 22% and 24% respectively, whilst the corresponding changes with the repeated very short duration sprints were

13% and 15%. Accumulating moderate-intensity exercise did not significantly lower postprandial triacylglycerol concentrations; however, the 11% (P = 0.183) and 16% (P = 0.088) reduction in the total and incremental area under the triacylglycerol concentrations versus time curve may be important and have physiological relevance.

4. No significant correlations were observed between postprandial FMD and triacylglycerol concentrations in any of the studies described in this thesis.

9.2 Effect of high-fat meals and exercise on postprandial endothelial function and triacylglycerol concentrations

In all of the studies described in this thesis lower FMD was observed in the control trials, where no exercise was performed, 3 h following the ingestion of the high-fat breakfast, indicating endothelial dysfunction. The mean declines, relative to fasting, in FMD following breakfast in this thesis were 45% (chapter 5), 32% (chapter 6), 20% (chapter 7) and 30% (chapter 8). Similarly, postprandial declines in FMD ranging between 17% (Nicholls et al., 2006) and 70% (Marchesi et al., 2000) have been reported in previous research, with the majority of studies reporting changes between 30-50% (Vogel et al., 1997; Bae et al., 2001; Gokce et al., 2001; Tushuizen et al., 2006; Tyldum et al., 2009). The ingestion of a second consecutive meal, lunch, later in the day, maintained but did not further exacerbate this reduced endothelial function in all of the studies described in this thesis. The only other study to investigate the effect of a second consecutive high-fat meal on endothelial function reported that the second meal further exacerbated endothelial dysfunction (Tushuizen et al., 2006).

As none of the studies described in this thesis included a trial where the effect of fasting or a meal containing little or no fat on endothelial function was investigated, it cannot be firmly concluded that the high-fat meals induced endothelial dysfunction as the responses may merely be a consequence of diurnal rhythms or eating. Research has identified that a diurnal rhythm for FMD exists, such that FMD increases during the waking day and decreases during sleep (Gaenzer et al., 2000; Otto et al., 2004). Such a diurnal rhythm would clearly not explain the dysfunction observed in the studies described in this thesis,

in fact it would suggest that the postprandial endothelial dysfunction is underestimated. It also seems unlikely that the observed postprandial endothelial dysfunction is merely related to the ingestion of food as previous research has consistently observed that an isoenergetic meal containing little or no fat (Vogel et al., 1997) or a period of fasting (Etsuda et al., 1999; Harris et al., 2005; Tushiezen et al., 2006) have negligible effects on endothelial function.

It has been argued that the postprandial decline in FMD, and thus endothelial dysfunction seen as a result of high fat meal ingestion, is purely a consequence of a concomitant increase in basal diameter (Raitakari et al., 2000; Gokce et al., 2001). This is due to the fact that previous research has identified that arteries with large diameters exhibit lower FMD than arteries with small diameters (Herrington et al., 2001; Silber et al., 2001; Pyke et al., 2004; Thijssen et al., 2008) primarily because the post occlusion shear stress stimulus (the stimulus for post-occlusion dilation) is inversely related to vessel diameter (Herrington et al., 2001; Pyke et al., 2004). A postprandial increase in basal artery diameter was observed in all of the studies described in this thesis. As these postprandial increases in basal artery diameter could influence the FMD responses, an attempt was made to account for this by normalising FMD to the area under the shear rate versus time curve. Normalised FMD is not associated with basal diameter (Pyke et al., 2004; Thijssen et al., 2008). As a postprandial decline in FMD was evident both before and after normalisation for the post-occlusion shear rate in all of the control trials described in this thesis, it is likely that the lower FMD observed in the control trial is not solely a consequence of an increase in basal diameter. In addition, only weak, non-significant correlations were observed between the postprandial change in basal diameter and FMD in the studies undertaken in adolescent boys described in this thesis, supporting the assertion that the postprandial decline in FMD is not purely a consequence of an increase in basal diameter.

Many measures of endothelial function, including FMD, are dependent on the endothelium producing nitric oxide. This endothelium derived nitric oxide travels to the underlying smooth muscle and then causes the smooth muscle to relax leading to dilation. Therefore, any change in endothelial function could represent a change in smooth muscle function. Smooth muscle function can be determined by measuring the resulting dilation to a nitric

oxide donor. Typically, when FMD is used to measure endothelial function, this smooth muscle function is assessed as the dilation of a blood vessel following administration of a single, high dose of nitroglycerin (a nitric oxide donor) (Corretti et al., 2002). However, given the participants described in this thesis were healthy and adolescent it was decided not to administer nitroglycerin for ethical reasons. Also, it has been argued that repeated administration of nitroglycerin could influence subsequent FMD measurements (Gori et al., 2004). Furthermore, the dilation following a dose of nitroglycerin has been shown previously to be similar before and after the ingestion of high-fat meals (Vogel et al., 1997; Tyldum et al., 2009; Gokce et al., 2001) indicating that the observed postprandial endothelial dysfunction is unlikely to be a consequence of altered smooth muscle function.

The studies presented in this thesis therefore demonstrate that the ingestion of high-fat meals induces a state of endothelial dysfunction which appears not to simply be an artefact of postprandial increases in basal diameter or changes in smooth muscle function. All previous research investigating the effect of high-fat meals on postprandial endothelial function has been undertaken with adults. Therefore, the studies described in chapters 6, 7 and 8 demonstrate, for the first time, that the ingestion of meals containing substantial quantities of fat induce a state of endothelial dysfunction in healthy, active young people. As endothelial dysfunction has been found to be a prerequisite for atherogenesis (Juonala et al., 2004), the observations of the studies described in this thesis suggest that the postprandial period following the ingestion of meals containing substantial quantities of fat is a time in which the body is susceptible to atherosclerotic development and progression in both adult and adolescent males. These studies are all acute and a single exposure to this postprandial endothelial dysfunction may not have huge clinical relevance; however, with food readily available in western societies this postprandial endothelial dysfunction is likely to be experienced on a regular, if not daily, basis and the resulting strain placed on the endothelial could have an accumulative effect and as a result have lasting deleterious consequences. Therefore, interventions which attenuate or prevent this postprandial endothelial dysfunction could be of clinical importance.

All three of the exercise modes employed in the studies described in this thesis (continuous moderate-intensity exercise, repeated very short duration sprints and accumulated moderate-intensity exercise) resulted in higher postprandial FMD than in the control trial

in adolescent boys. The repeated very short duration sprints and accumulated moderate-intensity exercise resulted in a significant attenuation in the postprandial decline in FMD (interaction effect trial x time, $P < 0.005$). The mean change, relative to fasting, in FMD following breakfast and lunch was only 2% and 5% when preceded by the repeated very short duration sprints (chapter 7) and 1% and 3% when 60 min of moderate-intensity exercise was accumulated (chapter 8). Although 60 min of continuous moderate-intensity exercise did not significantly attenuate the postprandial decline in FMD (interaction effect trial x time, $P = 0.088$), the mean change, relative to fasting, in FMD following breakfast and lunch was small at 8% and 10% in the exercise trial. The corresponding changes in the control trial were 32% and 24% (chapter 6). These observations of attenuated postprandial endothelial dysfunction following acute exercise are in agreement with previous studies which have demonstrated that continuous moderate-intensity exercise (Gill et al., 2004; Tyldum et al., 2009) and high-intensity intermittent exercise (Tyldum et al., 2009) attenuate postprandial endothelial function. Of the three studies investigating prior exercise on postprandial endothelial function, only one has failed to observe an effect of exercise (Silvestre et al., 2006); however, in this study postprandial endothelial dysfunction was not apparent in a control trial where no exercise was performed.

The design of the studies conducted in this thesis does not allow for a direct comparison between the exercise modalities as each participant sample was independent and therefore may be affected by each individual's specific response to exercise. However, Tyldum and colleagues (2009) suggested that exercise-intensity may influence the effect of prior exercise on postprandial endothelial function as they observed greater postprandial endothelial function when the high-fat breakfast was proceeded by high-intensity intermittent exercise compared to an isoenergetic bout of continuous moderate-intensity exercise. This concept is supported to some degree by the studies conducted in this thesis, where the very short duration sprints and 60 min of accumulated exercise (70% $\dot{V}O_2$peak), but not the 60 min of continuous exercise (60% $\dot{V}O_2$peak), prevented the postprandial decline in FMD. Future research is therefore required to establish any role of exercise intensity on postprandial endothelial function and also to directly compare the effectiveness of different exercise modes.

In addition to observing that prior exercise attenuated the postprandial decline in FMD, the continuous and accumulated moderate-intensity exercise were also shown to attenuate the postprandial decline in normalised FMD. Surprisingly no effect of repeated very short duration sprints on postprandial normalised FMD was apparent. The approach taken to normalise FMD for the post-occlusion shear rate in this thesis follows current recommendations (Thijssen et al., 2011) and allows for the comparison with other literature; however, the method used to normalise FMD is currently under debate (Atkinson et al., 2009; McCully 2012; Stoner et al., 2013). Atkinson and colleagues (2009) have questioned the validity of the approach taken to normalise FMD in this thesis, as the statistical assumptions inherent for such an approach are not met. These assumptions are that there should be a linear relationship between FMD and the area under the shear rate versus time curve, the intercept for the regression slope of this relationship is zero, data (including the residuals from any inferential test) are normally distributed, variances are similar (homogeneous) between groups and normalised FMD does not lead to spurious correlations with other variables. McCully and colleagues (2012) also question whether the total area under the shear rate versus time curve is a true representation of the dilatory stimulus which normalisation aims to account for. The use of allometric scaling (Atkinson & Batterham, 2013; Atkinson et al., 2013), multi-level modelling (Raudenbush & Bryk, 2001; Stoner et al., 2007) and dose-response curves (Stoner & Sabatier, 2011) have all recently been proposed as alternative approaches to account for differences in basal diameter and the dilatory shear rate stimulus. For these reasons, FMD, not normalised FMD, has been used as the main measure of endothelial function in this thesis.

Coincident to the postprandial endothelial dysfunction following the ingestion of high-fat meals is an increase in triacylglycerol concentrations. Postprandial triacylglycerol concentrations are an independent risk factor for CHD (Patsch et al., 1992; Stensvold et al., 1993; Stampfer et al., 1996; Nordestgaard et al., 2007; Bansal et al., 2007; Langsted et al., 2011; Nordestgaard & Freiberg, 2011) and may be a cause of the postprandial endothelial dysfunction (for review see Wallace et al., 2010). Consequently, any reduction in postprandial triacylglycerol concentrations could have clinical importance by directly lowering CHD risk or by reducing postprandial endothelial dysfunction. Therefore, the studies described in chapters 6, 7 and 8 sought to investigate the effect of prior exercise on postprandial triacylglycerol concentrations in addition to endothelial function. Each of the

studies described in this thesis observed that the respective prescribed exercise lowered postprandial triacylglycerol concentrations; however, this reduction was only significant when the prior exercise was continuous moderate-intensity exercise or repeated very short duration sprints.

The continuous moderate-intensity exercise (60 min of walking at 60% $\dot{V}O_2$peak) employed in the study described in chapter 6 was designed to replicate an exercise stimulus previously shown to lower postprandial triacylglycerol concentrations in adolescent boys (Barrett et al., 2007) but the effect of this exercise on postprandial endothelial function had yet to be investigated. In chapter 6 continuous moderate-intensity exercise significantly lowered fasting triacylglycerol concentration (21%, P = 0.001) and the total area under the triacylglycerol concentrations versus time curve (22%, P = 0.018). The incremental area under the triacylglycerol versus time curve was also lowered although this did not reach significance (24%, P = 0.169). The corresponding changes for fasting triacylglycerol concentration, total and incremental area under the triacylglycerol concentrations versus time curve in the study conducted by Barrett and colleagues (2007) were 15% (P = 0.139), 14% (P = 0.049) and 5% (P = 0.519), respectively. The larger percentage reductions in the study presented in chapter 6 may be because the adolescent boys were younger (13.6 (sd 0.6) versus 15.3 (sd 0.3) years) or because of the ingestion of a second high-fat meal later in the day. Other studies that have investigated the effect of prior continuous moderate-intensity exercise on postprandial triacylglycerol concentrations in adolescent boys have reported exercise-induced reductions in the total and incremental area under the triacylglycerol concentrations versus time curve of between 14 to 22% and 5 to 38% (Barrett et al., 2007; Tolfrey et al., 2008; MacEneaney et al., 2009; Tolfrey et al., 2012a).

Due to the study design it is not possible to determine the mechanisms for the observed exercise-induced reduction in triacylglycerol concentration but, based on other research literature, it appears that exercise reduces triacylglycerol concentration through two complementary pathways: an increased clearance of triacylglycerol rich lipoproteins and/or a reduced hepatic secretion of VLDL triacylglycerol (Gill & Hardman, 2003). Recent data from kinetic studies suggest the predominant cause of exercise induced reductions in triacylglycerol concentration is the increased clearance of triacylglycerol rich

lipoproteins, with this increased clearance being mediated by the secretion of fewer but more triacylglycerol-rich VLDL particles by the liver following exercise (Magkos et al., 2006; Tsekouras et al., 2007). These triacylglycerol rich VLDL particles are hydrolysed faster by lipoprotein lipase and removed faster from the circulation (Streja, 1979; Fisher et al., 1995). In addition, triacylglycerol clearance may be enhanced by increases in skeletal muscle lipoprotein lipase activity after exercise (Kiens & Lithell, 1989; Malkova et al., 2000).

In addition to investigating the effect of continuous moderate-intensity exercise on postprandial triacylglycerol concentrations, the studies described in chapters 7 and 8 investigated, for the very first time, the effect of repeated very short duration sprint exercise and accumulating moderate-intensity exercise on postprandial triacylglycerol concentrations in adolescent boys. Repeated very short duration sprints were shown to significantly lower the total and incremental area under the plasma triacylglycerol concentration versus time curve by 13% ($P = 0.023$) and 15% ($P = 0.036$) in chapter 7. Previous research in adults investigating the effect of repeated sprint exercise on postprandial triacylglycerol concentrations has used an exercise protocol consisting of repeated 30 s maximal sprints interspersed by 4 to 4.5 min of active, low-intensity recovery (Freese et al., 2011; Gabriel et al., 2012). Interestingly, Gabriel and colleagues (2012) observed repeated sprint exercise (5 x 30 s maximal sprints, with each sprint separated by 4 min of low-intensity recovery) to be more effective at reducing postprandial triacylglycerol concentrations than moderate-intensity exercise (30 min brisk treadmill walking such that the participants were "out of breath, but still able to talk"). Simulated games activity, which incorporates very short duration sprints (15 m), has also been shown to lower postprandial triacylglycerol concentrations in adults (Barrett et al., 2006) and adolescents (Barrett et al., 2007), and again this exercise mode was shown to be more effective than continuous moderate-intensity exercise. The greater reductions in postprandial triacylglycerol concentrations following sprint exercise could be a result of sprint exercise inducing greater increases in skeletal muscle lipoprotein lipase activity than moderate-intensity exercise. Exercise-induced increases in skeletal muscle lipoprotein lipase activity have been shown to be localised to the contracting muscles (Hamilton et al., 1998) and the vigorous nature of contractions and the larger recruitment of muscle mass seen during sprint exercise may increase skeletal muscle lipoprotein lipase activity to a

greater extent than moderate-intensity exercise. Sprint exercise is also associated with large increases in the concentrations of catecholamines and growth hormone (Ekbolm et al., 1971; Kindermann et al., 1982; Weltman et al., 1994; Pullinen et al., 1998; Stokes, 2003) which in turn are associated with increases in skeletal muscle lipoprotein lipase activity (Eckel et al., 1996; Oscarsson et al., 1999; Pedersen et al., 1999). The study described in chapter 7 advances our current knowledge of the effect of exercise on postprandial triacylglycerol concentrations by demonstrating that sprints alone (no active, low-intensity recovery) can lower postprandial triacylglycerol concentrations.

In chapter 8, accumulating 60 min of moderate-intensity exercise was also shown to lower the total and incremental area under the plasma triacylglycerol concentration versus time curve by 11% and 16% respectively when the exercise trial was compared with the control, although these differences were not statistically significant (P = 0.183 and P= 0.088 respectively). The percentage reductions observed in this study are consistent with the other studies in this thesis and with previous research in adolescent boys. Therefore, despite the changes in postprandial triacylglycerol concentrations induced by the accumulated exercise not reaching statistical significance, the reduction in the total and incremental area under the triacylglycerol concentration versus time curve is in line with previous research in adolescents, and may still be meaningful and have physiological importance.

At present there are no defined clinical cut-off points for postprandial triacylglycerol concentrations, making it challenging to define a clinically important change. However, it is recognised that minimum clinically important changes are needed to determine whether experimental changes are meaningful. Nordestgaard and colleagues (2007) and Langsted and colleagues (2011) reported, in prospective studies, that men with a postprandial triacylglycerol concentration between 1.00 and 1.99 $mmol.L^{-1}$ were 30 to 40% more likely to experience myocardial infarction and 10% more likely to experience ischemic heart disease than men with a postprandial triacylglycerol concentration of less than 1 $mmol.L^{-1}$. In addition, beyond these concentrations, greater postprandial triacylglycerol concentrations were associated with even greater risk of developing myocardial infarction and ischemic heart disease. Therefore, it would appear that any reduction in postprandial triacylglycerol concentrations can reduce the risk of future coronary heart disease,

suggesting that the reductions in triacylglycerol concentrations seen in the present study, following exercise the day before ingestion of the high fat meals, while not statistically significant, were likely to be meaningful. Furthermore, the importance of maintaining low postprandial triacylglycerol concentrations during adolescence was highlighted by Morrison and colleagues (2009) where adolescents with high postprandial triacylglycerol concentrations, independent of adult postprandial triacylglycerol concentrations, were more likely to experience cardiovascular disease in the fourth and fifth decade of life.

In adults it appears that a dose-response relationship exists between energy expenditure induced by exercise and the subsequent reductions seen in postprandial triacylglycerol concentrations, such that greater energy expenditures correspond to greater reductions in postprandial triacylglycerol concentrations (Petitt and Cureton, 2003). Such a dose-response relationship has yet to be established in adolescent boys. Tolfrey and colleagues (2008, 2012a) reported similar reductions in postprandial triacylglycerol concentrations when 60 min of running at 53% or 75% $\dot{V}O_2$peak (Tolfrey et al., 2008) and 30 min or 60 min of running at 55% $\dot{V}O_2$peak (Tolfrey et al., 2012a) was completed 15 h before the ingestion of a high-fat milkshake in adolescent boys. In addition to the dose-response relationship apparent in adults, Maraki and Sidossis (2010) argue that an expenditure of at least 2000 kJ is required to observe an effect of exercise on postprandial triacylglycerol concentrations. It may therefore perhaps be surprising that the repeated very short duration sprints lowered postprandial triacylglycerol concentrations as the energy expenditure associated with the exercise (533 or 1077 kJ depending how energy expenditure was estimated) was certainly much lower than the 2000 kJ threshold. This 2000 kJ threshold must be viewed sceptically as a number of studies have observed an effect of exercise on postprandial triacylglycerol concentrations when the exercise induced an energy exercise of less than 2000 kJ (Miyashita, 2008; Miyashita & Tokuyama, 2008; Miyashita et al., 2008; Peddie et al., 2012). Therefore, the findings of the present and previous studies (Freese et al., 2011; Gabriel et al., 2012) suggest that the effect of sprint exercise on postprandial triacylglycerol concentrations may not be dependent on energy expenditure, or that, when elicited by sprint exercise, much lower energy expenditures are sufficient to lower postprandial triacylglycerol concentrations.

As the adolescent boys spent all of day 1 of the exercise trial in the laboratory during the study investigating the effect of accumulated exercise but were in school (following the instruction to refrain from physical activity) on day 1 of the control trial, it was decided to monitor each boy's physical activity in this study. Analysis of the accelerometer data collected in the two days (day 0 and day 1) preceding the day when the high-fat meals were consumed demonstrated that the time spent in low-, moderate- and vigorous-intensity physical activity was similar during day 0 of the control and exercise trials. However, during day 1 of the exercise trial the boys spent more time in vigorous-intensity physical activity, as expected, but they also spent less time in low- and moderate-intensity physical activity than in the control trial. Consequently, although the boys expended 1900 (sem 79) kJ during the prescribed exercise, the estimated difference in total daily energy expenditure between the exercise and control trials was substantially less than this (estimated to be 1128 (205) kJ greater on day 1 of the exercise trial compared with day 1 of the control trial). Therefore, although the participants were instructed to be as physically inactive as possible and to refrain from any form of exercise whilst in their free-living environment, their adherence to these instructions, perhaps not unexpectedly, was poorer under free-living conditions compared with that achieved in the more controlled laboratory conditions where physical inactivity was ensured. The small net increase in total energy expenditure between the exercise and control trial in chapter 8 may explain the smaller than expected and non-significant reduction in postprandial triacylglycerol concentrations evident following the accumulated exercise. This study highlights the importance of monitoring physical activity and energy expenditure in the days preceding the ingestion of the high-fat meals in future studies.

A number of studies have reported significant correlations between some quantification of postprandial endothelial (dys)function and triacylglycerol concentrations (Vogel et al., 1997; Marchesi et al., 2000; Bae et al., 2001; Wallace et al., 2010). The presence of such correlations led to the formation of the hypothesis that prior exercise could attenuate postprandial endothelial dysfunction through its ability to concomitantly lower postprandial triacylglycerol concentrations (Chandrruangphen & Collins, 2002; Gill et al., 2004). With each mode of exercise in this thesis shown to attenuate postprandial endothelial dysfunction and lower postprandial triacylglycerol concentrations, these observations qualitatively support such a hypothesis. However, the presence of such

significant correlations is not universal with some studies (Williams et al., 1999; Steer et al., 2003; Tushuizen et al., 2006; Rudolph et al., 2007; Wallace et al., 2010; Johnson et al., 2011), including all of the studies described in this thesis, observing no significant correlations between postprandial endothelial dysfunction and triacylglycerol concentrations. This discrepancy in findings may be due to differences in the methods used to quantify variables (e.g. absolute postprandial values, postprandial changes, total area under the variable versus time curve and incremental area under the concentration time curve), differences in the timing of postprandial events and differences in the lipid load (Wallace et al., 2010; Johnson et al., 2011). In addition many studies that have previously reported significant correlations between postprandial FMD and triacylglycerol concentration responses have pooled the data from a high-fat test meal trial and a low-fat test meal trial, by doing so the range of values within the data set was increased enhancing the chance of finding significant correlations (Bates et al., 1996). The studies described in this thesis appear to suggest that the observed effect of exercise on postprandial endothelial function may be independent of the changes in postprandial triacylglycerol concentrations. This notion is supported by the studies conducted by Gill and colleagues (2003) and Tyldum and colleagues (2009) where a dissociation between changes in postprandial endothelial dysfunction and triacylglycerol concentrations was evident. However, future research should continue to consider the influence of postprandial triacylglycerol concentrations on postprandial endothelial function because it has been reported that only those individuals who experience high postprandial triacylglycerol concentrations experience postprandial endothelial dysfunction (Gaezner et al., 2001). In addition, dietary or pharmacological interventions which reduced postprandial triacylglycerol concentrations have also been found to attenuate postprandial endothelial dysfunction (Ceriello et al., 2002; Berry et al., 2008). Such future research might include investigating endothelial function following the infusion of different doses of intralipid, in order to investigate the effects of varying triacylglycerol concentration on endothelial function. As intralipid does not induce an insulinaemic response, co-infusion of insulin should also be considered.

There is also compelling evidence in adults linking the postprandial decline in endothelial function with the postprandial increase in oxidative stress. This link is based on the presence of strong correlations between the magnitude of postprandial endothelial

(dys)function and oxidative stress (Bae et al., 2001; Tsai et al., 2004; Wallace et al., 2010). Furthermore, the co-ingestion of anti-oxidant vitamins with a high-fat meal which reduces postprandial oxidative stress, has been shown to prevent postprandial endothelial dysfunction (Plotnick et al., 1997; Ling et al., 2002; Bae et al., 2003). Oxidative stress was not determined in the studies described in this thesis due to the use of capillary blood sampling which resulted in a limited supply of plasma. However, in adults a single bout of prior exercise has been shown to increase total antioxidant capacity (Tyldum et al., 2009) and attenuate postprandial oxidative stress (Gabriel et al., 2012). In these studies, it would appear that higher intensity exercise results in greater increases in total antioxidant capacity and reductions in postprandial oxidative stress, which could explain why the effect of exercise on postprandial endothelial dysfunction was greater in the studies investigating repeated very short duration sprints and accumulated exercise (70% $\dot{V}O_2$peak) than continuous moderate-intensity exercise (60% $\dot{V}O_2$peak). The effect of a single bout of exercise on total antioxidant capacity or postprandial oxidative stress in the young has not been established. Clearly, further research is required to establish the effect of food ingestion, and the added effect of exercise, on triacylglycerol concentrations, oxidative stress and endothelial function.

9.3 Efficacy and implications of the exercise interventions

All of the modes and patterns of exercise investigated in this thesis were effective at attenuating postprandial endothelial dysfunction and lowering postprandial triacylglycerol concentrations in adolescent boys. Consequently, the experimental evidence from the studies presented in this thesis indicates that exercise offers an acceptable, non-pharmacological means of influencing CHD risk when individuals are young. Current physical activity guidelines for the young are heavily dependent on research in adults; however, as the young have different metabolic and hormonal responses to physical activity and exercise to adults (Riddell, 2008), it is not approriate to simply extrapolate data collected in adults to the young. Therefore, the studies contained in this thesis provide experimental evidence that can be used in the development of physical activity guidelines for the young. Importantly, each of the exercise modes / patterns investigated in this thesis have specific aspects that may make them particularly appealing or suitable for the young.

Continuous, moderate-intensity physical activity has been a fixed recommendation in the physical activity guidelines for good health. Previous research has established that continuous, moderate-intensity exercise has positive health benefits for both adults and adolescents. Chapter 6 supported this previous research identifying that 60 min of continuous, moderate-intensity exercise attenuated postprandial endothelial dysfunction, lowered postprandial triacylglycerol concentrations and improved insulin sensitivity in adolescent boys. Interestingly, this was the only mode of exercise to result in improved insulin sensitivity.

As young people's activity patterns are usually intermittent in nature and characterised by multiple short-duration bouts of vigorous activity typically lasting less than 10 s, with a median duration of 4 s (Stone et al., 2009), repeated very-short duration sprints may offer a better and more attractive exercise model than continuous moderate-intensity exercise for the young. In addition, short intense bursts of activity, a characteristic of repeated sprint exercise, has been reported to be more enjoyable than continuous moderate-intensity exercise (Bartlett et al., 2011). Previous research in adults has typically investigated the effect of longer duration (30 s) repeated sprints, reporting similar health benefits to continuous, moderate intensity exercise (Gibala et al., 2012). These longer duration (30 s) repeated sprint protocols require high levels of motivation to complete and are often associated with feelings of nausea and light-headedness (Richards et al., 2010). It was thought that the repeated very short duration (6 s) repeated sprints provided in chapter 7 would be less likely to elicit these types of responses. However, of the 15 active adolescent boys who participated in this study, 5 proved unable to tolerate the exercise pattern even though the sprints were only of 6 s duration. Typically these participants stopped exercising because they felt light-headed and nauseous; in 2 cases participants vomited. These responses occurred after only 7 (sd 2) sprints, which suggests that some individuals cannot easily tolerate sprinting per se. It should be acknowledged that a low-intensity active recovery between the repeated sprints may be one method to increase tolerance to this type of sprint exercise, as it may reduce venous pooling in the legs and reduce feelings of light-headiness and nausea (Rakobowchuk et al., 2008), but this was not done in this study in order to investigate the physiological effects of repeated short sprints alone. The tolerance to this type of sprint exercise may also be improved by reducing the length of each sprint (e.g. 3 s) or my completing intense, but not maximal, sprints. Even

given these changes to the sprint protocol it is important to recognise that sprint exercise may be unsuitable for many individuals, in particular those with pre-existing medical conditions or susceptibility to injury and for this reason should not be recommended to all populations.

In addition the short-duration of actual exercise undertaken in repeated sprint protocols has led to this type of exercise regimen being deemed time-efficient (Burgomaster et al., 2008). This feature may be attractive to the larger population as lack of time is often cited as an obstacle for exercising (Tappe et al., 1989). While the duration of sprint exercise in the study described in chapter 7 was only 4 min (11 min of exercise in total if the warm-up was included) the total duration of the repeated sprint protocol was 90 min (the length of an association football game), which is 50% greater in duration than the 60 min of moderate-intensity exercise currently recommended in the physical activity guidelines for adolescents (Department of Health, 2011).

Young people have also been reported to perceive prolonged exercise (>15 min) to be more demanding than adults (Timmons & Bar-Or, 2003) potentially making continuous, moderate-intensity exercise an unattractive mode of exercise for this age group. The accumulated exercise employed in chapter 8 partitioned the 60 min of moderate-intensity exercise out into six, 10 min bouts spread throughout the day. By reducing the duration of each bout this pattern may be more appealing to the young as they will perceive the exercise to be less demanding. Furthermore, the multiple shorter bouts may be easier to structure around the school day. The results of the study presented in chapter 8 demonstrate that accumulating exercise in 10 min bouts can have health benefits in the young. In fact the benefits seen as a result of this accumulated exercise in this thesis may be underestimated as the participants failed to fully comply with the instruction to refrain from physical activity in the days preceding the ingestion of the high-fat meals.

9.4 Limitations

The high-fat mixed meals provided throughout this thesis can be criticised as not being representative of the normal dietary intake of people in western societies. However, the ingestion of meals containing substantial quantities of fat is not uncommon in adolescents

with 60% of teenagers in the United States of America reported to eat fried food at least once a week (Taveras et al., 2005), and 30% reported to eat "fast foods" (which is typically high in fat) daily (Schmidt et al., 2005). In addition to the meals containing substantial quantities of fat, they also provided a larger amount of energy with an average adolescent boy (45 kg) receiving 4176 kJ for breakfast and 3798 kJ for lunch resulting in a total intake of 7974 kJ (excluding dinner which was likely to have been consumed at home after leaving the laboratory). Although this intake would be higher than the 10000 kJ recommended for an adolescent boy (aged 13 years with medium height and body mass and physical activity level) (Scientific Advisory Committee on Nutrition, 2012), the energy intake of adolescent boys between 1999 and 2010 in the United States of America was reported to be higher ranging between 12000 and 13000 kJ (Ervin & Ogden, 2013). The meals provided in this thesis may have exaggerated the postprandial responses; however, the energy content of the foods provided in this study is not unrepresentative of regular daily intakes indicating that the postprandial endothelial dysfunction and elevations in plasma triacylglycerol concentrations observed in this thesis, and previous research, are likely to be experienced on a regular basis. Furthermore, the effect of exercise seems to be consistent, regardless of the extent of fat consumed with Hurren and colleagues (2011b) reporting that a bout of moderate-intensity exercise (60% $\dot{V}O_2max$) completed 12 h before the ingestion of a high-fat meal (1.2 g fat·kg body mass^{-1} (66% fat)) and a moderate-fat meal (0.64 g fat·kg body mass^{-1} (35% fat)) resulted in similar reductions in postprandial triacylglycerol concentrations (29% and 26%, respectively). Research investigating the effects of meals of a lower fat and energy content and those rich in carbohydrate is still warranted and could further contribute to the better understanding of the atherosclerotic nature of the postprandial state. Such research could aid in the development of dietary guidelines.

As dietary intake was controlled in the days before the ingestion of the high-fat meals, the participants were in a state of negative energy balance in the exercise trial compared to the control trial. The effect of exercise on postprandial endothelial function and triacylglycerol concentrations observed in this thesis may simply be a result of the negative energy balance induced in the exercise trials. Gill and colleagues (2000) reported that only a negative energy balance induced by moderate-intensity exercise, not by dietary restriction, resulted in lower postprandial triacylglycerol concentrations, indicating that the

effect is specific to an exercise induced energy deficit. Also as similar reductions in postprandial triacylglycerol concentrations were observed following the repeated very-short duration sprints and moderate-intensity exercise, despite sizable differences in energy expenditure, in this thesis it seems unlikely that a negative energy balance is solely responsible for the attenuated postprandial endothelial dysfunction and lower postprandial triacylglycerol concentrations. However, studies which have replenished the energy expended during exercise in the period after exercise cessation have observed that the effect of exercise is reduced (Burton et al., 2008; Harrison et al., 2009; Singhal et al., 2009; Freese et al., 2011) indicating some role of a negative energy balance in the exercise induced reduction in postprandial triacylglycerol concentrations. It seems however, that individuals do not fully replenish the energy expended through acute exercise (Moore et al., 2004; Dodd et al., 2008; King et al., 2010) and therefore these studies may also lack external validity. The effect of negative energy balance, or replenishment of this energy, on postprandial endothelial function has yet to be investigated.

All the studies investigating the effects of exercise and high-fat meals in this thesis have been undertaken with normal weight adolescent boys. Therefore, caution must be taken when extrapolating the observations made with different populations including children (those yet to reach puberty), female adolescents, and young people at an increased risk of CHD (e.g. those who are overweight or obese, insulin resistant or diabetic and those who are sedentary). However, exercise has been shown to induce similar reductions in postprandial triacylglycerol concentrations in normal weight and overweight adolescent boys (MacEneaney et al., 2009) and recently, research has also identified that prior exercise can lower postprandial triacylglycerol concentrations in adolescent girls (Tolfrey et al., 2012b). Exercise has also been shown to be similarly effective at attenuating postprandial endothelial dysfunction in normal and overweight adults (Gill et al., 2004). No study has investigated the effect of high-fat meals, or the added effect of exercise, on endothelial function in children or adolescent girls.

The sample sizes for the studies described in chapters 6, 7 and 8 were selected such that each study had sufficient power to detect a large effect of exercise on postprandial FMD and triacylglycerol concentrations (ES = 0.9; $\alpha = 0.05$, $\beta = 0.8$, two tailed). A large effect was expected based on previous research in our laboratory and other laboratories.

Conseqently the studies conducted in this thesis lack statistical power to identify small or moderate effects of exercise (ES < 0.9; $\alpha = 0.05$, $\beta = 0.8$, two tailed), and therefore the selected sample size may have introduced type II error for the more moderate changes in postprandial triacylglycerol concentrations (chapter 8) and a number of the secondary outcome variables. Future research should consider using a sample size that provides sufficient power to detect a moderate effect size or even a small effect, given that Samsa and colleagues (1999) have argued that an effect size of 0.2 is the minimum important difference when a clinical anchor is not available (as is the case for postprandial triacyglycerol and FMD).

9.5 Directions for future research

- To investigate the effect of ingesting a high-fat mixed meal and exercise on postprandial endothelial function and triacylglycerol concentrations in populations other than healthy active adolescent boys such as girls, children (and to establish the effect of maturity on these responses) and those at an increased risk of CHD (e.g. those who are overweight or obese, insulin resistant or diabetic and those who are sedentary).
- Research should continue to investigate the effect of different modes and patterns of exercise on postprandial endothelial function and triacylglycerol concentrations in both adults and the young in order to inform future physical activity guidelines. Based on the studies described in this thesis, research should identify and investigate the effect of a more tolerable repeated sprint, or high-intensity, exercise session and investigate the effect of accumulating sprint exercise throughout the day.
- This thesis focused on the effect of exercise on CHD risk factors; however, sedentary behaviour has been argued to be as important as physical activity on CHD risk (Biddle et al., 2004). Therefore, research should examine the acute effect of physical inactivity on postprandial endothelial function and triacylglycerol concentrations in both adults and the young.
- The effect of meals of more moderate fat content and those rich in carbohydrates, and the additional effect of exercise on postprandial endothelial function and triacylglycerol concentrations should be investigated in both adults and the young.

- The studies described in this thesis did not investigate the mechanisms through which the ingestion of the high-fat mixed meals induced endothelial dysfunction and through which exercise attenuated this dysfunction. Therefore, future research should investigate the mechanisms behind the high-fat meal induced endothelial dysfunction and the protective effect of exercise.
- The effect of exercise may be a result of the participants being in a negative energy balance. Therefore, future research should compare the effect of negative energy balances induced by exercise and dietary restriction on postprandial endothelial function and triacylglycerol concentrations. Research should also investigate the effect of compensating the energy expended during exercise on postprandial endothelial function and triacylglycerol concentrations.
- Future research investigating the effect of exercise and / or meals on endothelial function should be conducted with measures of endothelial function other than FMD to confirm the endothelial function results of this thesis

9.6 Conclusions

The studies contained in this thesis demonstrate that a state of endothelial dysfunction is evident following the ingestion of a high-fat breakfast and the ingestion of a high-fat lunch later in the day maintains this endothelial dysfunction in both healthy adults men and adolescent boys. A prior session of exercise (continuous moderate-intensity exercise, repeated very short duration sprints or accumulated moderate-intensity exercise) can attenuate this postprandial endothelial dysfunction, and lower postprandial triacylglycerol concentrations in adolescent boys. The effect of exercise on postprandial endothelial function appears not to be related to the effect on postprandial triacylglycerol concentrations.

The experimental evidence from this thesis emphasises that exercise might offer an acceptable, non-pharmacological means of influencing CHD risk when individuals are young, despite CHD typically being a chronic problem in which the potentially terminal manifestations, such as myocardial infarction and stroke, usually occur later in the life cycle. It also implies that interventions which begin early in an individual's life (such as encouraging regular physical activity or exercise) are likely to be beneficial.

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Appendix 1: Participant information

Influence of a 60-minute bout of walking on responses to high-fat meals in adolescent males

Background

Although coronary heart disease occurs later in life, atherosclerotic (hardening of the blood vessel walls) development has been shown to begin during childhood and progress throughout life. Coronary heart disease risk factors have been observed in children and these risk factors have been shown to follow into adulthood. Therefore interventions that can reduce these risk factors, even in young people, could theoretically delay the development and progression of atherosclerosis, ultimately reducing the risk of developing coronary heart disease. Two risk factors that we are interested in are triacylglycerol (one of the main fats in the blood) concentrations and endothelial function (a measure of how healthy your blood vessels are and how well they can expand and contract) with a particular focus on these risk factors in a fed state.

Previous research in adults has shown that 60 minutes of running or walking the afternoon before a high fat meal can help the body clear the fat consumed more rapidly from the blood stream. This means the fat has less contact time with the blood vessel walls and therefore less time to damage them. The limited number of studies which have been conducted in young people have reported a similar response to adults; however, the effects on the endothelium (the blood vessel walls) have not been determined in young people.

Research in adults has observed a reduction in endothelial function in the period after a meal. The magnitude of this reduction has been related to the (in)ability of the body to clear fat from the blood stream, however, this has not been investigated in young people. The ability of exercise to enhance fat clearance could indicate that exercise may also be able prevent the reduction in endothelial function. Therefore this study seeks to investigate the effect of 60 minutes of walking (4 x 15 min with 3 min rest between each block) on postprandial triacylglycerol concentrations, blood pressure and endothelial function in adolescent males.

Screening

Prior to your son participating in the study, we ask that you complete a health screen questionnaire about them. This is to ensure that your son has no health problems that could prevent them from participating in the study. We will also ask your son to complete this health screen questionnaire during their first visit to the university and then verbally ask if they are free from illness before participation in the remaining visits.

Preliminary visit

The following measures will be taken:

- Height
- Sitting height
- Weight
- Skinfold and circumference measurements
- Self-assessment of maturity
- Peak oxygen uptake test

Your son will also be familiarised with the measurement of blood pressure and flow-mediated dilation

Additional information regarding the measures to be taken

Self-assessment of maturity

The reason for this assessment is that young people of the same age can be at very different stages of maturity, e.g. 13-14 year old boys may look slim and slight or tall and thicker-set depending on whether or not they have gone through puberty. It is a better comparison to examine the test results of young people of the same maturity rather than of the same age.

The assessment procedure requires your son to enter an enclosed room on their own and carefully study some pictures of different stages of development (e.g. genital development and amount of pubic hair). Your son should then hold-up or pull-down their clothing (there is no need to completely remove clothing) and look in the mirror and decide which picture most closely matches their own stage of development. They will write the number of that picture down on the form, place the form in the envelope and seal it. Finally they will fully replace all of their clothing before leaving the room and hand the envelope to the person leading the testing.

Peak oxygen uptake test

This test measures the maximum amount of oxygen (the substance in air we require to be able to breathe) you can take into your body and use in your muscles to produce energy. This test involves walking on a treadmill at different gradients. A starting speed which your son finds comfortable will be determined at the beginning of the test and this speed will not change during the test.

During the last minute of each 4 minutes at a particular gradient your son will be asked to breathe through a mouthpiece so that we can collect the air that your son breathes out. The treadmill gradient will then be increased and your son will start the next stage. This process will be repeated until your son feels they can only keep walking for one more minute. Your son will indicate this to the tester by raising one finger clearly in the air. An air sample will then be collected during this minute and the test is complete.

Main trials

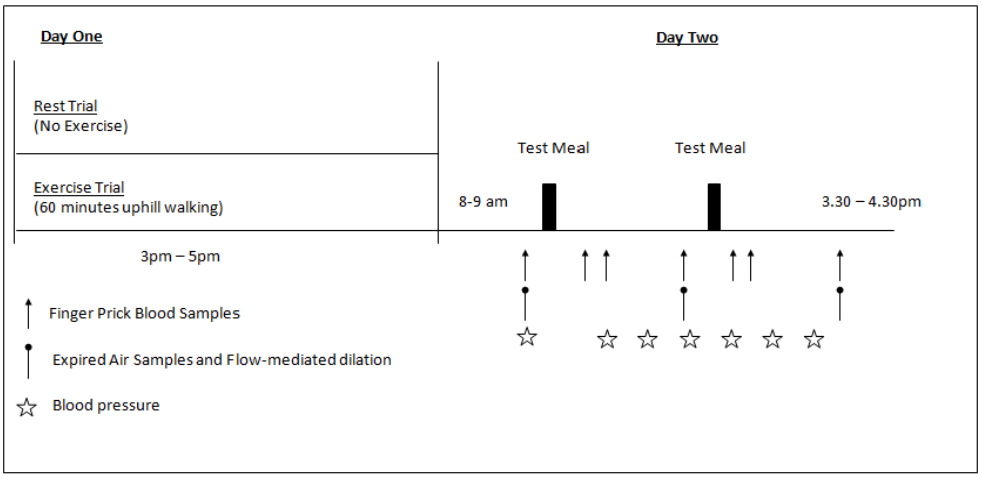
This study involves two main trials with each trial covering two days (see figure below).

Day 1 of each trial differs depending on the trial.

Control trial – This does not involve your son visiting the university and all we ask is that he is as inactive as possible on this day.

Exercise trial – This requires your son to visit the university after school (1500 – 1700) and complete 60 minutes of walking

Day 2 of each trial is identical and requires your son to visit the university arriving between 0800 – 0900 and finishing between 15:30 – 16:30. During this time he will complete an oral fat tolerance test.



In preparation for each main trial we ask that your son is as inactive as possible for the two days prior to each oral fat tolerance test and only participates in the exercise that we prescribe as part of the main trials. During this time we also ask that your son with your assistance keep a record of what he eats prior to the first main trial and then replicate this prior to the second main trial.

Additional information regarding the measures to be taken

Oral Fat Tolerance Test

Your son will report to the laboratory after an overnight fast (nothing to eat or drink, other than water, after 10 pm) and will be asked to lie quietly for approximately twenty minutes after which a technique called flow-mediated dilation (a measure of the endothelial function) will be completed. Following this a finger prick blood sample, a measurement of blood pressure and an expired air sample will be collected (as in the peak oxygen uptake test).

Next your son will be given a high fat meal to eat; this will comprise of croissant, chocolate spread, chocolate milkshake powder, whole milk and double cream. A second high fat meal will be given 3.5 hours later; this will consist of white bread, butter, cheddar cheese, ready salted crisps, chocolate milkshake powder and whole milk.

Further blood samples (5) and expired air samples (2) will be obtained and blood pressure (6) and flow-mediated dilation (2) will be assessed at intervals during the 6.5 hour observation period.

Following the meals your son will not be allowed to eat or drink anything other than water and he will be asked to rest quietly throughout the observation period. Your son will be allowed to read, watch television/videos or play on a games console.

Finger prick blood sampling

A small finger prick blood sample will be taken at regular time points and analysed for triacylglycerol, glucose and insulin concentration.

All samples will be disposed of in an appropriate manner following analysis.

The finger prick blood sample may be uncomfortable and may cause the finger to feel a little sore for a day or so.

Flow-mediated dilation

Flow-mediated dilation is a standardized technique used to assess endothelial function (which is a measure of how healthy your blood vessels are and how well they can expand and contract). It has been designed to be used with young people.

The technique involves your son lying down for approximately 20 minutes and images of an artery in the arm being recorded using ultrasound (the same way images are taken of babies in the womb during pregnancy). Images are taken before and after the inflation of a blood pressure cuff around the forearm (duration of inflation = 5 minutes).

A slight numbness may be felt in the fingers whilst the cuff is inflated and a warm tingling feeling may be felt immediately after the release of the cuff as blood flow to the forearm and hand is enhanced.

Time Requirements

Visit 1 – Preliminary visit – approximately 2 h (after school)
Visit 2 – Exercise session – approximately 2 h (after school the evening before visit 3 or 4)
Visit 3 – Oral fat tolerance test – approximately 7.5 h (during school)
Visit 4 - Oral fat tolerance test – approximately 7.5 h (during school)

Important information

- Although your son's school has agreed to take part in this project, it is **NOT** compulsory for your son to take part.
- Your son's school has agreed to participate in this project, thus taking time off normal lessons to participate has been cleared with the school.
- Your son can withdraw from the study at any time without providing a reason.
- All staff involved in the study have received training in the measures involved and have undertaken a CRB check to clear them to work with children and young people.
- All information will be stored anonymously and no individual data will be reported in the findings of the study.
- The study has been approved by Loughborough University's Ethical Advisory Committee.

Contact details

If you have any additional questions please do not hesitate to contact:

Mr Matthew Sedgwick	01509 226351	M.J.Sedgwick@lboro.ac.uk
Dr Laura Barrett	01509 226395	L.A.Barrett@lboro.ac.uk

Appendix 2: Statement of informed consent (adult)

Loughborough University

School of Sport, Exercise and Health Sciences

Statement of Informed Consent

Comparison of analyte concentration obtained when using capillary and venous blood sampling

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethical Advisory Committee.

- I have read and understood the information sheet and this consent form.
- I have had an opportunity to ask questions about my participation.
- I understand that I am under no obligation to take part in the study.
- I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.
- I understand that all the information I provide will be treated in strict confidence.
- I agree to participate in this study.

Your name _____

Your signature _____

Date _____

Phone Number _____

Email Address _____

Date of Birth _____

Signature of investigator _____

Appendix 3: Statement of informed assent (adolescent)

Influence of a 60-minute bout of walking on postprandial triacylglycerol concentrations, blood pressure and endothelial function in adolescent males

Young Person's Willingness to Participate Form

Please read the statements below and indicate whether you are willing to participate in this study.

Willingness to Participate Statement:

- I have read the information about the study and the tests involved have also been explained to me. I have had the opportunity to ask questions and I understand what is required of me. I know that I can say that I do not wish to continue with the testing at any time and I do not have to give a reason.

- I agree to take part in the tests (please tick): ☐

Signed: _____ Date: _____

Witnessed by: _____

Appendix 4: Statement of informed consent (parent)

Influence of a 60-minute bout of walking on postprandial triacylglycerol concentrations, blood pressure and endothelial function in adolescent males

Parent/Guardian Consent

- I have been invited to observe procedures.
- I have been given the opportunity to ask questions and I understand what is required from my son.
- I have seen the information sheet and fully understand what the tests entail.

I give permission for my son to be involved in the testing (please print your son's name) _____

Parent/guardian's signature _____

Parent/guardian's name (please print) _____

Contact details:

Telephone _____

E-mail _____

Appendix 5: Heath screen questionnaire

Health Screen Questionnaire for Study Volunteers

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research

Please complete this brief questionnaire to confirm your fitness to participate:

1.	**At present,** do you have any health problem for which you are:				
	(a) on medication, prescribed or otherwise.....	Yes		No	
	(b) attending your general practitioner	Yes		No	
	(c) on a hospital waiting list	Yes		No	
2.	**In the past two years,** have you had any illness which required you to:				
	(a) consult your GP.....	Yes		No	
	(b) attend a hospital outpatient department.....	Yes		No	
	(c) be admitted to hospital	Yes		No	
3.	**Have you ever** had any of the following:				
	(a) Convulsions/epilepsy	Yes		No	
	(b) Asthma	Yes		No	
	(c) Eczema	Yes		No	
	(d) Diabetes	Yes		No	
	(e) A blood disorder	Yes		No	
	(f) Head injury	Yes		No	
	(g) Digestive problems	Yes		No	
	(h) Heart problems	Yes		No	
	(i) Problems with bones or joints	Yes		No	
	(j) Disturbance of balance/coordination	Yes		No	
	(k) Numbness in hands or feet	Yes		No	
	(l) Disturbance of vision	Yes		No	
	(m) Ear / hearing problems	Yes		No	
	(n) Thyroid problems	Yes		No	
	(o) Kidney or liver problems	Yes		No	
	(p) Allergy to nuts	Yes		No	

4. **Has any,** otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? ..... Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....
.....

5. Allergy Information

(a) are you allergic to any food products? Yes ☐ No ☐

(b) are you allergic to any medicines? Yes ☐ No ☐

(c) are you allergic to plasters? Yes ☐ No ☐

If YES to any of the above, please provide additional information concerning your allergy

……
……

6. Supplements

(a) Have you ingested vitamin supplements over the past 6 months? Yes ☐ No ☐

(b) Have you ingested any other supplements over the past 6 months? Yes ☐ No ☐

If YES to any of the above, please provide additional information regarding your supplement usage

……
……

7. Do you currently smoke?

Yes ☐ No ☐

If YES , how many cigarettes do you smoke each week?

……
……

8. Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name: ……
…

TelephoneNumber: ……
……

Work ☐ Home ☐ Mobile ☐

Relationship::……
…

9. Are you currently involved in any other research studies at the University or elsewhere?

Yes ☐ No ☐

If yes, please provide details of the study

……
……

Appendix 6: Determination of analyte concentrations: analytical techniques

Determination of plasma triacylglycerol concentration

Plasma triacylglycerol concentrations were determined by an enzymatic calorimetric method using a commercially available kit (Triglycerides CP, HORIBA ABX Diagnostics, Montpellier, France). Samples were analysed on an automated centrifugal analyser (Pentra 400, HORIBA ABX Diagnostics, Montpellier, France).

Reagents

The commercially available reagent was composed of:

Pipes free acid	50 $mmol \cdot L^{-1}$
Sodium hydroxide	3.36 $g \cdot L^{-1}$
Triton X-100	1 $mL \cdot L^{-1}$
Magnesium salt	14.8 $mmol \cdot L^{-1}$
p-chlorophenol	2.69 $mmol \cdot L^{-1}$
ATP	3.14 $mmol \cdot L^{-1}$
Sodium azide	7.99 $mmol \cdot L^{-1}$
Potassium ferrocyanide	9.94 $\mu mol \cdot L^{-1}$
4-aminoantipyrine	0.31 $mmol \cdot L^{-1}$
Lipoprotein lipase	1.90 $U \cdot L^{-1}$
Glycerokinase	0.5050 $KU \cdot L^{-1}$
Glycerol phosphate Oxidase	4.15 $KU \cdot L^{-1}$
Peroxidase	0.4950 $KU \cdot L^{-1}$
Distilled water	qs $1L \cdot lL^{-1}$

Standards

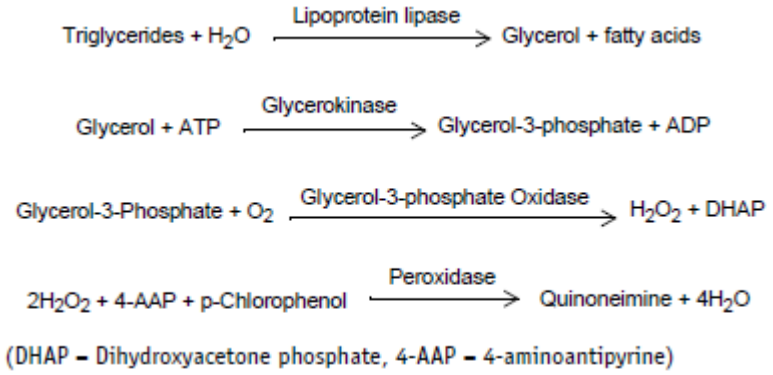
A single commercially available standard was used (Multical, HORIBA ABX Diagnostics, Montpellier, France). This standard is a lyophilized standard based on human serum. To reconstitute this sample 3 mL of distilled water was added and left to stand at room

temperature for 30 min. During this time the vial was agitated slowly avoiding the formation of foam.

Quality controls

Two commercially available quality controls were used (N-control and P-control, HORIBA ABX Diagnostics, Montpellier, France). These quality controls are a lyophilized control based on human serum. To reconstitute this sample 5 mL of distilled water was added and left to stand at room temperature for 30 min. During this time the vial was agitated slowly avoiding the formation of foam.

Method



Procedure

1. Samples were defrosted for 60 min and vortexed before being loaded into the Pentra 400.
2. 3 µL of sample (standard, control or plasma), 290 µL of reagent and 10 µL of distilled water were pipetted into a cuvette
3. The sample was then mixed, incubated and read at a wavelength of 505 nm.
4. Plasma and control triacylglycerol concentrations were determined by the analyser using a regression equation derived from analysis of the standard.

Determination of plasma glucose concentration

Plasma glucose concentrations were determined by an enzymatic calorimetric method using a commercially available kit (Glucose PAP CP, HORIBA ABX Diagnostics, Montpellier, France). Samples were analysed on an automated centrifugal analyser (Pentra 400, HORIBA ABX Diagnostics, Montpellier, France).

Reagents

The commercially available reagent was composed of:

Phosphate buffer, pH 7.40	13.8 $mmol \cdot L^{-1}$
Phenol	10 $mmol \cdot L^{-1}$
4-aminoantipyrine	0.3 $mmol \cdot L^{-1}$
Glucose oxidase ≥	10,000 $U \cdot L^{-1}$
Peroxidase	≥ 700 $U \cdot L^{-1}$
Sodium azide	< 0.1 %

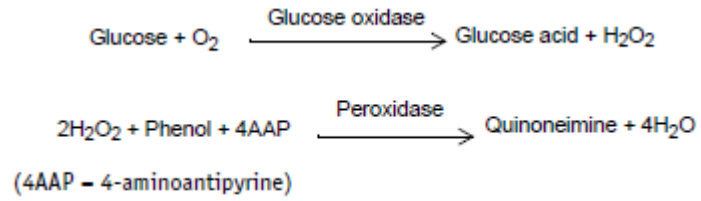
Standards

A single commercially available standard was used (Multical, HORIBA ABX Diagnostics, Montpellier, France). This standard is a lyophilized standard based on human serum. To reconstitute this sample 3 mL of distilled water was added and left to stand at room temperature for 30 min. During this time the vial was agitated slowly avoiding the formation of foam.

Quality controls

Two commercially available quality controls were used (N-control and P-control, HORIBA ABX Diagnostics, Montpellier, France). These quality controls are a lyophilized control based on human serum. To reconstitute this sample 5 mL of distilled water was added and left to stand at room temperature for 30 min. During this time the vial was agitated slowly avoiding the formation of foam.

Method



Procedure

1. Samples were defrosted for 60 min and vortexed before being loaded into the Pentra 400.
2. 4 µL of sample (standard, control or plasma), 290 µL of reagent and 15 µL of distilled water were pipetted into a cuvette
3. The sample was then mixed, incubated and read at a wavelength of 505 nm.
4. Plasma and control glucose concentrations were determined by the analyser using a regression equation derived from analysis of the standard.

Determination of plasma total cholesterol concentration

Plasma total cholesterol concentrations were determined by an enzymatic calorimetric method using a commercially available kit (Cholesterol CP, HORIBA ABX Diagnostics, Montpellier, France). Samples were analysed on an automated centrifugal analyser (Pentra 400, HORIBA ABX Diagnostics, Montpellier, France).

Reagents

The commercially available reagent was composed of:

Good's buffer pH 6.7	50 mmol L^{-1}
Phenol	5 mmol L^{-1}
4-Aminoantipyrine	0.3 mmol L^{-1}
Cholesterol esterase	$\geq$ 200 U L^{-1}
Cholesterol oxidase	$\geq$ 50 U L^{-1}
Peroxidase	$\geq$ 3 kU L^{-1}
Sodium azide	0.95 g L^{-1}

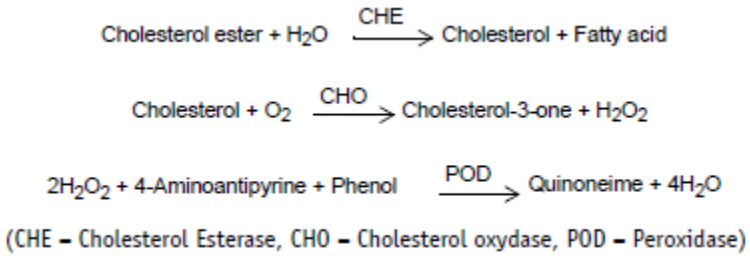
Standards

A single commercially available standard was used (Multical, HORIBA ABX Diagnostics, Montpellier, France). This standard is a lyophilized standard based on human serum. To reconstitute this sample 3 mL of distilled water was added and left to stand at room temperature for 30 min. During this time the vial was agitated slowly avoiding the formation of foam.

Quality controls

Two commercially available quality controls were used (N-control and P-control, HORIBA ABX Diagnostics, Montpellier, France). These quality controls are a lyophilized control based on human serum. To reconstitute this sample 5 mL of distilled water was added and left to stand at room temperature for 30 min. During this time the vial was agitated slowly avoiding the formation of foam.

Method



Procedure

1. Samples were defrosted for 60 min and vortexed before being loaded into the Pentra 400.
2. 3 µL of sample (standard, control or plasma), 250 µL of reagent and 20 µL of distilled water were pipetted into a cuvette
3. The sample was then mixed, incubated and read at a wavelength of 505 nm.
4. Plasma and control total cholesterol concentrations were determined by the analyser using a regression equation derived from analysis of the standard.

Determination of plasma high-density lipoprotein-cholesterol concentration

Plasma HDL-cholesterol concentrations were determined by an enzymatic calorimetric method using a commercially available kit (HDL Direct CP, HORIBA ABX Diagnostics, Montpellier, France). Samples were analysed on an automated centrifugal analyser (Pentra 400, HORIBA ABX Diagnostics, Montpellier, France).

Reagents

The commercially available kit consisted of two reagents. The reagents were composed of:

Reagent 1

Good's Buffer	
Cholesterol oxidase	< 1000 U L^{-1}
Peroxidase	< 1300 ppg U L^{-1}
N,N-bis(4-sulphobutyl)- m-toluidinedisodium	< 1 mM
Accelerator	< 1 mM
Preservative	< 0.06 %
Ascorbic acid oxidase	< 3000 U L^{-1}

Reagent 2:

Good's Buffer	
Cholesterol esterase	< 1500 U L^{-1}
4-Aminoantipyrine	< 1mM
Detergent	< 2 %
Preservative	< 0.06 %

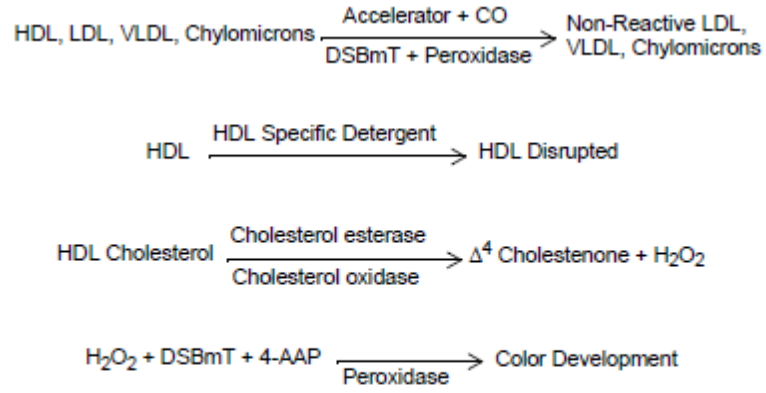
Standards

A single commercially available standard was used (HDL-Cal, HORIBA ABX Diagnostics, Montpellier, France). This standard is a lyophilized standard based on human serum. To reconstitute this sample 1 mL of distilled water was added and left to stand at room temperature for 20 min. During this time the vial was agitated slowly avoiding the formation of foam.

Quality controls

Two commercially available quality controls were used (N-control and P-control, HORIBA ABX Diagnostics, Montpellier, France). These quality controls are a lyophilized control based on human serum. To reconstitute this sample 5 mL of distilled water was added and left to stand at room temperature for 30 min. During this time the vial was agitated slowly avoiding the formation of foam.

Method



(4-AAP – 4-Aminoantipyrine, CO – Cholesterol Oxidase, DSBmT – N,N-bis(4-sulphobutyl)-m-toluidine-disodium)

Procedure

1. Samples were defrosted for 60 min and vortexed before being loaded into the Pentra 400.
2. 2.4 µL of sample (standard, control or plasma), 240 µL of reagent 1 and 10 µL of distilled water were pipetted into a cuvette
3. The sample was then mixed, incubated and 80 µL of reagent 2 added.
4. The sample was then mixed, incubated and read at a wavelength of 600 nm.
5. Plasma and control total cholesterol concentrations were determined by the analyser using a regression equation derived from analysis of the standard.

Determination of plasma low-density lipoprotein-cholesterol concentration

Plasma LDL-cholesterol concentrations were determined by an enzymatic calorimetric method using a commercially available kit (LDL Direct CP, HORIBA ABX Diagnostics, Montpellier, France). Samples were analysed on an automated centrifugal analyser (Pentra 400, HORIBA ABX Diagnostics, Montpellier, France).

Reagents

The commercially available kit consisted of two reagents. The reagents were composed of:

Reagent 1

MES Buffer (pH 6.3)	
Detergent 1	< 1.0 %
Cholesterol Esterase	< 1500 U L^{-1}
Cholesterol Oxidase	< 1500 U L^{-1}
Peroxidase	< 1300 ppg U L^{-1}
4-aminoantipiryrine	< 0.1 %
Ascorbic Acid Oxidase Preservative	< 3000 U L^{-1}

Reagent 2:

MES Buffer (pH 6.3)	
Detergent 2	< 1.0 %
N,N-bis(4-sulfobutyl)-toluidine, disodium	< 1.0 mM
Preservative	

Each reagent was covered with a protective cap to prevent evaporation of the reagent

Standards

A single commercially available standard was used (LDL-Cal, HORIBA ABX Diagnostics, Montpellier, France). This standard is a lyophilized standard based on human serum. To reconstitute this sample 1 mL of distilled water was added and left to stand at room temperature for 20 min. During this time the vial was agitated slowly avoiding the formation of foam.

Quality controls

Two commercially available quality controls were used (N-control and P-control, HORIBA ABX Diagnostics, Montpellier, France). These quality controls are a lyophilized control based on human serum. To reconstitute this sample 5 mL of distilled water was added and left to stand at room temperature for 30 min. During this time the vial was agitated slowly avoiding the formation of foam.

Method

The reactions involved in this assay were not provided by the manufacturer but was described as the following:

"The method is in a two reagent format and depends on the properties of a unique detergent. This detergent (Reagent 1) solubilizes only the non LDL lipoprotein particles. The cholesterol released is consumed by cholesterol esterase and cholesterol oxidase in a non color forming reaction. A second detergent (Reagent 2) solubilizes the remaining LDL particles and a chromogenic coupler allows for color formation. The enzyme reaction with LDL-C in the presence of the coupler produces color that is proportional to the amount of LDL cholesterol present in the sample."

Procedure

1. Samples were defrosted for 60 min and vortexed before being loaded into the Pentra 400.
2. 2.4 µL of sample (standard, control or plasma), 240 µL of reagent 1 and 10 µL of distilled water were pipetted into a cuvette
3. The sample was then mixed, incubated and 80 µL of reagent 2 added.
4. The sample was then mixed, incubated and read at a wavelength of 600 nm.
5. Plasma and control total cholesterol concentrations were determined by the analyser using a regression equation derived from analysis of the standard.

Determination of plasma insulin concentration

Plasma insulin concentrations were determined using using a commercially available ELISA assay (Insulin ELISA, Mercodia, Uppsala, Sweden). Plate reader to measure absorbance (Expert Plus, ASYS Atlantis, Eugendorf, Austria).

ELISA Kit

The commercially available ELISA kit was composed of:

1 x 96 well coated plate
1 x 5 ml calibrator 0
1 x 1 mL calibrator 1
1 x 1 mL calibrator 2
1 x 1 mL calibrator 3
1 x 1 mL calibrator 5
1 x 1 mL calibrator 6
1 x 1.2 mL enzyme conjugate 11X
1 x 12 mL enzyme conjugate buffer
1 x 50 mL wash buffer 21X
1 x 22 mL substrate 3,3',5,5'-tetramethylbenzidine (TMB)
1 x 7 mL stop solution (0.5 M H_2SO_4)

Standards

A 6 level standard provided in the ELISA kit was used. Each standard was used in duplicate.

Quality controls

Two commercially available quality controls were used (Mercodia Diabetes Antigen Hi and Lo, Mercodia, Uppsala, Sweden). These quality controls are a lyophilized control based on human serum. To reconstitute this sample 500 µL of distilled water was added and left to stand at room temperature for 30 min. During this time the vial was agitated slowly avoiding the formation of foam.

Method

This ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microplate wells. A simple washing step removes unbound enzyme labelled antibody. The bound conjugate is detected by reaction with TMB. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

Procedure

1. Samples were defrosted for 60 min and vortexed before the start of the assay.
2. Enzyme conjugate was prepared by mixing 1.2 ml of enzyme conjugate 11X and 12 ml of enzyme conjugate buffer for each full plate.
3. Wash solution was prepared by mixing 50 ml wash buffer with 1000 ml distilled water for each full plate. Once prepared this was poured into a plate washer ready for later use.
4. 25 µL of sample (standard, control or plasma) and 100 µL of enzyme conjugate was added to each well
5. The plate was sealed and incubated on a plate shaker (700 rpm) for 60 min at room temperature.
6. The plate was then washed 6 times with 700 µL of wash solution per well using an automatic plate washer overflow-wash function. Following the final wash, any

remaining wash solution was blotted out by tapping the plate several times against absorbent paper.

7. 200 µL of substrate TMB was then pipetted into each well and incubated for 15 min at room temperature.
8. 50 µL of stop solution was then added and the plate was gently shook.
9. The plate was read at an optical density of 450 nm.
10. Plasma and control insulin concentrations were determined by the plate reader with the software calculations set to concentration and absorbance and the advanced calculation set to long result – catox(avg(od)).

Determination of haemoglobin concentration

Haemoglobin concentrations were determined in duplicate using Drabkin's solution and a spectrophotometer (Digital Grating Spectrophotometer, Cecil Instruments Ltd, Cambridge, UK).

Reagent (Drabkin's solution)

Drabkin's solution was prepared as follows using commercially available chemicals.

1 g Sodium bicarbonate (Na HCO_3)[MW 84.01]
0.2 g Potassium ferricyanide ($K_3Fe(CN)_6$) [MW 329.3]
0.05 g Potassium cyanide (KCN) [MW65.12]
1 L distilled water

* Sodium bicarbonate is light sensitive
* Potassium ferricyanide is toxic

Method

Haemoglobin is oxidised to methaeglobin by the potassium ferricyanide which is then converted to cyanmethaeglobin by the potassium cyanide. The absorbance of cyanmethaeglobin is then measured spectrophotometrically at a wavelength of 546 nm.

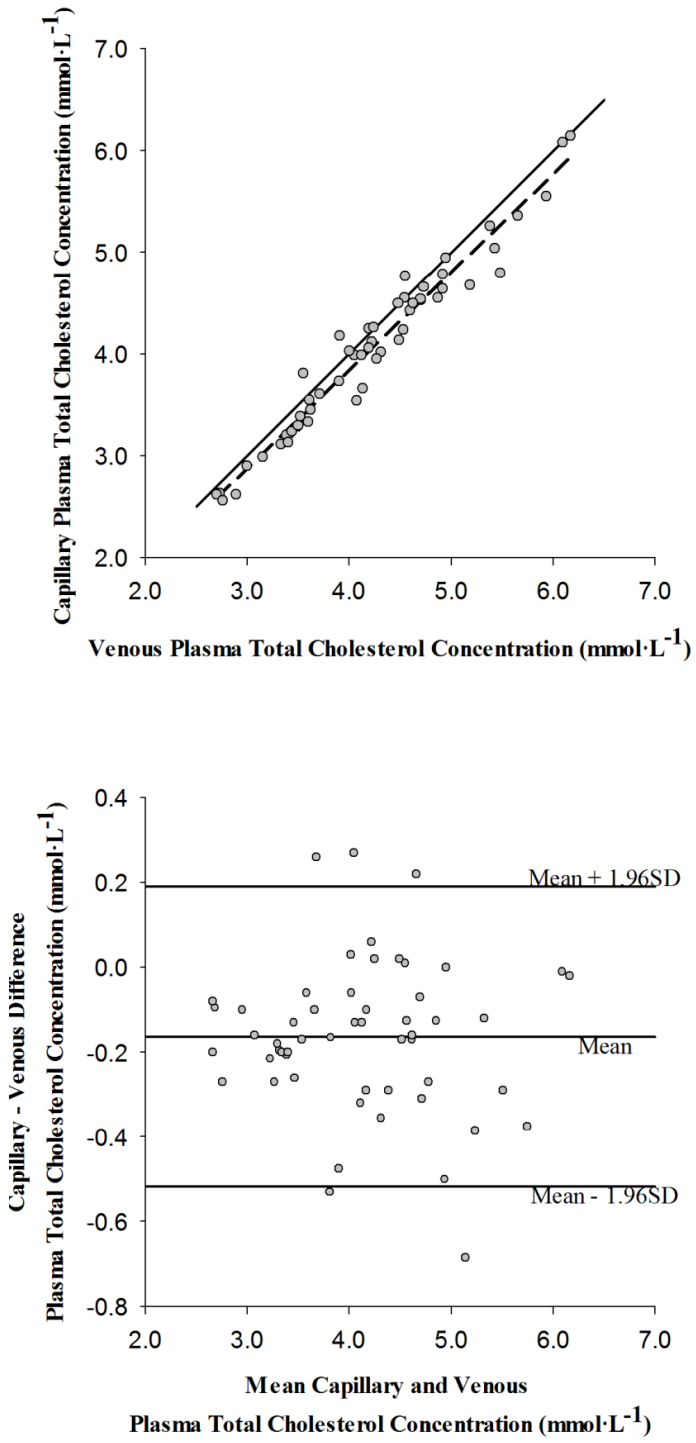
Haemoglobin + Potassium ferricyanide + Potassium cyanide → Cyanmethaeglobin

Procedure

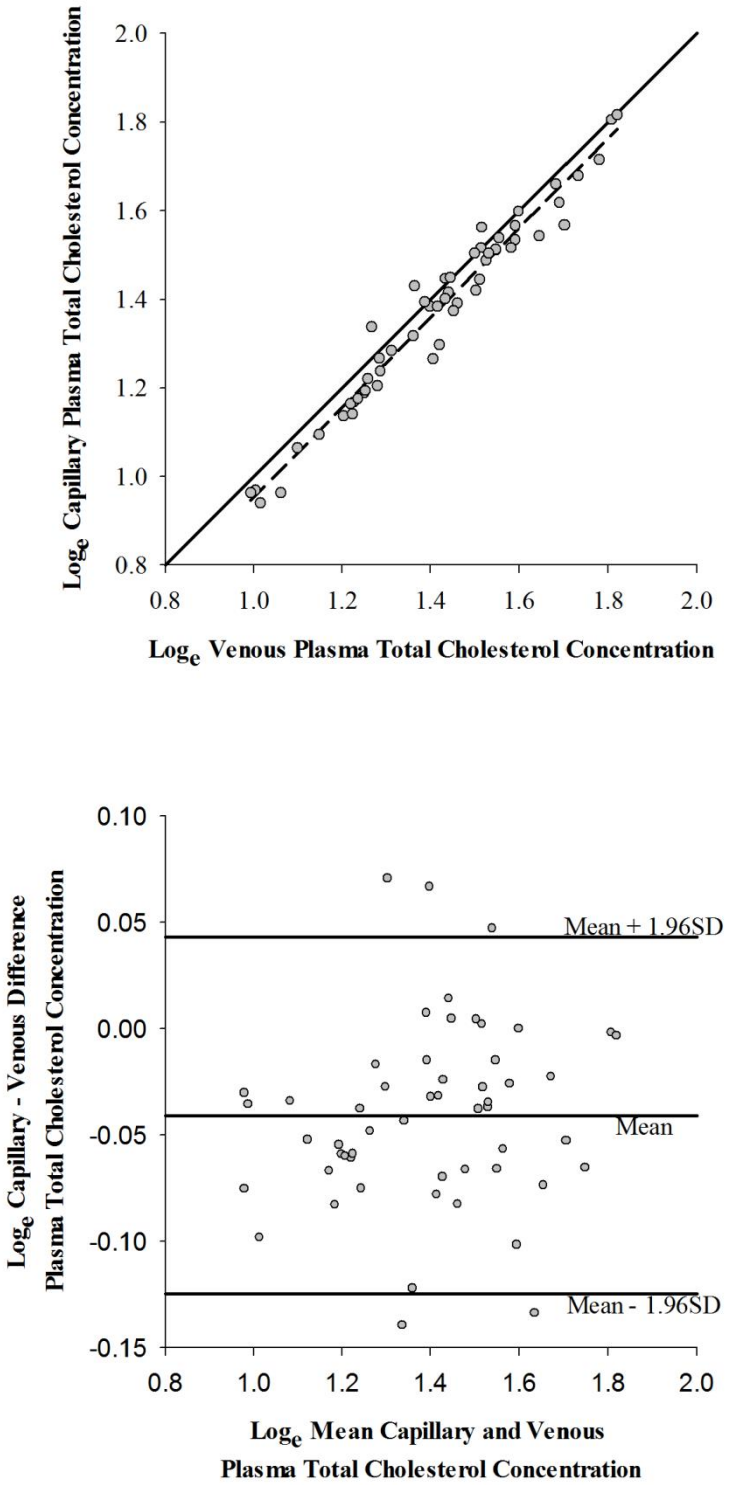
1. 20 µL of whole blood was dispensed in to 5 mL of Drabkin's solution and mixed.
2. The spectrophotometer was then zeroed against a Drabkin's solution blank before the sample absorbance was read.
3. Haemoglobin concentrations ($g \cdot dL^{-1}$) was calculated by multiplying the obtained absorbance by 36.77.

Appendix 8: Relationship between venous and capillary analyte concentration and Bland and Altman plots for data presented in Chapter 4

Total cholesterol

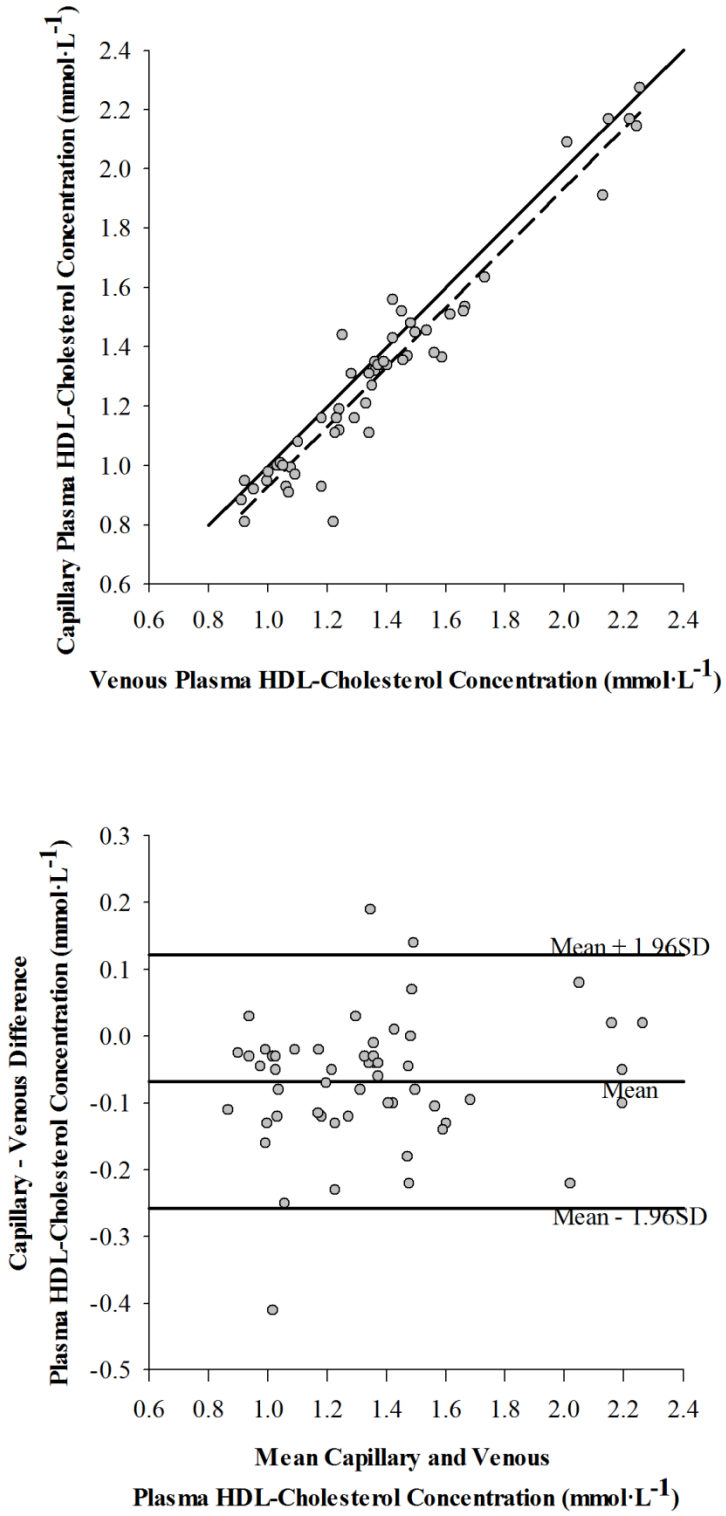


Relationship between venous and capillary plasma total cholesterol concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

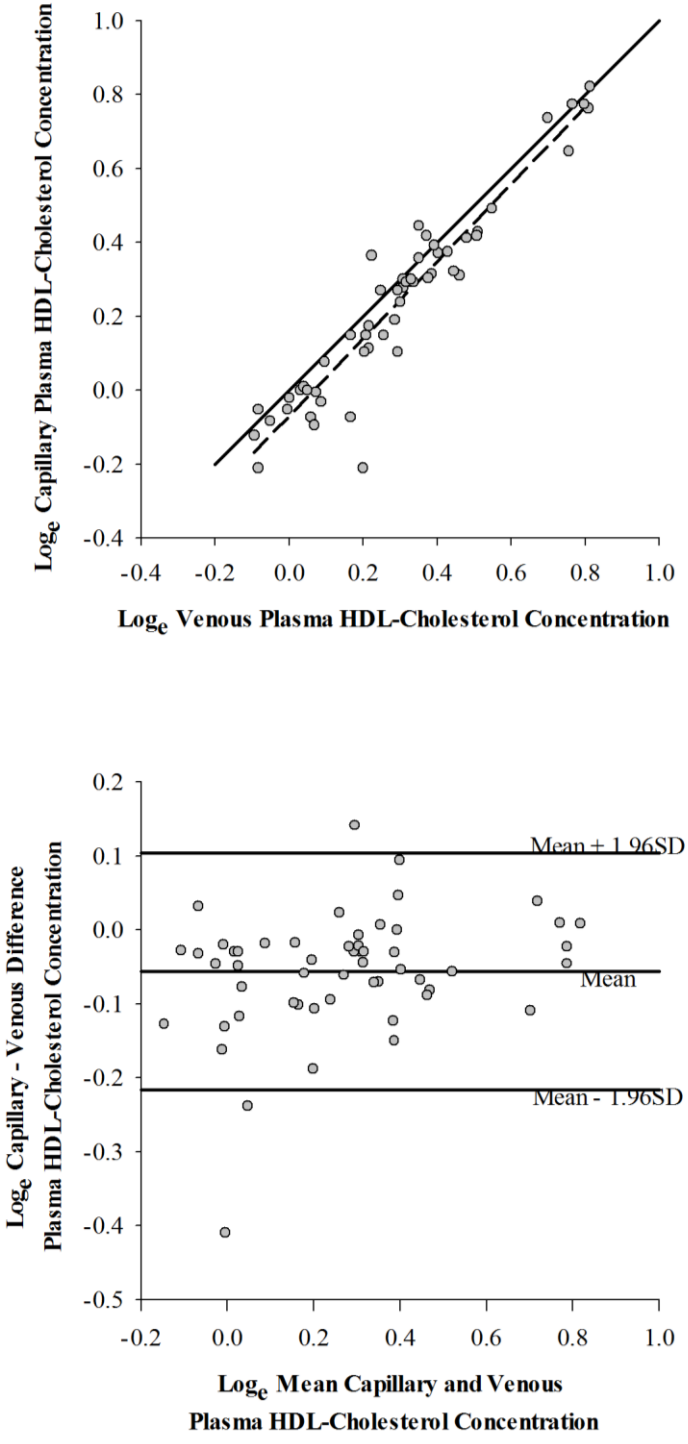


Relationship between $\log_e$ transformed venous and capillary plasma total cholesterol concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

High-density lipoprotein-cholesterol

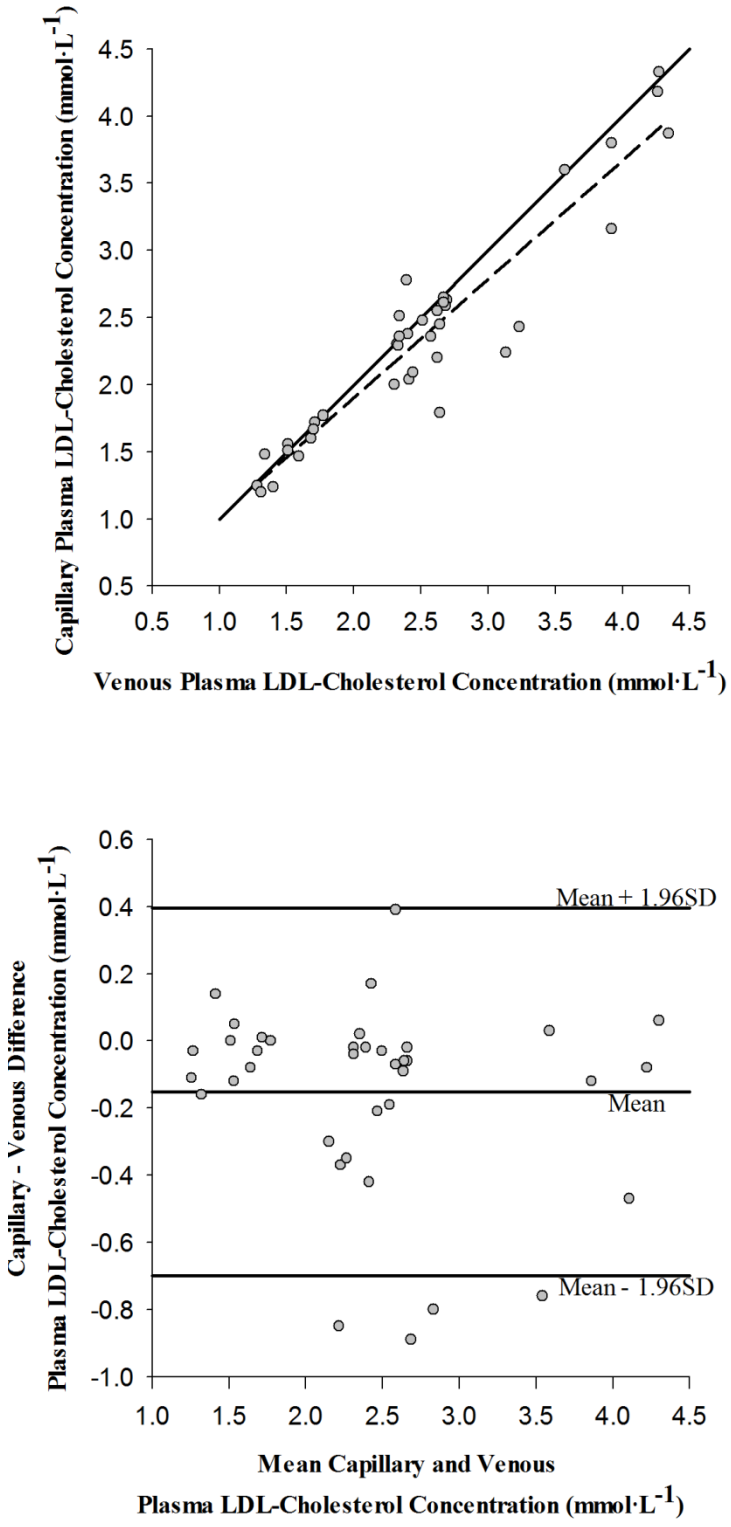


Relationship between venous and capillary plasma high-density lipoprotein (HDL) cholesterol concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

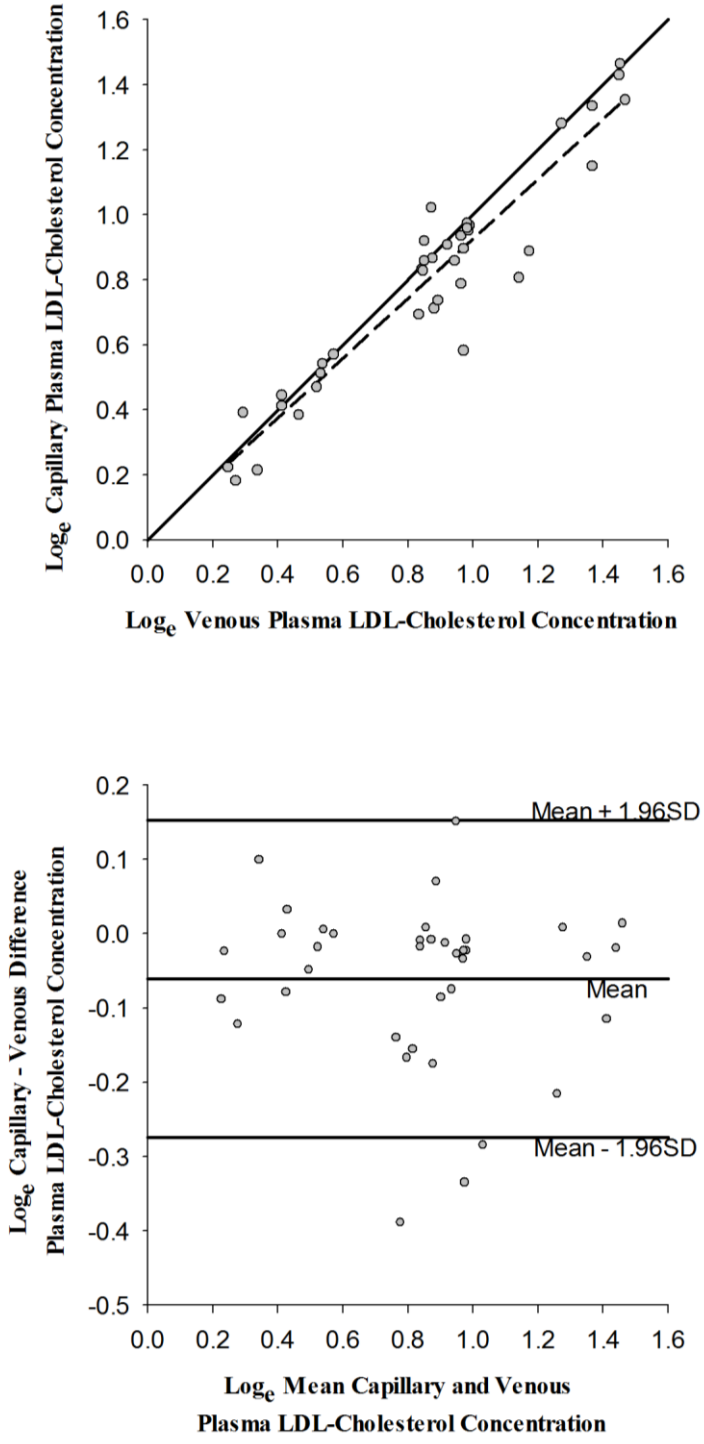


Relationship between $\log_e$ transformed venous and capillary plasma high-density lipoprotein (HDL) cholesterol concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

Low-density lipoprotein-cholesterol

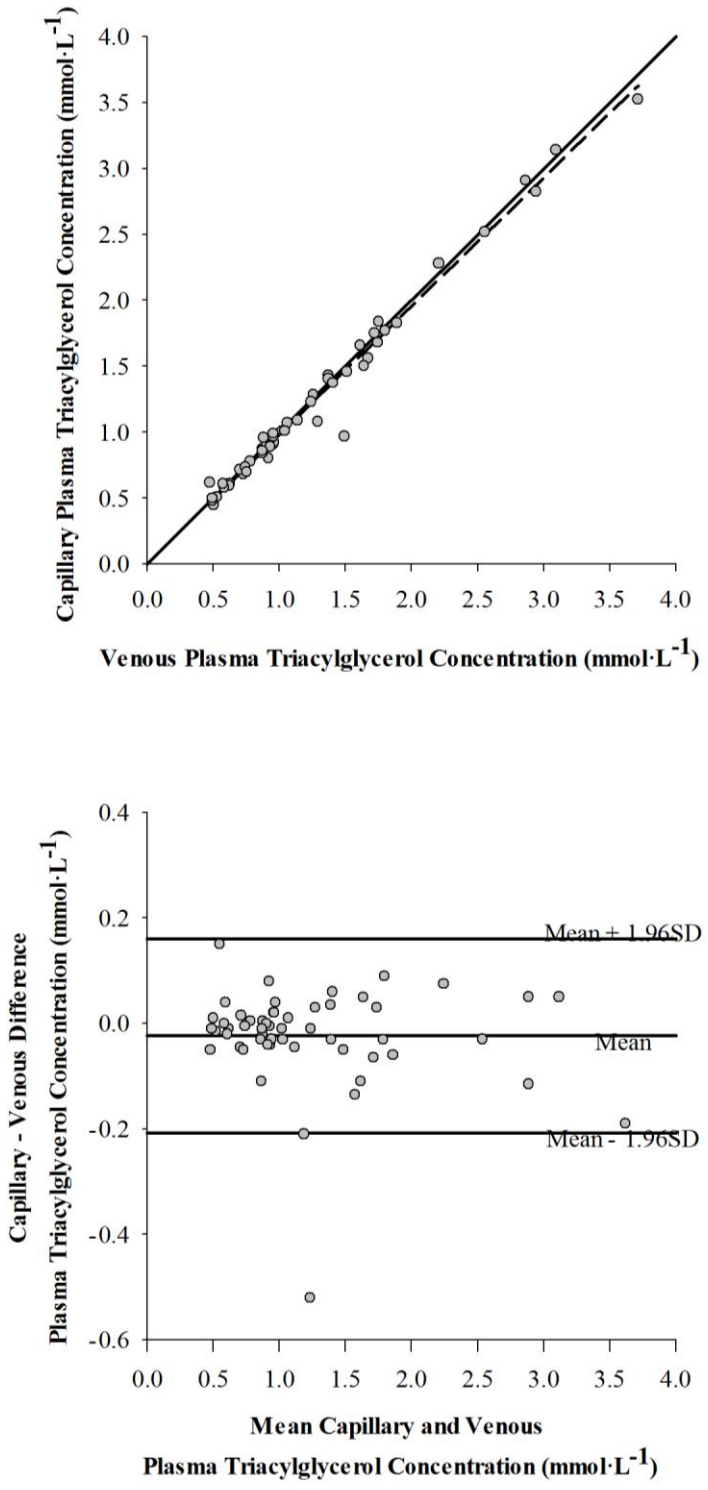


Relationship between venous and capillary plasma low-density lipoprotein (LDL) cholesterol concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

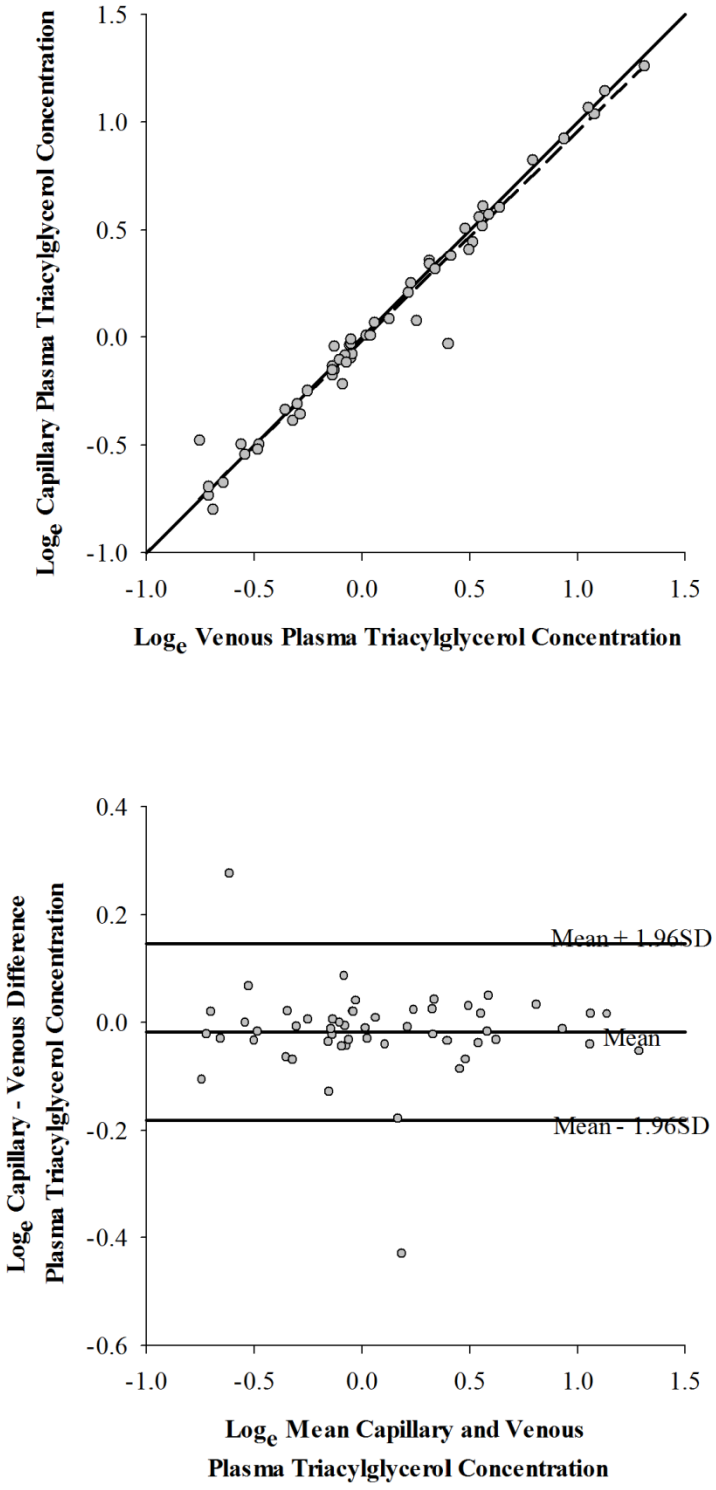


Relationship between $\log_e$ transformed venous and capillary plasma low-density lipoprotein (LDL) cholesterol concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

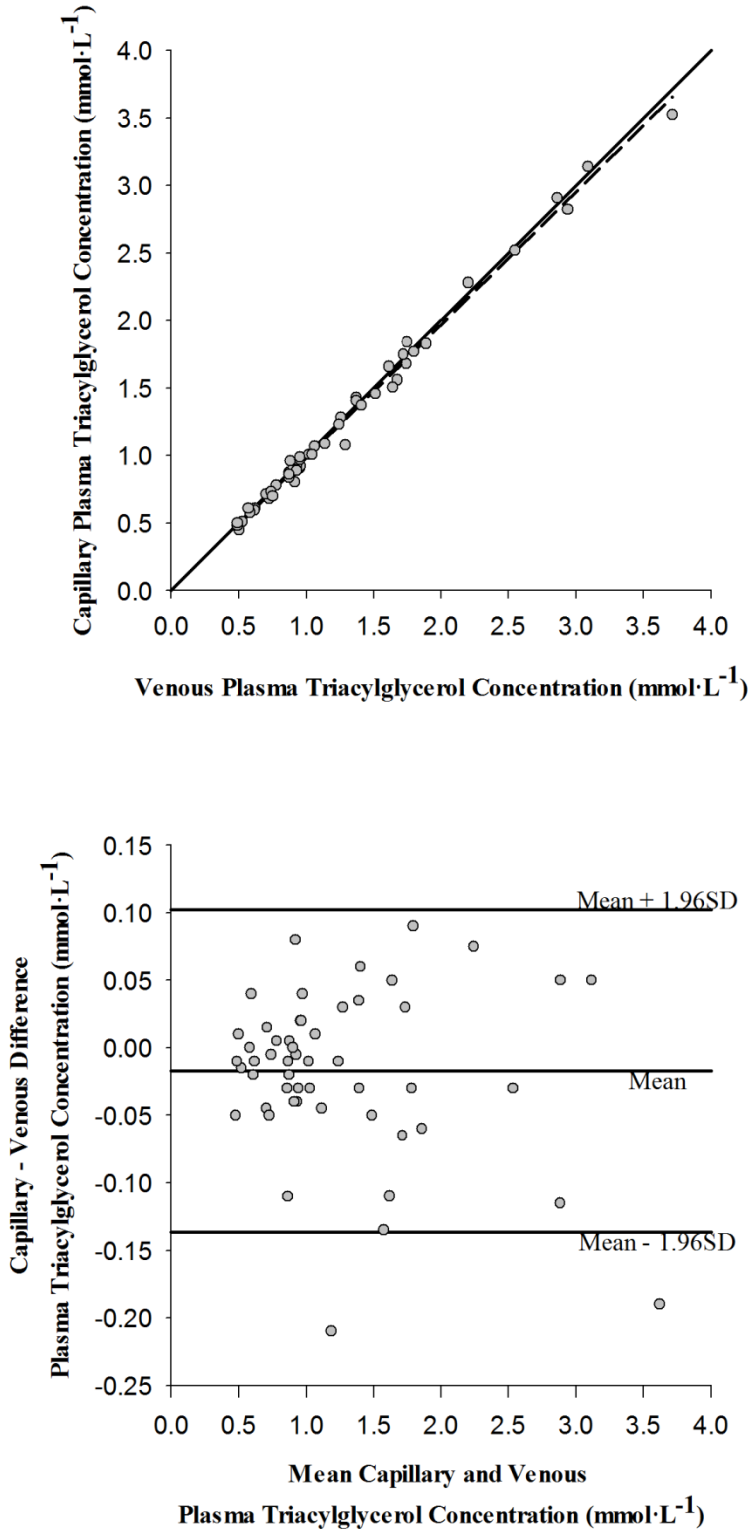
Triacylglycerol



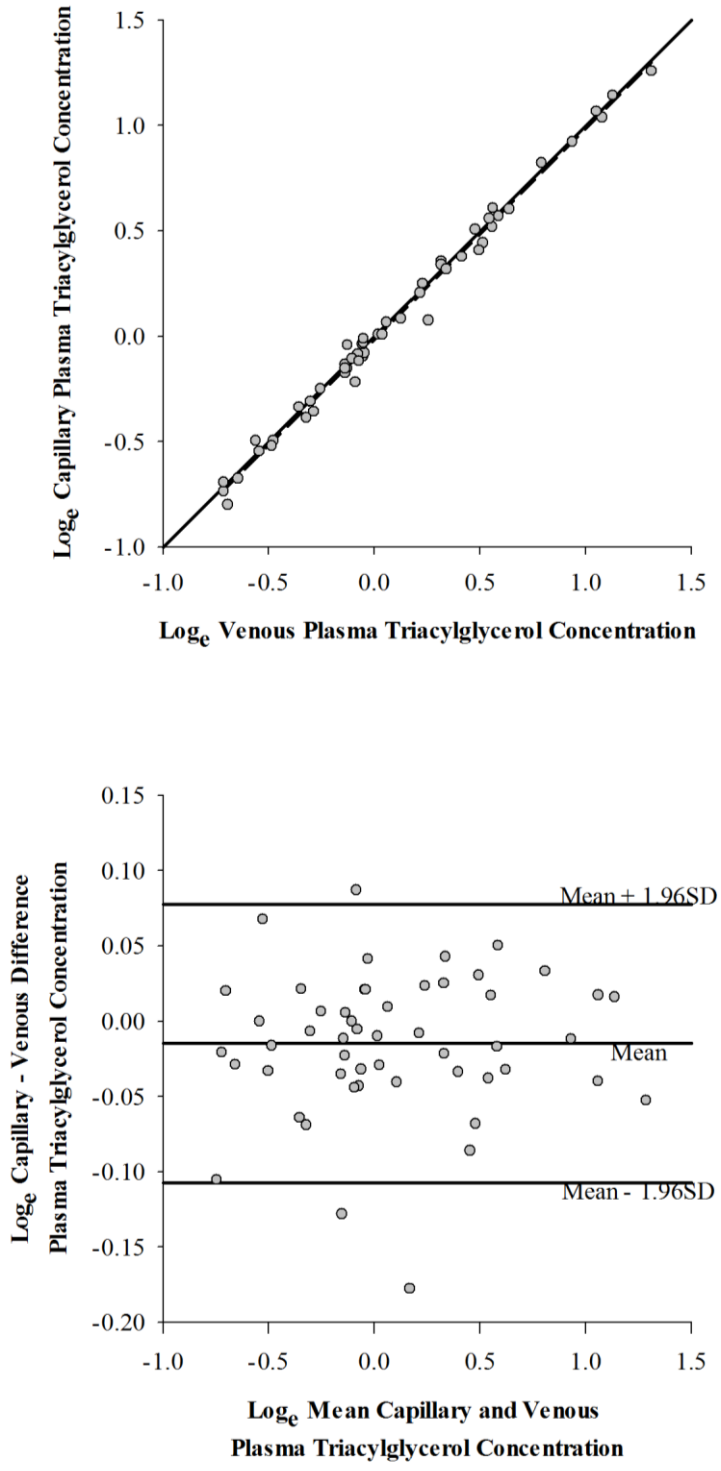
Relationship between venous and capillary plasma triacylglycerol concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].



Relationship between $\log_e$ transformed venous and capillary plasma triacylglycerol concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

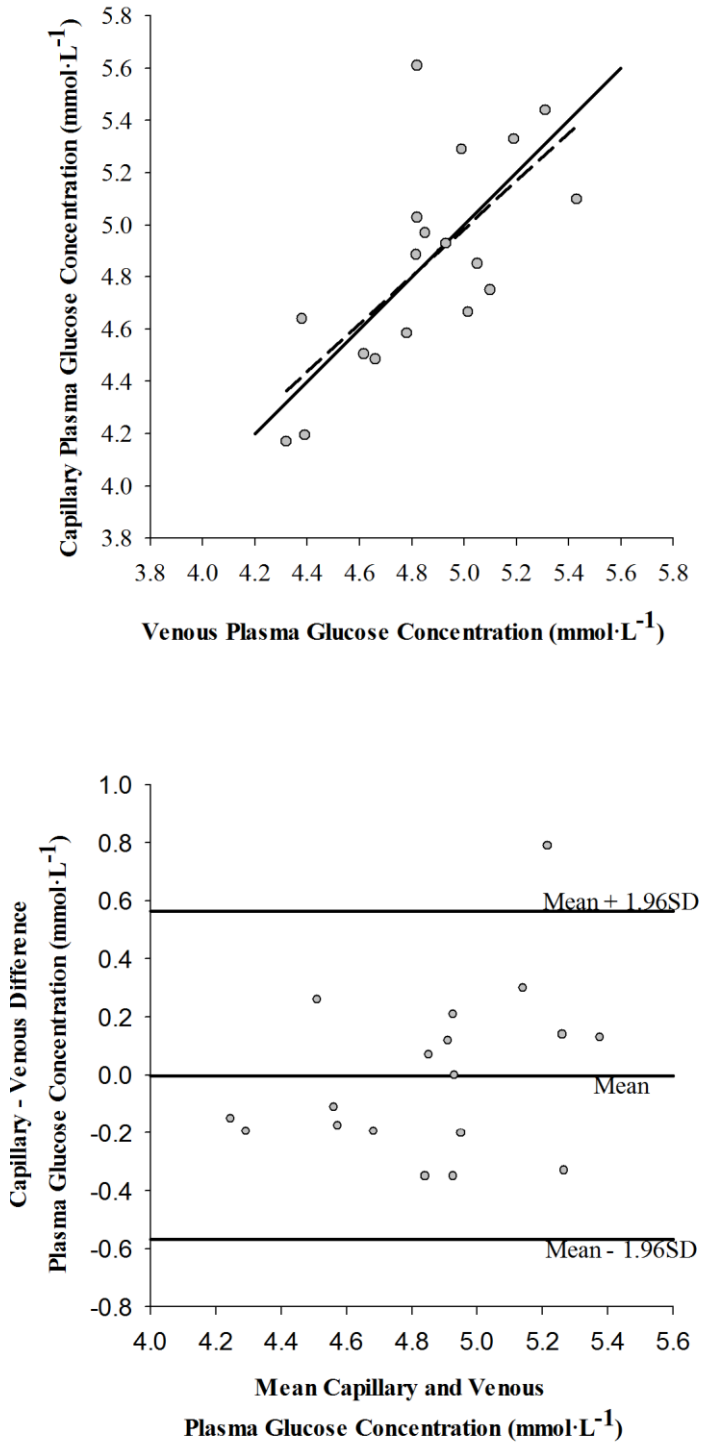


Relationship between venous and capillary plasma triacylglycerol concentration (outliers removed) (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

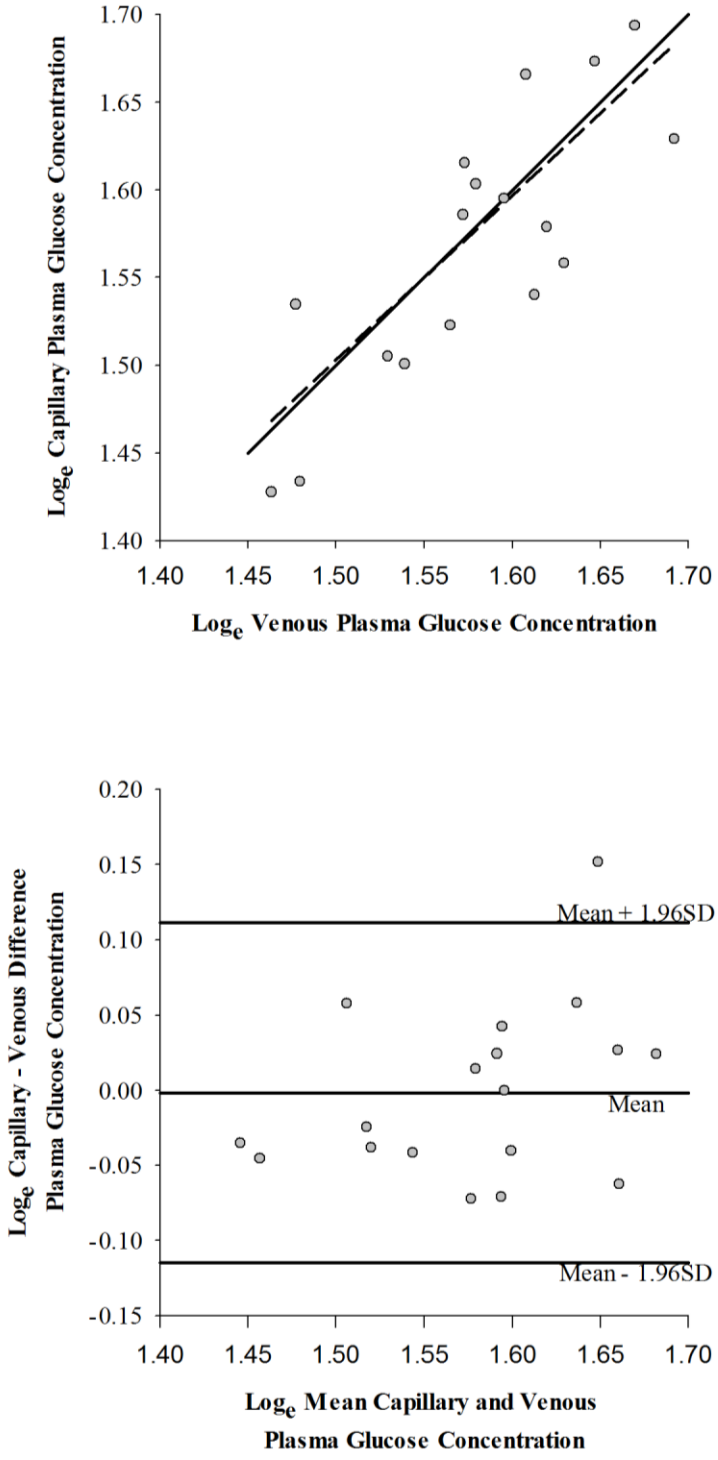


Relationship between $\log_e$ transformed venous and capillary plasma triacylglycerol concentration (outliers removed) (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

Fasting glucose

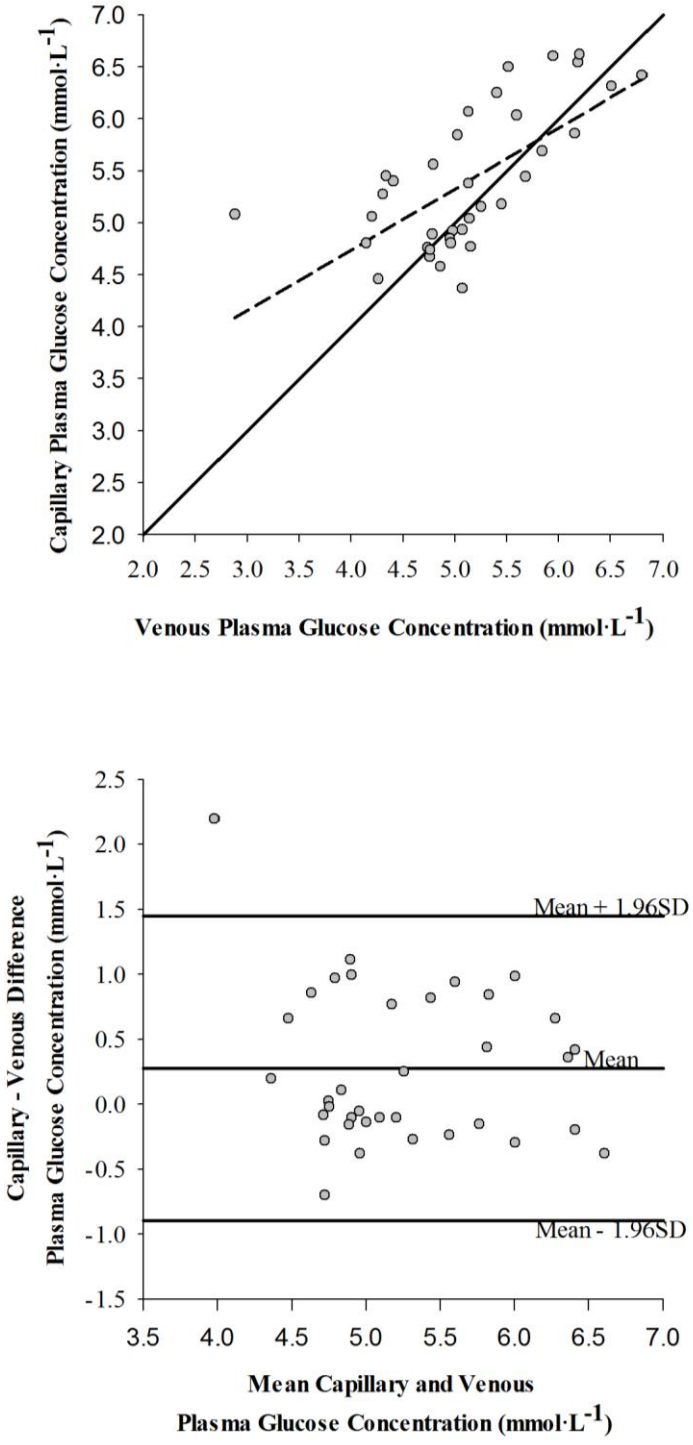


Relationship between venous and capillary plasma fasting glucose concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

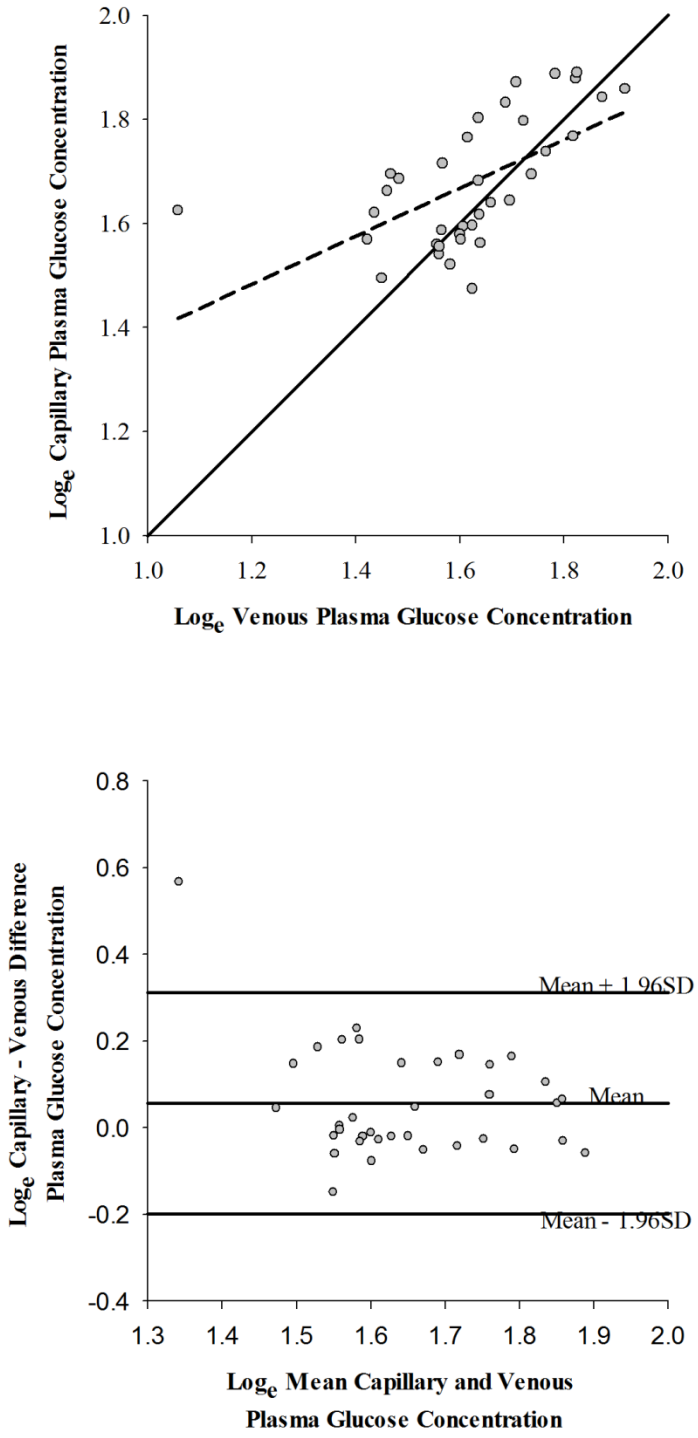


Relationship between log$_e$ transformed venous and capillary plasma fasting glucose concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

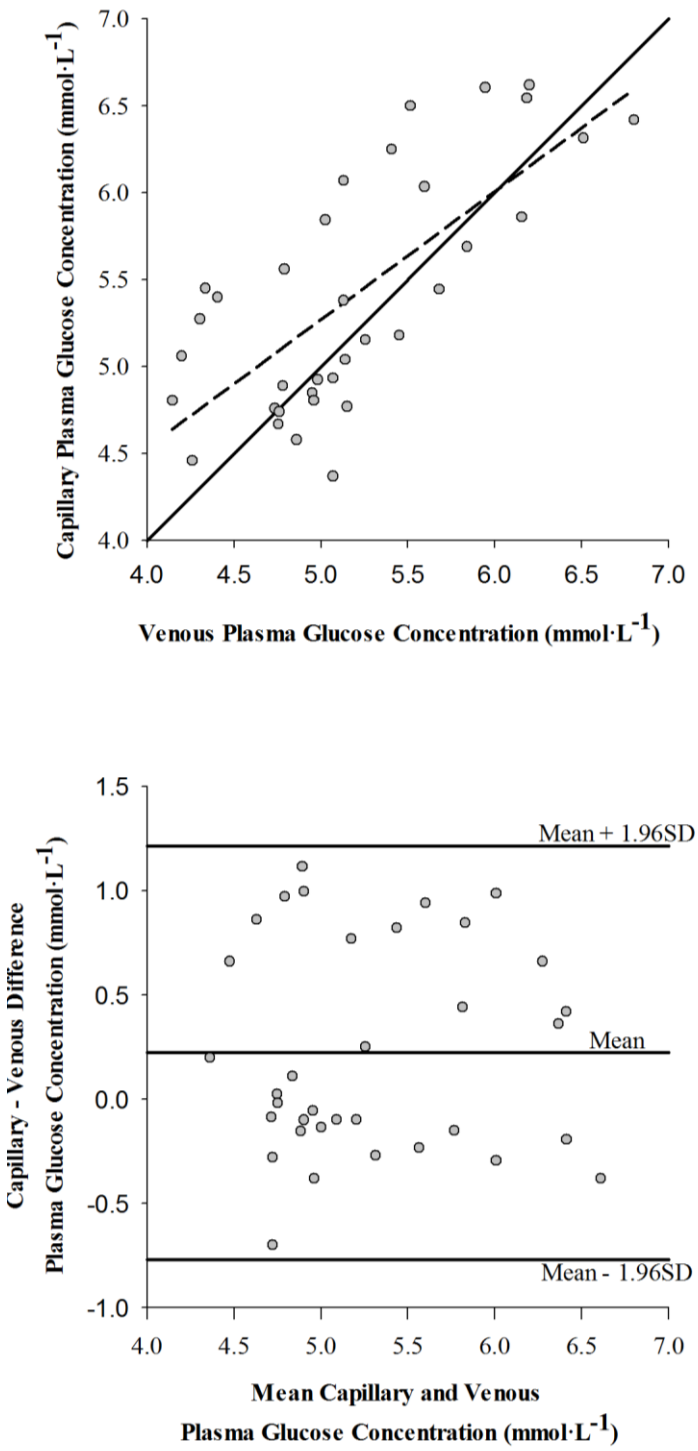
Postprandial glucose



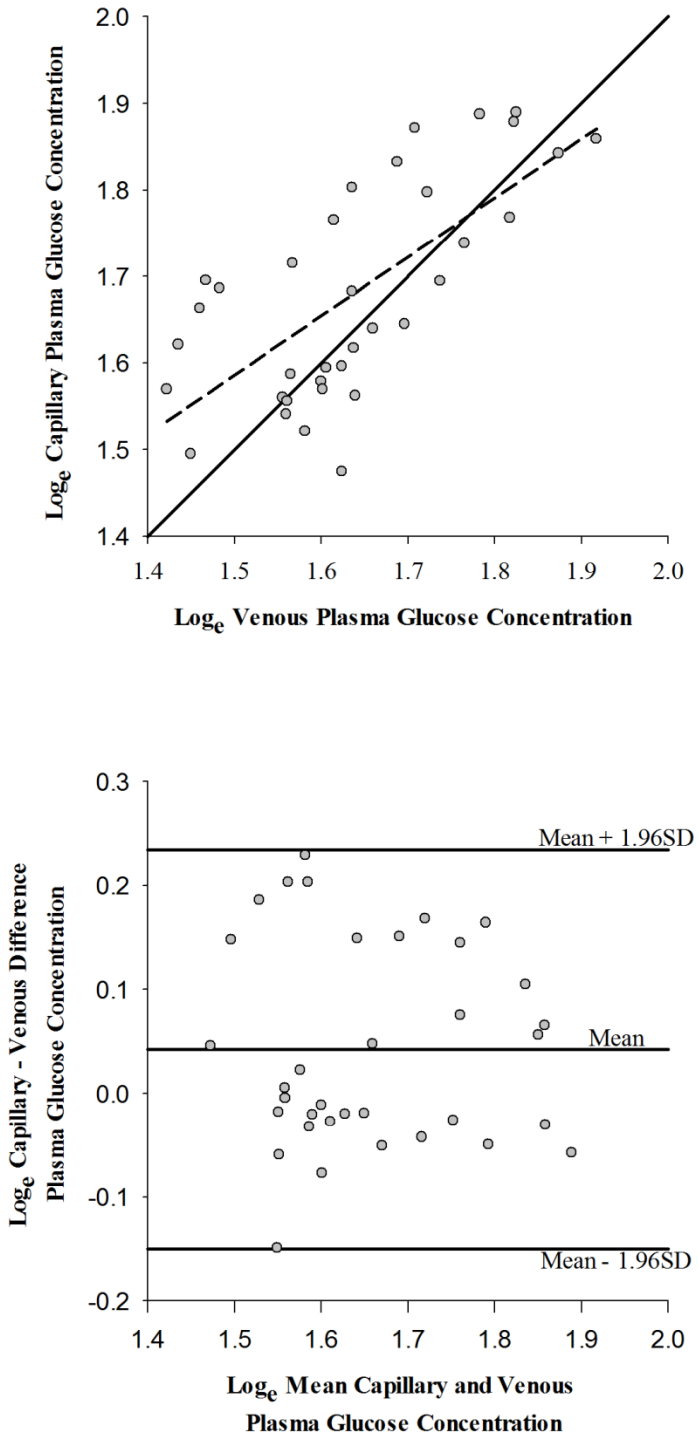
Relationship between venous and capillary plasma postprandial glucose concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].



Relationship between log$_e$ transformed venous and capillary plasma postprandial glucose concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

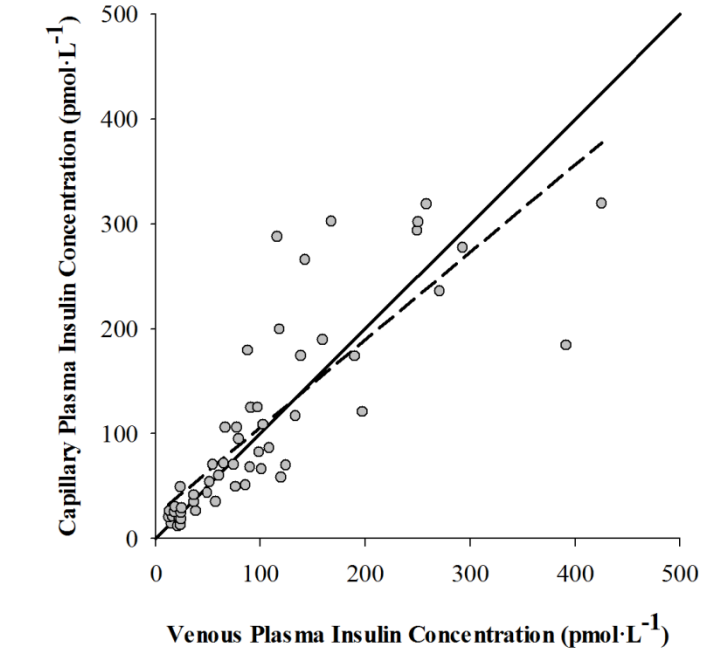


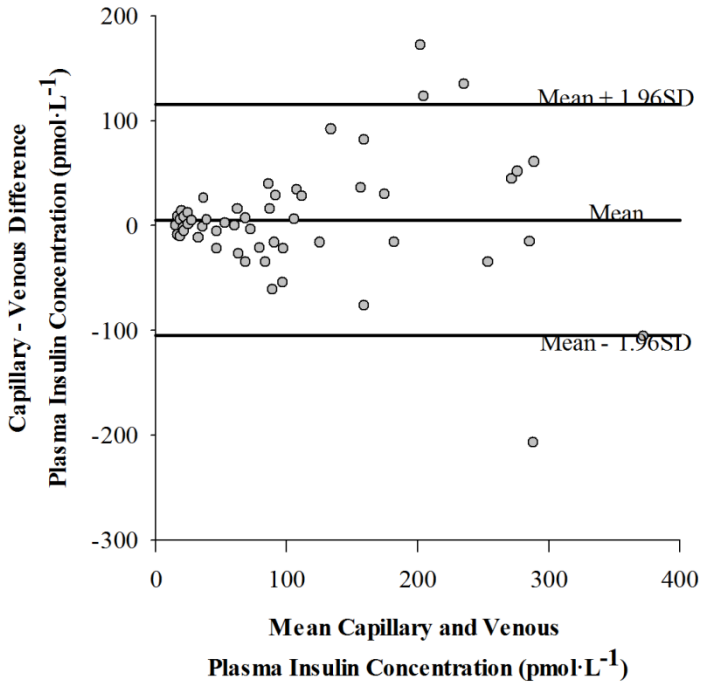
Relationship between venous and capillary plasma postprandial glucose concentration (outliers removed) (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].



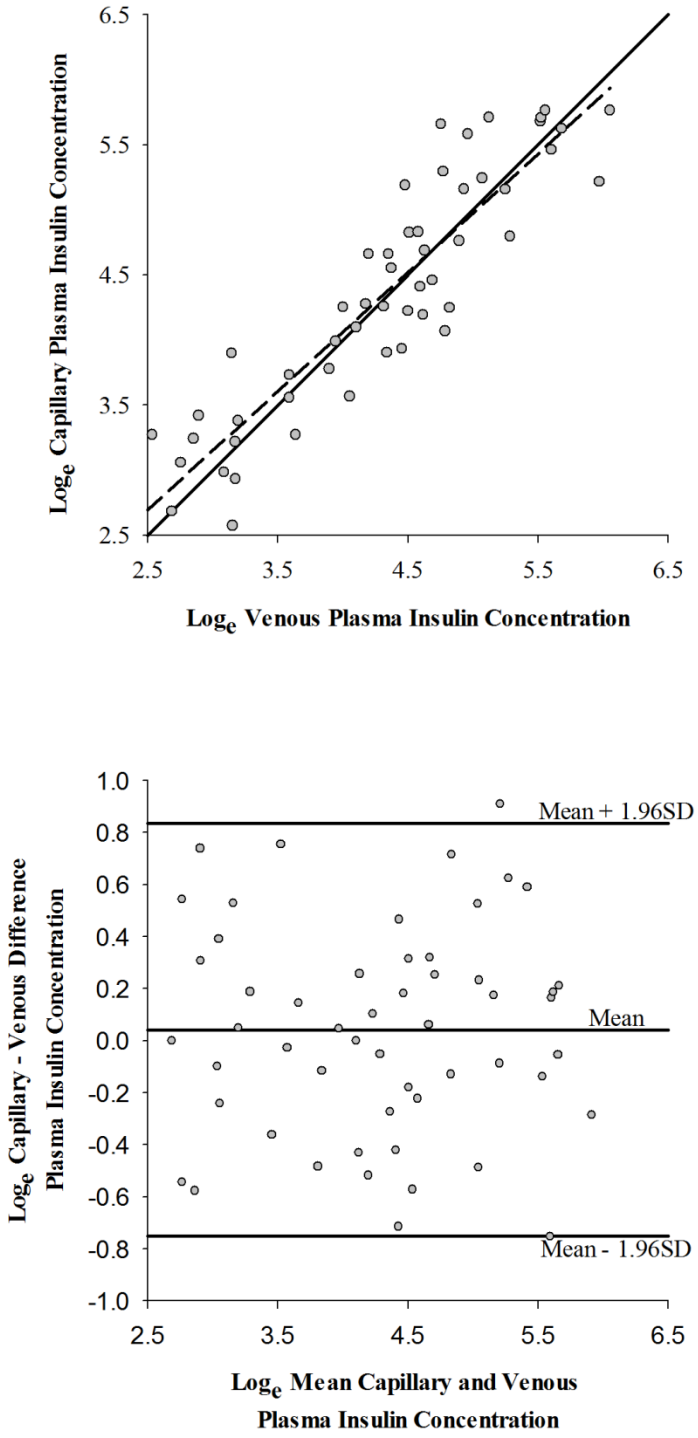
Relationship between log$_e$ transformed venous and capillary plasma postprandial glucose concentration (outliers removed) (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].

Insulin





Relationship between venous and capillary plasma insulin concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].



Relationship between log$_e$ transformed venous and capillary plasma insulin concentration (dashed line) with the line of equality (solid line) [top] and the Bland and Altman plot [bottom].