VASCULAR AND METABOLIC ADAPTATIONS TO EXERCISE IN NON-ALCOHOLIC FATTY LIVER DISEASE

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is characterised by the accumulation of fat in the liver and is associated with liver-related morbidity and mortality. Nevertheless, the leading cause of death in these patients is cardiovascular disease (CVD). Excess abdominal visceral adipose tissue (VAT) is frequently expressed in NAFLD and is considered a pivotal feature in the pathogenesis of NAFLD which is predictive of CVD. Endothelial dysfunction is an early manifestation in the development of atherosclerosis and is characterised by a diminished bioavailabilty of the anti-atherogenic molecule NO, which is secreted by the endothelium of all blood vessels throughout the vascular tree. Limited pharmacological treatment is available to reduce hepatic fat, therefore, lifestyle modification interventions comprised of structured exercise and diet are recommended as a non-pharmacological management strategy to reduce hepatic fat in NAFLD. The primary aim of this thesis was to explore nitric oxide (NO)-mediated endothelial function at different levels of the vascular tree in NAFLD patients and to establish whether supervised exercise training has a sustained therapeutic impact on endothelial function.

Thirty-two NAFLD patients (21 males, 11 females, 48±2yrs, BMI 31±1kg/m^2) and eighteen matched controls (8 males, 10 females, 48±2yrs, BMI 30±1kg/m^2) underwent magnetic resonance imaging (MRI) to quantify abdominal VAT and proton magnetic resonance spectroscopy (^{1}H-MRS) to determine intrahepatocellular triglyceride content (IHTC). Brachial artery flow mediated dilatation (FMD) (as an index of endothelial NO function) was also assessed. IHTC (27.2±3.0 vs. 2.9±0.4%) and abdominal VAT (5.4±0.3vs. 3.4±0.2 l) were elevated in NAFLD patients when compared with controls (P<0.0005). FMD was significantly impaired in NAFLD patients when compared with controls (4.8±0.3 vs. 8.3±0.7%, P<0.0005). A significant inverse correlation was observed between FMD and abdominal VAT (r = -0.48, P=0.01) in NAFLD patients, but no relationship was observed between FMD and IHTC (P>0.05). Impairment in FMD remained in NAFLD patients following independent covariate adjustment for abdominal VAT (5.0±0.5 vs. 7.3±0.7%, P=0.01). These findings indicate that excess IHTC and abdominal VAT do not explain endothelial dysfunction in NAFLD.

Twenty NAFLD patients were randomly assigned to either 16-weeks of supervised moderate intensity (30-60% HRR, 30-45 min, 3-5 times per week) exercise training (n=13, 50±3yrs, BMI 30±1kg/m^2) or to 16-weeks of conventional care lifestyle advice (n=7, 47±6yrs, BMI 31±2kg/m^2). Supervised exercise training induced a greater improvement in FMD when compared with conventional care (3.6±0.6 vs. 0.3±0.8%, P=0.004). There was no significant difference between the effect of exercise and conventional care on IHTC or abdominal VAT (P>0.05). These data suggest that supervised exercise training is an effective management strategy in NAFLD capable of improving conduit artery endothelial function independent of IHTC and abdominal VAT.

In order to explore the longevity of the exercise-induced improvements in conduit artery endothelial function, a 12-month follow up assessment was performed in 9 of the NAFLD patients (5 males, 4 females, 50±5yrs, BMI 30±1kg/m^2) who completed the 16-week supervised exercise training intervention. The exercise-induced improvement in FMD (5.1±0.8 vs. 7.9±0.8%; P=0.004) was abolished 12 months following the cessation of supervised exercise training (7.9±0.8 vs.5.0±0.5%; P=0.02), returning to a similar level observed at baseline (5.1±0.8 vs. 5.0±0.5%; P=0.95).These findings indicate that in order to chronically sustain exercise-induced improvements in

endothelial function in NAFLD patients, long-term exercise supervision and guidance is required.

Cutaneous NO-mediated microvessel function reflects generalised microvascular function and provides a translational model to investigate pre-clinical disease, but has not been previously investigated in NAFLD. NO-mediated vasodilatation in the cutaneous microvessels was examined in 13 NAFLD patients (7 males, 6 females, 50±3yrs, BMI 31±1kg/m^2) and 7 matched controls (3 males, 4 females, 48±4yrs, BMI 30±2 kg/m^2). Microdialysis fibres were embedded into the skin of the forearm and laser Doppler probes placed over these sites. Both sites were then heated to 42°C, with saline solution infused in one probe and L-N^G-monomethyl arginine (L-NMMA) through the second. Following baseline assessment, 11 NAFLD patients were randomly assigned to 16-weeks of supervised moderate intensity exercise training (*n*=6, 45±5yrs, BMI 31±1kg/m^2) or to 16-weeks of conventional care (*n*=5, 51±3yrs, BMI 30±21kg/m^2). The NO contribution to skin blood flow in response to incremental heating was not different between NAFLD patients and controls (*P*=0.47) at baseline. However, significant differences were evident in NO contribution between the exercise training and conventional care group (*P*=0.01), suggesting that supervised exercise training improves cutaneous NO-mediated microvascular endothelial function in NAFLD patients.

This thesis suggests that supervised exercise training has a direct therapeutic impact on endothelial function in NAFLD which may decrease the risk of future heart disease and stroke. As a cardioprotective management strategy in NAFLD, exercise training is superior to that of current conventional care pathways, however, in order to chronically sustain the exercise-induced improvements in endothelial function, long term exercise supervision and guidance is required.

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Declaration

I declare that the work contained within this thesis is entirely my own.

Publications directly associated with this thesis

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C.J.A. Pugh., H. Jones., V.S. Sprung., G.J. Kemp., A. Irwin., V.L. Adams., *et al.* (2011). A 16-week moderate intensity exercise intervention reduces body mass, hepatic triglyceride and abdominal subcutaneous fat in NAFLD patients. *Journal of Diabetes, 3, S1, p201.*

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List of abbreviations

ACh	Acetylcholine
ALT	Alanine transaminase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AUC	Area under the curve
AST	Aspartate transaminase
β-ox	β-oxidation
BMI	Body mass index
BP	Blood pressure
CC	Conventional care
CAD	Coronary artery disease
CVD	Cardiovascular disease
CVC	Cutaneous vascular conductance
DBP	Diastolic blood pressure
DICOM	Digital imaging and communications in medicine
DNL	*de novo* lipogenesis
eNOS	Endothelial nitric oxide synthase
Ex	Exercise
FFA	Free fatty acid
FMD	Flow mediated dilatation
GGT	Gamma-glutamyltransferase
GTN	Glycerl trinitrate
HDL	High density lipoprotein
HOMA	Homeostatic model assessment
HR	Heart rate
HRR	Heart rate reserve

Hz	Hertz
IMT	Intima-media thickness
LDF	Laser Doppler flowmetry
LDL	Low density lipoprotein
L-NAME	*N*-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-$_L$-arginine
MAP	Mean arterial pressure
MRI	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NO	Nitric oxide
PU	Arbitrary perfusion units
RPE	Rate of perceived exertion
RER	Respiratory exchange ratio
ROI	Region of interest
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SBP	Systolic blood pressure
SE	Standard Error
SKBF	Skin blood flow
SNP	Sodium Nitroprusside
SR	Shear rate
TNF	Tumour necrosis factor
VAT	Visceral adipose tissue
VLDL	Very low density lipoprotein
$VO_{2\ peak}$	Maximal oxygen consumption
^{1}H-MRS	Proton magnetic resonance spectroscopy

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Chapter 1

INTRODUCTION & AIMS

Non-alcoholic fatty liver disease (NAFLD) is characterised by excessive triglyceride accumulation within the liver and represents a spectrum of histopathological disorders ranging from simple steatosis, to inflammatory non-alcoholic steatohepatitis (NASH) with increasing levels of fibrosis and ultimately cirrhosis. Over recent years, NAFLD has emerged as the most common form of chronic liver disease in western society, affecting approximately 20-30% (Bedogni *et al.*, 2005) of the general population and ~70-90% of obese and type 2 diabetic individuals (Adams & Angulo, 2005). Alarmingly, given the rapid rise of obesity in children globally, NAFLD is now also recognized as the most common cause of liver disease in paediatric population (Schwimmer *et al.*, 2006).

NAFLD is closely associated with obesity, insulin resistance and type 2 diabetes and is now universally regarded as the hepatic manifestation of the metabolic syndrome (Marchesini *et al.*, 2001). Like all prediabetic conditions, insulin resistance is an integral pathological feature of NAFLD, nevertheless, recent studies indicate that body fat distribution also plays an integral role in the pathogenesis of NAFLD (Kelley *et al.*, 2003; Nguyen-Duy *et al.*, 2003; van der Poorten *et al.*, 2008). Specifically, excess abdominal visceral adipose tissue (VAT) seems to be a key determinant of NAFLD, as it is potent source of circulatory free fatty acids (FFA) (Eguchi *et al.*, 2006) and is closely associated with the severity of NAFLD independently of insulin resistance (van der Poorten *et al.*, 2008).

Although NAFLD is primarily a hepatic disorder, cardiovascular disease (CVD) accounts for a greater number of deaths than that of liver disease (Ong *et al.*, 2008; Soderberg *et al.*, 2010) and some studies report CVD to be the leading cause of mortality in NAFLD (Ekstedt *et al.*, 2006; Ong *et al.*, 2008). These findings strongly

imply that NAFLD patients are at high risk of atherosclerosis, a progressive disease that precedes overt CVD and subsequent cardiovascular events. Importantly, several studies have demonstrated that NAFLD confers CVD risk independently of underlying metabolic syndrome risk factors such as obesity and hypercholesterolaemia, indicative that NAFLD is not merely a marker of CVD but may also be directly involved in its pathogenesis (Ruttmann *et al.*, 2005; Targher, 2005b, a; Schindhelm *et al.*, 2007b; Targher, 2007; Targher & Arcaro, 2007).

Endothelial dysfunction is the earliest detectable change in the atherosclerotic disease process and thus represents a barometer for CVD risk (Vita & Keaney, 2002). Endothelial dysfunction is characterised by impaired production of the anti-atherogenic molecule nitric oxide (NO), a vital component in the maintenance of efficient vasomotor function and the endogenous defence against atherosclerosis. Consequently, efficient endothelial function is essential in order to maintain the health of vessels throughout the arterial tree. Previous studies have demonstrated that conduit artery endothelial dysfunction is present in NAFLD patients (Villanova *et al.*, 2005; Senturk *et al.*, 2008); however, the impact of body fat deposition, specifically abdominal VAT, on endothelial dysfunction in NAFLD is yet to be investigated. Moreover, cutaneous microvessel endothelial function has not been investigated in this high risk group. Given that microvascular disease is well documented in associated conditions such as type 2 diabetes (Fowler, 2008), examination of microvessel endothelial function in NAFLD is warranted.

Currently, there is limited pharmacological treatment to reduce hepatic fat, therefore, alternative management strategies such as exercise training are recommended as a non-pharmacological treatment to reduce hepatic fat in NAFLD patients (Bonekamp *et al.*,

2008; Johnson *et al.*, 2009; van der Heijden *et al.*, 2010). Nevertheless, no previous research study has compared the therapeutic effect of exercise with that of the general lifestyle advice (weight loss via increased physical activity and diet) provided as part of conventional clinical care. Moreover, the effect of exercise training on conduit artery and cutaneous microvessel endothelial function has not been previously investigated in NAFLD.

In summary, conduit artery endothelial dysfunction is present in NAFLD, yet the mechanisms contributing to this dysfunction are incompletely understood. Excess abdominal VAT is highly prevalent in NAFLD and may contribute to endothelial dysfunction. NAFLD is a prediabetic condition, and therefore these patients are at high risk of microvascular complications that are associated with type 2 diabetes, such as neuropathy and retinopathy. Nevertheless, no research studies have been conducted on cutaneous microvessel endothelial function in this high risk group. Critically, CVD is the leading cause of death in NAFLD, however, the effect of exercise training on conduit artery and microvessel endothelial function as a cardio-protective management strategy has not been previously investigated. Therefore, the overall aim of this thesis is to explore the contributing factors to endothelial dysfunction in NAFLD and investigate the potential therapeutic effects of exercise training on endothelial function. The specific aims are:

1. To examine the impact of intrahepatocellular triglyceride content (IHTC) and abdominal VAT on endothelial function in NAFLD patients compared with matched controls.

2. To utilise a randomised controlled intervention to compare the therapeutic effect of a supervised exercise intervention with conventional clinical care on conduit artery endothelial function in NAFLD patients.

3. To examine the long-term effects of supervised exercise training on conduit artery endothelial function in NAFLD patients 12 months following the cessation of a supervised exercise training intervention.

4. To investigate cutaneous microvessel endothelial function in NAFLD patients compared with matched controls; and to utilise a randomised controlled intervention to compare the effect of supervised exercise training with conventional clinical care on cutaneous microvessel endothelial function in NAFLD patients.

Chapter 2

REVIEW OF LITERATURE

2.1 Non-Alcoholic Fatty Liver Disease

The accumulation of triglycerides in the liver, in the absence of excess alcohol intake, was first described in the early sixties (Thaler, 1962). Nevertheless, the presence of fatty liver in the obese and/or diabetic populations was initially considered an incidental and benign pathological finding, with little or no clinical significance. Non-alcoholic fatty liver disease (NAFLD) has recently emerged as a disease afflicting our increasingly obese society. The condition refers to a spectrum of histopathological changes in the liver identical to those seen in alcoholic liver disease, but in individuals who do not consume significant amounts of alcohol (<10g daily) (Ludwig *et al.*, 1980; Szczepaniak *et al.*, 2005). NAFLD represents a spectrum of disorders which may manifest as simple steatosis, non-alcoholic steatohepatitis (NASH) or cirrhosis (Figure 2.1). NASH is characterized by hepatic fat accumulation accompanied by hepatocellular damage, inflammation and/or fibrosis and represents a more aggressive form of NAFLD with an increased prevalence of cirrhosis and end-stage liver disease. However, the factors that influence the rate of transition between the different stages of NAFLD are still unclear. Thus, the natural history of the disease remains uncertain.

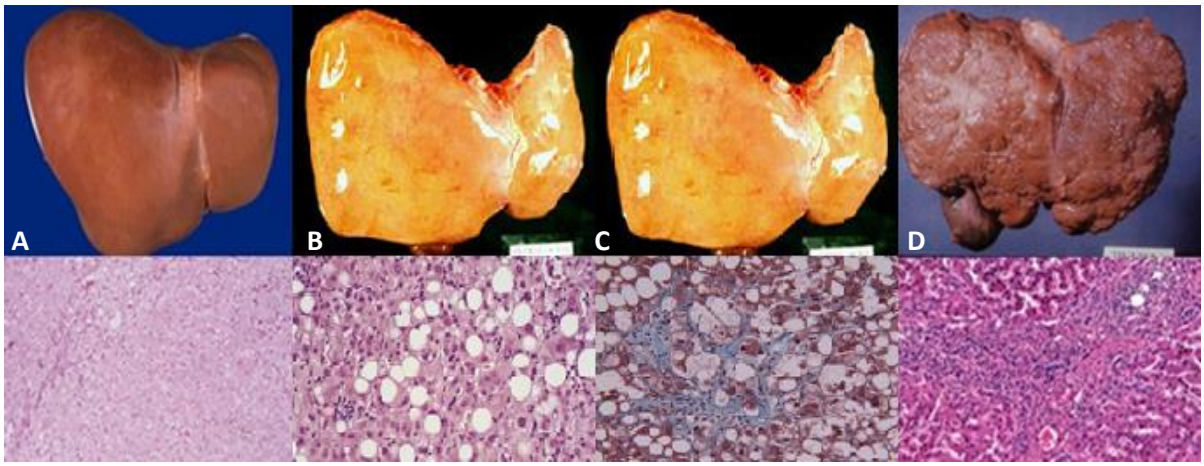


Figure 2.1 The histological spectrum of NAFLD. Upper panels represent the physical appearance of the liver and lower panels represent liver biopsy images ranging from a healthy liver (A), simple steatosis (B), NASH (C) through to cirrhosis (D).

2.1.1 Aetiology & Pathogenesis

Within the healthy human body, the liver plays an integral role in regulating fatty-acid and triglyceride metabolism by synthesizing, storing, secreting and oxidizing free fatty acids (FFA). FFA are produced naturally within the liver via *de novo* lipogenesis (synthesis of FFA's from non-fat precursors), however, dietary lipid intake and lipolysis of peripheral adipose tissue generate an external source of plasma FFA's which are subsequently absorbed by the liver. Within hepatocytes, FFA's either undergo esterification to form triglycerides which are stored and ultimately exported in the form of very low density lipoproteins (VLDL) or β-oxidation within the mitochondria and peroxisomes. Collectively, these mechanisms maintain equilibrium between supply, formation, consumption and disposal of FFA.

The exact mechanisms that cause hepatic triglyceride accumulation and subsequent hepatocellular damage within NAFLD patients are incompletely understood. As a consequence, the current theories alluding to the aetiology and pathogenesis of NAFLD remain hypothetical. However, insulin resistance, oxidative stress and an inflammatory cascade are believed to play integral roles in the progression of NAFLD. The most widely accepted hypothesis explaining the pathophysiology and progression of NAFLD is the "two hit theory" proposed by Day and James (1998). In brief, the primary insult relates to insulin resistance and the subsequent development of steatosis; the secondary insult of increased oxidative stress and the generation of pro-inflammatory cytokines (principally tumour necrosis factor; TNF-α) causes an inflammatory reaction, steatohepatitis, with eventual progression to fibrosis.

Simple steatosis is a consequence of a disruption to normal hepatocyte lipid metabolism. In the insulin resistant state, the inhibitory effect of insulin on peripheral

adipose lipolysis is diminished which causes an influx of circulatory plasma FFA and subsequently an increase in FFA absorption by the liver. Moreover, hyperinsulinaemia, which is commonly associated with insulin resistance, provokes an increase in *de novo* lipogenesis (Browning & Horton, 2004), which further adds to FFA accumulation within the hepatocytes. Clearly, high dietary fat intake is also an important factor that will add to the overall supply of plasma FFA to the liver. This increased FFA availability exceeds the mitochondrial β-oxidation capacity (Browning & Horton, 2004) and therefore, in order to dispose of the excess FFA, esterification is up-regulated. Nevertheless, in the presence of insulin resistance, synthesis and disposal of VLDL is also down regulated (Paschos & Paletas, 2009), causing esterified triglycerides to accumulate within the hepatocytes and thus promote steatosis ("first hit") (Figure 2.2).

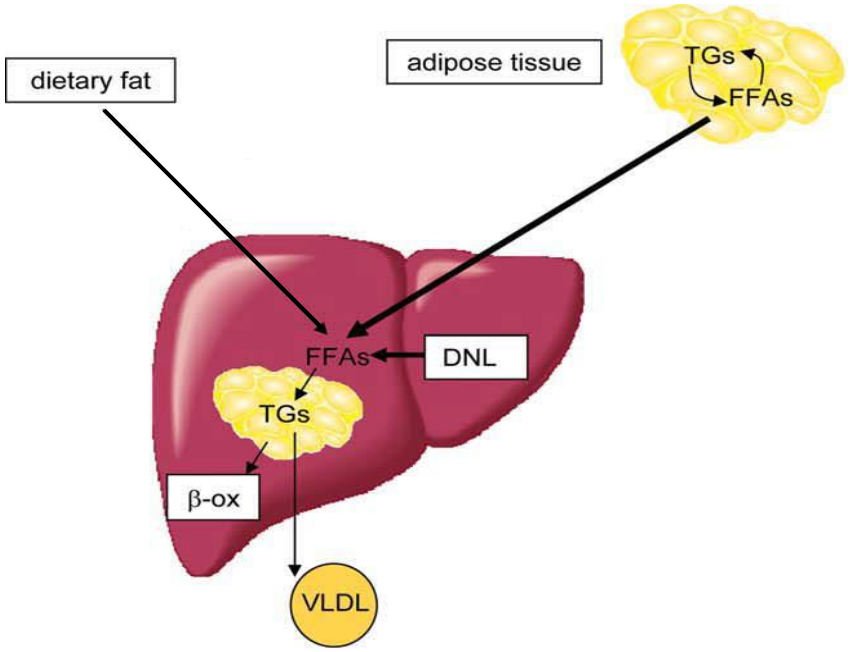


Figure 2.2 Processes involved in the development of hepatic steatosis. Thick lines indicate dominant effect on triglyceride concentration. FFA's from adipose tissue are the dominant source of fatty acids for hepatic triglyceride, followed by *de novo* lipogenesis (DNL) and derived fatty acids from dietary fat. Hepatic triglyceride concentration is also a function of hepatic β-oxidation (β-ox) and the synthesis, removal and clearance of very low density lipoproteins (VLDL) (Johnson & George, 2010).

Over time, increased fat accumulation promotes hepatic insulin resistance as part of lipotoxicity, which may exacerbate overall insulin resistance and consequently advance the progression of steatosis to NASH. It is still unclear why some patients progress past a steatosis stage and develop inflammation and fibrosis. However, factors thought to be involved in the progression of steatosis to NASH ("second hit") include lipid metabolism abnormalities, increased hepatic lipid peroxidation, production of reactive oxygen species (ROS), activated stellate cells, and abnormal patterns of cytokine production (Younossi, 1999; Chitturi & Farrell, 2001). Indeed, when FFA accumulation within the hepatocytes exceeds the metabolic capacity of the mitochondria, the rate of β-oxidation increases, which results in the formation of ROS and provokes subsequent oxidative stress. Excessive ROS formation promotes the release of inflammatory cytokines (TNF-α and leptin) and can reduce the availability of anti-inflammatory substances such as adiponectin (Nishida *et al.*, 2008). The interplay between inflammatory mediators and the activation of stellate cells promotes fibrosis, which in turn can reduce hepatocyte regeneration and cause the liver to become cirrhotic.

2.1.2 Diagnosis and Clinical Manifestations

Most individuals with NAFLD are asymptomatic and show no clinical signs of liver disease. Indeed, diagnostic markers such as ultrasound and serum liver enzyme abnormalities are often identified unintentionally at routine check-up or when patients seek medical consultation regarding a different disease. Nevertheless, some patients report fatigue and/or vague right upper quadrant abdominal discomfort, but these symptoms are not reported frequently. The diagnosis of NAFLD requires evidence of fatty infiltration of the liver in the absence of excessive alcohol consumption. The volume of alcohol consumption required to promote hepatic steatosis and steatohepatitis is uncertain, but a daily limit of 20g (approximately 2 standard units) is commonly

recognised as the threshold. It is imperative that excessive alcohol consumption is excluded as possible aetiology as the histological features of NAFLD are indistinguishable from alcoholic fatty liver disease (Adams & Angulo, 2005). Additionally it is crucial to exclude all other liver related pathologies, as the histological manifestations of NAFLD are similar to those observed in secondary causes of chronic liver disease, such as viral hepatitis, autoimmune hepatitis, or the use of hepato-toxic drugs.

Mild or moderate elevation of serum liver enzymes, usually restricted to alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) (Angulo *et al.*, 1999; Sorbi *et al.*, 1999; Clark *et al.*, 2003), are the most common and often the only laboratory abnormality found in NAFLD patients. However, it has been reported that liver enzymes may be within the normal range in up to 78% of NAFLD patients (Browning *et al.*, 2004) Indeed, several studies have reported ALT to be an insensitive and non-specific marker of NAFLD and therefore an inappropriate diagnostic tool (Mofrad *et al.*, 2003; Browning & Horton, 2004; Zelber-Sagi *et al.*, 2006). These findings suggest that measurement of serum liver enzyme in isolation cannot be reliably used to exclude the presence of steatosis and more advanced forms of NAFLD.

Liver biopsy is considered the gold standard technique used to diagnose NAFLD, whereby histological confirmation requires a minimum of 5% steatosis to be present relative to the total liver weight (Adams & Angulo, 2005). Currently, liver biopsy is the only method capable of differentiating between NASH and steatosis with or without inflammation (Bianchi, 2001; Saadeh *et al.*, 2002). Furthermore, liver biopsy allows clinicians to exclude other causes of liver disease, estimate prognosis and determine progression of fibrosis over time. Importantly, it has been suggested that a liver biopsy

is integral in establishing a true reflection of disease progression or response to therapy as serum liver enzymes improve over time regardless of whether hepatic fibrosis progresses or improves (Adams *et al.*, 2005b). Nevertheless, liver biopsy is an invasive procedure that many patients are reluctant to endure as part of their clinical care, and incurs a risk of infection, haematoma formation or more significant internal bleeding. Together, these factors make the use of liver biopsy ethically difficult in a research context and impractical for longitudinal studies. Furthermore, biopsies are subject to sampling error, providing quantitative information on only a small isolated volume of liver (0.01–0.05 ml), which may be of limited use in the presence of heterogeneous intrahepatocellular lipid distribution (lipid accumulation within hepatocytes) (Thomas *et al.*, 2005).

In most cases, the clinical diagnosis of NAFLD is made upon persistently elevated liver enzyme levels and the presence of an 'echo-bright' liver on ultrasound. The sensitivity and specificity of ultrasound for detecting >33% steatosis is between 60-94% and 88-95% respectively, although this accuracy falls with increasing BMI and in cases with <33% steatosis (Joy *et al.*, 2003). Furthermore, ultrasonography is highly operator dependent and is unable to quantify the degree of steatosis present. In contrast, both magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (^{1}H-MRS) give a non-invasive and quantitative diagnosis of NAFLD. Indeed, a good correlation has been reported between the diagnostic findings of MRI, ultrasound and biopsy in NAFLD patients (Fishbein *et al.*, 2005), whereas, findings from ^{1}H-MRS correlate closely with ultrasonographic confirmation of steatosis (Lewis & Mohanty, 2010). ^{1}H-MRS has a higher sensitivity in detecting hepatic fat content in healthy people and in the quantitative analysis of steatosis than cross-sectional MRI (Thomas *et al.*, 2005; Machann *et al.*, 2006; Guiu *et al.*, 2009). It is likely that ^{1}H-MRS reflects the

severity of steatosis with greater accuracy because of its direct measurement of the triglyceride content within hepatocytes. Moreover, ^{1}H-MRS is able to implement multi-voxel (i.e. site) rather than single-voxel measurements of intrahepatocellular lipid, allowing for reproducible assessment of the heterogeneous distribution of triglyceride present in steatosis (Irwan *et al.*, 2008). Consequently, ^{1}H-MRS is now commonly regarded as the non-invasive 'gold standard' technique for quantifying hepatic steatosis (Cusi, 2009), whereby diagnosis of NAFLD is confirmed upon ≥5.56% hepatic triglyceride concentration (Szczepaniak *et al.*, 2005). Nevertheless, both MRI and ^{1}H-MRS are currently too expensive to be frequently utilised in a clinical setting so are currently only implemented as research tools.

2.1.3 Prevalence & Epidemiology

NAFLD is the most common form of chronic liver disease in western society, affecting approximately 20-30% of individuals (Bedogni *et al.*, 2005). The prevalence of NAFLD is increasing steadily and is extremely common in obese individuals, type 2 diabetic patients and individuals displaying components of the metabolic syndrome with a prevalence of ~70-90% (Adams & Angulo, 2005; Marchesini *et al.*, 2005; Neuschwander-Tetri, 2005). The association between NAFLD and obesity is also present in the paediatric population, with an overall prevalence of NAFLD in 2.6% of children, increasing up to 40% in obese children, and is consequently the leading cause of chronic liver disease in childhood (Schwimmer *et al.*, 2006). Although both sexes seem to be equally affected by NAFLD (Bedogni *et al.*, 2005), a large multi-ethnic population based study reported that the prevalence of NAFLD varies greatly with ethnicity (45% in Hispanic, 33% in Whites, 24% in Blacks). Furthermore, Jimba *et al.* (2005) demonstrated a 29% prevalence of NAFLD among healthy Japanese adults.

Collectively, these data indicate that NAFLD has reached epidemic proportions in different populations around the world.

Numerous studies (Fassio *et al.*, 2004; Adams *et al.*, 2005b; Ekstedt *et al.*, 2006) have demonstrated that ~20-30% of patients with NAFLD develop NASH, and 5-8% with NASH will progress to cirrhosis (Lee, 1989; Powell, 1990; Cortez-Pinto *et al.*, 2003). Alarmingly, it has also been reported that NAFLD accounts for at least 13% of hepatocellular carcinoma cases (Marrero *et al.*, 2002). In contrast, other studies have demonstrated that patients with simple steatosis who show no evidence of steatohepatitis have a relatively benign liver-related prognosis with 1.5% developing cirrhosis and 1% dying from liver-related causes over one or two decades (Teli *et al.*, 1995; Matteoni *et al.*, 1999; Dam-Larsen *et al.*, 2004). Nevertheless, Matteoni *et al.* (1999) reported an increase of up to 11% in liver-related death among patients with biopsy diagnosed NASH.

Importantly, Adams *et al.* (2005a) conducted a seven year follow up study involving 420 individuals with NAFLD and compared all-cause mortality rates with those of the general population. In this cohort, liver disease was the third leading cause of death compared with the thirteenth leading cause in the general population. Interestingly, ischaemic heart disease accounted for a greater number of deaths (25%) than liver disease (13%) in the NAFLD population. This finding is supported by (Ong *et al.*, 2008) who reported cardiovascular disease to be the leading cause of death in a cohort of 80 NAFLD patients. Taken together, the current evidence indicates that NAFLD patients have an increased risk of all-cause mortality compared to the general population (Jepsen *et al.*, 2003). But, it is likely that this is only in part due to liver related death with a larger proportion due to cardiovascular related mortality.

2.2 Metabolic features of NAFLD

NAFLD patients appear to encompass several co-morbidities, which seem to influence the severity of NAFLD as well as increasing the risk of cardiovascular disease (CVD). Insulin sensitivity and body fat deposition are key pathogenic factors associated with NAFLD, which appear to have an intricate and complex relationship in the disease pathogenesis. If the balance between these two factors is disrupted, the risk of hepatic steatosis increases, as well as the likelihood of the disease progressing to NASH, fibrosis and cirrhosis.

2.2.1 Obesity and body fat deposition

It is well established that NAFLD is strongly associated with global obesity (Fabbrini *et al.*, 2010) and elevated waist circumference (Targher & Arcaro, 2007) with the severity of NAFLD likely to increase with the degree of obesity (Wanless & Lentz, 1990). In keeping with these findings, numerous studies have reported a reduction in steatosis following gradual weight loss (Adams & Angulo, 2005). It appears that body fat distribution plays an important role in the pathogenesis of NAFLD, and specifically, excess abdominal VAT (adipose tissue stored around the internal organs of the abdomen) seems to be a key determinant, due to its strong association with insulin resistance and possibly as a source of FFA (Eguchi *et al.*, 2006). Indeed, even in NAFLD patients with a healthy weight and BMI, there is a strong association between abdominal VAT accumulation and insulin resistance (Chitturi *et al.*, 2002; Park *et al.*, 2008). Furthermore, abdominal visceral adiposity is positively correlated with steatosis (Kelley *et al.*, 2003; Nguyen-Duy *et al.*, 2003) and hepatic insulin resistance (Miyazaki *et al.*, 2002), and interestingly, is closely linked with the severity of NAFLD independently of insulin resistance (van der Poorten *et al.*, 2008).

Although most studies have found abdominal VAT to have a stronger relationship with insulin resistance (Cnop *et al.*, 2002; Miyazaki *et al.*, 2002), subcutaneous adipose tissue (SAT) is also associated with the development of NAFLD, as it has a greater overall mass that VAT and therefore contributes more circulating FFA. Indeed, 59% of triglyceride that accumulates in the liver of NAFLD patients is derived from circulating FFA's and the majority of this is from non-splanchnic sources (Donnelly *et al.*, 2005). Nevertheless, VAT is metabolically more active than SAT (Montague & O'Rahilly, 2000) and is therefore more susceptible to lipolysis. VAT and SAT can also act as an endocrine source by secreting numerous adipokines such as FFA, adiponectin, leptin, and TNF-α into the plasma circulation (Ronti *et al.*, 2006). Once absorbed by the liver, these adipokines play a pivotal role in the disruption of hepatocyte lipid metabolism and subsequently aid the development of NAFLD.

2.2.2 Insulin resistance

Insulin resistance can be defined as a condition in which higher than normal insulin concentrations are required to achieve normal metabolic responses, or when normal insulin concentrations fail to achieve a normal metabolic response. Adipose insulin resistance refers to a decreased suppression of lipolysis, whereas, hepatic insulin resistance is characterised by impaired glycogenesis and increased glucose production (Bugianesi *et al.*, 2005a). It is well established that insulin resistance is associated with NAFLD and its severity (Bugianesi *et al.*, 2005b). This relationship was first demonstrated by Marchesini *et al.* (1999), who observed an independent correlation between insulin resistance, evaluated by HOMA-IR (fasting glucose x fasting insulin / 22.5), and ultrasonographic NAFLD in non-diabetic patients. Since then, this relationship has been confirmed in biopsy proven NAFLD cohorts using the

euglycaemic clamp as a more robust method of assessing insulin resistance (Marchesini *et al.*, 2001; Sanyal, 2001).

Much like peripheral insulin resistance, NAFLD is also associated with adipose insulin resistance (Marchesini *et al.*, 2001), which promotes an upregulation in FFA secretion by adipocytes. Similarly, NAFLD patients also exhibit hepatic insulin resistance (Seppala-Lindroos *et al.*, 2002; Bugianesi *et al.*, 2005a), whereby the suppression of endogenous production of glucose is impaired. Indeed, it seems that insulin resistance not only provokes hepatic triglyceride accumulation via an increased supply of FFA to the liver, but also via hyperinsulinaemia-induced upregulation in hepatic *de novo* lipogenesis. Importantly, it appears that insulin resistance is also involved in the progression of steatosis to NASH as several clinical trials have demonstrated that insulin resistance is strongly correlated with the severity of fibrosis (Bugianesi *et al.*, 2005b). The current body of evidence suggests that insulin resistance is the key pathophysiological hallmark of NAFLD, promoting both hepatic fat accumulation and the progression to NASH. Alarmingly, excess hepatic fat accumulation can also exacerbate peripheral insulin resistance, via enhanced basal insulin secretion and decreased suppression of hepatic glucose output, and thus generate a vicious circle that can ultimately lead to diabetes (Taylor, 2008).

2.3 Type 2 diabetes and the metabolic syndrome

The association between obesity, insulin resistance, type 2 diabetes and the metabolic syndrome is now universally accepted, with NAFLD being increasingly recognised as the hepatic manifestation of the metabolic syndrome (Marchesini *et al.*, 2001). The term "metabolic syndrome" refers to a clustering of risk factors, including CVD risk factors, whose underlying pathophysiology is thought to be related to insulin resistance (Kahn *et*

al., 2005). An individual defined as having the metabolic syndrome exhibits abdominal obesity (waist circumference >102cm in men, >88cm in women) plus any two of the following factors; elevated triglycerides (≥150 mg/dL), reduced HDL cholesterol (<40 males, <50mg/dL females), raised blood pressure (systolic >130, diastolic >85mmHg) and raised fasting glucose (≥100 mg/dL) (Alberti, 2006). The pathogenesis of NAFLD and metabolic syndrome seem to share a common pathophysiological pathway, with insulin resistance and hyperinsulinaemia playing a central role (Paschos & Paletas, 2009). Nevertheless, the exact mechanisms underlying the development of NAFLD are not completely understood (see *2.1.1 Aetiology & Pathogenesis*).

Over 90% of patients with NAFLD demonstrate at least one feature of the metabolic syndrome, with approximately one-third having the complete syndrome (Marchesini *et al.*, 2003). Indeed, the prevalence of NAFLD has been consistently associated with obesity (60-95%), type 2 diabetes (~70%) and dyslipidaemia (30-90%), which are frequently expressed as components of the metabolic syndrome (Bugianesi *et al.*, 2005b; Cusi, 2009). Kotronen and Yki-Jarvinen (2008) utilised ^{1}H-MRS to elegantly demonstrate that all components of the metabolic syndrome correlate with hepatic fat, even after adjusting for BMI. Furthermore, with the addition of each of the components of metabolic syndrome the risk of steatosis increases exponentially (Marceau *et al.*, 1999). The metabolic syndrome in NAFLD also increases the likelihood of more severe forms of the disease, conferring an odds ratio of 3.2 for the presence of NASH and 3.5 for the onset of advanced fibrosis (Marchesini *et al.*, 2003).

In light of the pathogenic relationship between insulin resistance and triglyceride accumulation within the liver, it is not surprising that NAFLD is highly prevalent in type 2 diabetes. Compared with non-diabetic individuals, type 2 diabetic patients are at

greater risk of developing NAFLD, fibrosis and cirrhosis (Angulo, 2002; Day, 2006). Indeed, a study by Kotronen & Yki-Jarvinen (2008), which utilized H^1 MRS to determine hepatic fat content, reported that type 2 diabetic patients exhibit 80% more hepatic fat than age, weight and sex matched non-diabetic individuals. Importantly, a study with a cohort of 458 biopsy-proven NASH patients, reported that type 2 diabetes was the single most important predictor of NASH and fibrosis (Fracanzani *et al.*, 2008b). Conversely, NAFLD may also be an important early marker of type 2 diabetes, as 75% of prospective epidemiological studies have shown that elevated serum liver enzyme concentrations predict type 2 diabetes independent of obesity (Kotronen & Yki-Jarvinen, 2008). Nevertheless, considering that up to 40% of adults with NAFLD progress to NASH (Cusi, 2009) and that the majority of patients with cirrhosis are diabetic (Ekstedt *et al.*, 2006), it would seem that NASH in type 2 diabetes must be frequently left undiagnosed until end-stage liver disease develops. Consequently, greater surveillance and more intense screening of NAFLD in individuals at high risk of type 2 diabetes is required in order to diagnose this important pre-diabetic condition in its infancy.

2.4 NAFLD and Cardiovascular Disease Risk

As a consequence of the strong association NAFLD has with metabolic syndrome, as well as the mortality statistics for NAFLD patients, much attention has been drawn to the possible role of NAFLD in the development of CVD (Figure 2.3). Although, not a unanimous finding, most epidemiological studies have revealed that CVD accounts for a greater number of deaths than that of liver disease in NAFLD patients (Adams *et al.*, 2005a; Ong *et al.*, 2008; Soderberg *et al.*, 2010) and some report CVD to be the leading cause of mortality (Ekstedt *et al.*, 2006; Ong *et al.*, 2008). These findings strongly imply that NAFLD patients are at high risk of cardiovascular events. Indeed, the Hoorn Study reported that in 1439 individuals, elevated serum ALT at baseline increased the 10-year

risk of coronary heart disease events even after adjusting for components of the metabolic syndrome and other CVD risk factors (glucose tolerance status, HbA_{1c}, systolic blood pressure, low density lipoprotien cholesterol, BMI) (Schindhelm *et al.*, 2007a). Supportive epidemiological findings were later reported by Ong *et al.* (2008), who demonstrated that in 80 NAFLD patients (diagnosis confirmed via elevated serum liver enzymes); cardiovascular disease accounted for 25% of mortality, whereas, liver-related disease accounted for only 6% of deaths.

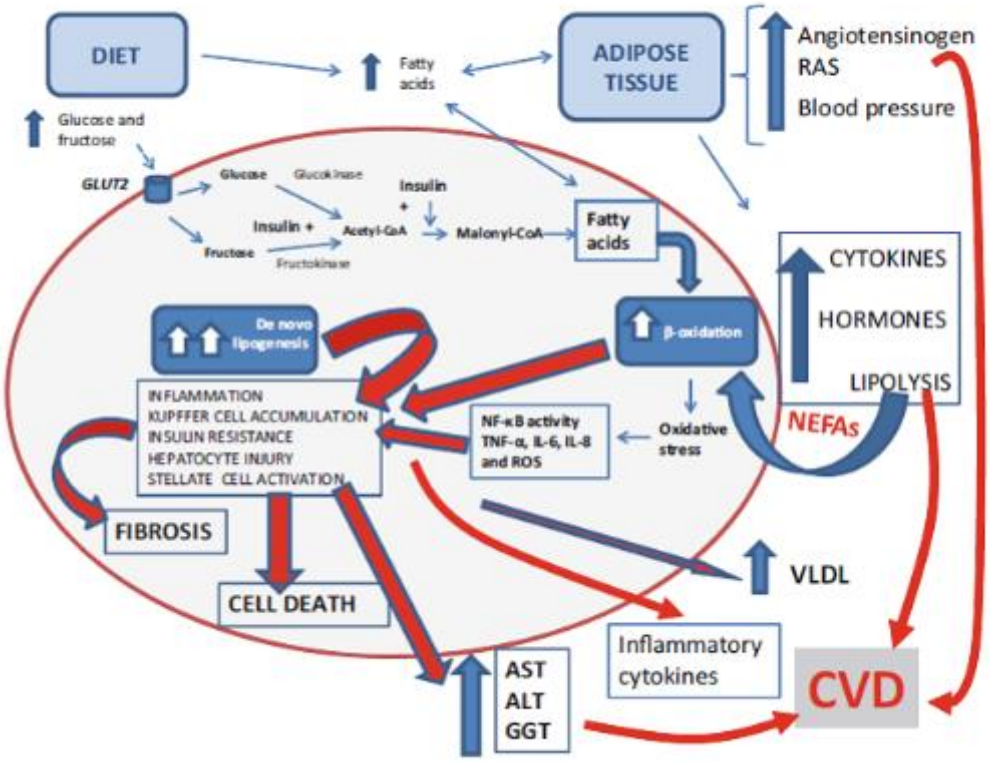


Figure 2.3 Schematic demonstrating potential mechanisms linking NAFLD with CVD (Scorletti *et al.*, 2011).

The association between NAFLD and cardiovascular related death has also been reported using more robust diagnostic techniques. A Swedish prospective study performed a ~28 year follow up in 256 individuals who had previously undergone liver biopsy and used the national death registry to obtain mortality data. Up to 118 individuals were diagnosed with NAFLD, of those, 67 were classified as steatosis and

51 as NASH. During the follow-up period 40% of the NAFLD cohort died and importantly, CVD was the leading cause of death for the entire NAFLD cohort (30%), as well as those exhibiting steatosis alone. CVD was a prominent cause of death in the NASH cohort second only to extrahepatic malignancy (Soderberg *et al.*, 2010). This data is corroborated by Ekstedt et al. (2006), who followed 129 biopsy-proven NAFLD patients for 13.7 years and reported that mortality from cardiovascular and liver related causes were significantly increased compared with matched controls and that CVD was a far more prominent cause of death than liver-related disease (16% vs. 3%).

Although the above studies provide an insight into the CVD related risk and mortality in NAFLD, there is currently paucity in epidemiological studies of this nature, due to the recent emergence of NAFLD as a risk factor for CVD. Consequently, clinical surrogate markers of CVD, such as intima media thickness (IMT) have been utilised to evaluate the CVD risk in NAFLD patients. Recent cross-sectional studies have demonstrated that NAFLD patients have a significantly greater carotid artery IMT than matched healthy controls (Targher *et al.*, 2004; Brea *et al.*, 2005; Targher *et al.*, 2006; Fracanzani *et al.*, 2008a). This finding is endorsed by the results of population (Volzke *et al.*, 2005) and meta-analysis (Sookoian & Pirola, 2008) based studies demonstrating increased carotid IMT and higher prevalence of carotid plaques in NAFLD patients.

Increased CVD risk could be due to the strong clinical association of NAFLD with features of metabolic syndrome, as it is known to confer an adverse CVD risk profile. However, it is plausible that NAFLD may also act as an independent CVD risk factor in addition to the underlying metabolic syndrome risk factors. Indeed, previous research (Targher, 2005b; Targher & Arcaro, 2007) has not only demonstrated carotid IMT to be significantly higher in individuals with NASH than in those with simple steatosis

(0.96±0.15 vs. 1.26±0.24) but, more importantly, that the histological severity of NAFLD (i.e., steatosis, necroinflammation and fibrosis) predicted carotid IMT independently of classical risk factors, insulin resistance and the metabolic syndrome. Moreover, population-based studies have demonstrated an independent association between serum liver enzymes and CVD risk (Schindhelm *et al.*, 2007a) and mortality (Ruttmann *et al.*, 2005). Consequently, it is hypothesised that NAFLD is not merely a marker of CVD but may also be involved in its pathogenesis (Targher, 2007).

2.5 The Role of the Vascular Endothelium in Cardiovascular Risk

The arterial tree consists of a complex network of blood vessels branching from the heart to large conduit arteries, arterioles and capillaries (microvessels). The contractility of conduit artery walls allows them to actively vasoconstrict and vasodilate, primarily under the control of the sympathetic division of the autonomic nervous system. These vessels are characterised by a thick tunica media that contains a greater amount of smooth muscle cells compared to other branches of the arterial tree (Figure 2.4). Arterioles have much smaller internal diameters than conduit vessels, which change in response to either sympathetic or endocrine stimulation. Arterioles branch out from conduit vessels and extend to the microcirculation. The capillaries, or microvessels, that constitute the microvasculature measure 5-10μm in diameter. Microvessels form an interconnected network, or capillary bed, containing direct connections between arterioles and venules. Microvessels represent the only component of the arterial tree which permit the diffusion of water, small solutes and lipid-soluble substances into the surrounding interstitial fluid. A squamous epithelial layer lines the inner surface of all blood vessels and is otherwise known as the endothelium (Figure 2.4).

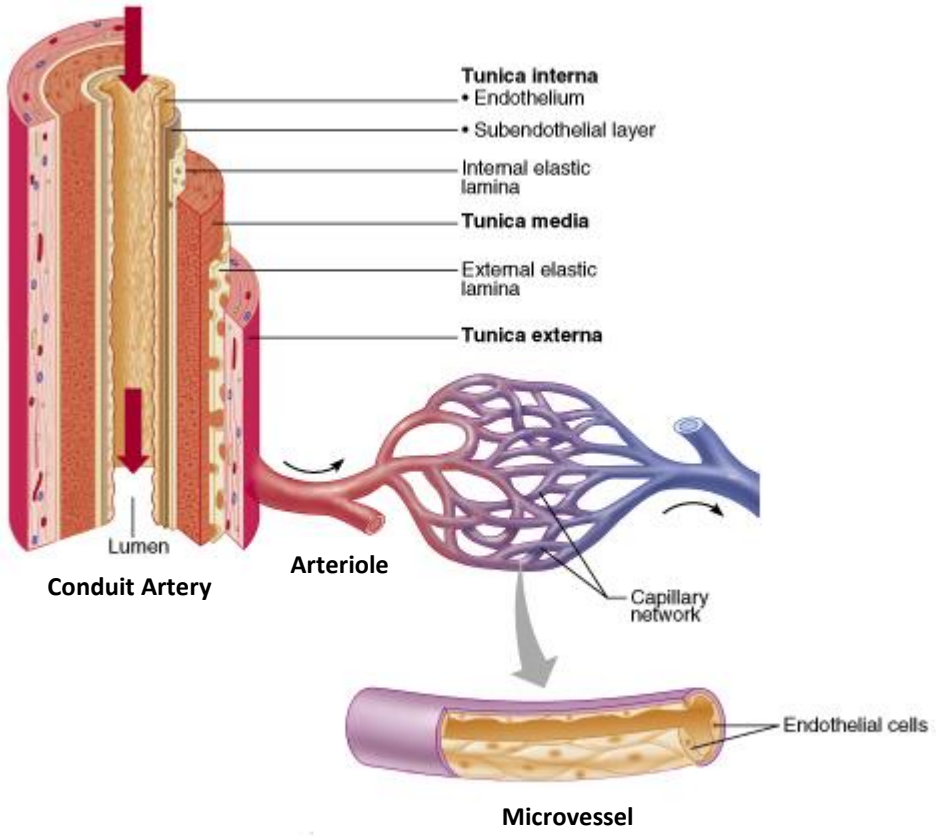


Figure 2.4 The arterial tree from the macrovessels through to microvessels.

Atherosclerosis is a progressive disease that precedes overt CVD. It is characterised by vascular inflammation and infiltration of lipids, cholesterol, calcium and cellular debris into the sub-intima of the arterial wall; resulting in plaque formation, vascular remodelling, acute and chronic luminal obstruction and blood flow abnormalities (Stary *et al.*, 1995). Clinical manifestations of CVD (e.g. myocardial infarction, angina and stroke) become apparent with age and evidence of risk factors, but, the atherosclerotic disease process precedes both evidence of risk factors and clinical outcomes (Chan *et al.*, 2003).

Endothelial dysfunction is the earliest detectable change in the atherosclerotic disease process and thus represents a barometer for CVD risk (Vita & Keaney, 2002). The vascular endothelium is a confluent, cellular monolayer that lines the entire vascular

compartment at the interface between blood and the vessel wall. Crucially, a healthy endothelium mediates anti-atherogenic properties that protect against vasoconstriction, smooth muscle cell growth and inflammatory responses (Davignon & Ganz, 2004). In the presence of endothelial dysfunction, the endothelium may adopt a phenotype that promotes inflammation, thrombosis, vasoconstriction and atherosclerotic lesion formation.

A healthy endothelium produces numerous paracrine substances, including nitric oxide (NO), which help maintain the health of the vascular wall and regulate vasomotor function (Green *et al.*, 2004a). NO is a labile, lipid soluble gas synthesised in endothelial cells from the amino acid L-arginine through the action of endothelial nitric oxide synthase (eNOS) (Palmer *et al.*, 1988). It rapidly diffuses into the vascular smooth muscle of the tunica media where it binds to the enzyme guanylate cyclase (Ignarro *et al.*, 1986), resulting in an increase in cyclic guanosine monophosphate, which induces smooth muscle relaxation and subsequent vasodilation (Furchgott & Jothianandan, 1991) (Figure 2.5).

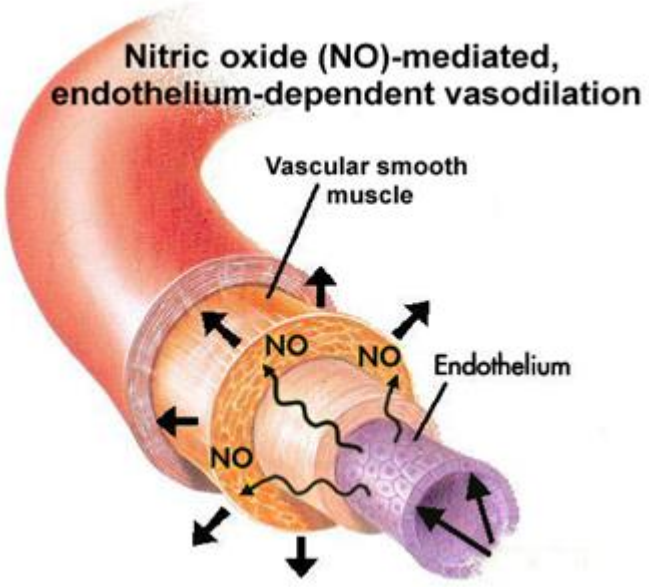


Figure 2.5 Nitric oxide (NO) mediated endothelium-dependent vasodilation (Green, 2009).

Additionally, by releasing NO, the endothelium inhibits platelet and leukocyte activation, inflammation and thrombosis while maintaining the vascular smooth muscle in a non-proliferative state. Collectively, these biological actions make NO a vital component in the endogenous defence against atherosclerosis. Efficient endothelial function is therefore essential in order to maintain the health of vessel walls throughout the arterial tree.

NO is tonically released by the endothelium at rest and contributes approximately 50% to basal vascular tone (Vallance *et al.,* 1989). Production of NO can be up-regulated via physiological stimuli such as increased flow and consequent shear stress, or pharmacologically utilising receptor agonists such as acetylcholine (ACh), to cause acute arterial vasodilation. Vasodilation is a physiological response to an acute release of NO during periods of increased flow (Hutcheson & Griffith, 1991). Although the preset signalling cascade linking mechanical stimulation to the secretion of NO remains incompletely understood, several mechanisms are thought to be involved. For example, increased flow and arterial shear stress have been reported to induce endothelial potassium channel activation (Oleson & Johnson, 1988), calcium influx in endothelial cells (Dull & Davies, 1991) and phosphorylation of serine residue (Groves *et al.*, 1995); all of which are thought to play a role in increasing bioavailability of NO. Nevertheless, it is important to note that other vasoactive substances can be released by the endothelium in response to shear stress, such as prostacyclin and endothelium-derived hyperpolarizing factor (Grabowski *et al.,* 1985; Kuchan & Frangos, 1993). NO readily reacts with free radicals, and increased oxygen degradation resulting in a reduced bioavailability of NO is considered a central feature of endothelial dysfunction. Thus, NO bioavailability is commonly utilised as a surrogate marker of endothelial function.

2.6 Measurement of Conduit Artery Endothelial Function

Non-invasive assessment of endothelial function *in vivo* is commonly utilised to assess NO-mediated vasodilator function. In the 1990's, high-frequency ultrasonographic imaging of the brachial artery was developed to assess endothelial function, using a technique called flow-mediated dilation (FMD). This method involves direct assessment of conduit artery dilator responses to reactive hyperaemia induced by a brief period of limb ischaemia (Green *et al.*, 2004a). This stimulus causes a shear stress that provokes the endothelium to release NO, which induces a subsequent vasodilation (i.e. increase in arterial diameter) that can be imaged using duplex ultrasonography (Dijkhorst-Oei *et al.*, 1999). On the assumption that the occluding cuff, which induces the ischaemia, is positioned distal to the artery (Doshi *et al.*, 2001) and that the period of ischaemia is not greater than five minutes (Mullen *et al.*, 2001), the arterial vasodilator response to this stimulus is largely mediated by NO (Joannides *et al.*, 1995; Doshi *et al.*, 2001). Thus, FMD provides an index of conduit artery endothelium-dependent NO function (Ganz & Vita, 2003).

FMD was first described by Schretzenmayr (1933), however, it was not until 1986 that the importance of the endothelium in mediating this response was demonstrated (Pohl *et al.*, 1986). Pohl and colleagues (1986) elegantly demonstrated that the femoral artery FMD response of canines is significantly attenuated if the endothelium is denuded. Soon after, Sinoway *et al*. (1989) first described the phenomenon of FMD *in-vivo* within humans, reporting a delayed dilation of the brachial artery after the time of peak blood flow, following a reactive hyperaemic challenge. Following this, Celermajer *et al.* (1992) demonstrated that an impaired FMD response is exhibited in populations with CVD risk factors. Importantly, endothelium-derived NO was confirmed as the primary mediator of FMD in humans by Joannides *et al.* (1995), who reported an attenuated

FMD response in the radial artery during the infusion of NO blocker; L-N^G-monomethyl Arginine (L-NMMA). This finding has also been demonstrated in the brachial (Doshi *et al.*, 2001; Mullen *et al.*, 2001) and femoral artery (Kooijman *et al.*, 2008).

Doshi *et al.* (2001) highlighted the importance of correct cuff placement during the FMD technique so to ensure a NO-mediated vasodilator response. This study elegantly demonstrated that NO blockade almost completely abolished the vasodilator response to FMD when the cuff was placed distal to the imaged site. Distinct from this was the observation that when the cuff was positioned proximally, NO blockade had much less of an effect on the vasodilator response. Similarly, the duration of the hyperaemic stimulus is also an important factor in the validity of FMD. Indeed, Mullen *et al.* (2001) reported that the FMD response to transient reactive hyperaemia (5 minutes) was almost completely abolished by NO blockade, however, following a period of sustained reactive hyperaemia (>5 minutes) the subsequent FMD response was unaffected by NO blockade. Accordingly, in order to produce a vasomotor response that is reflective of NO bioavailability, FMD must adhere to stringent guidelines, with any deviation in protocol potentially increasing the contribution of alternative vasodilator pathways (Green *et al.*, 2005).

2.7 Prognostic Relevance of FMD

The concept that FMD provides a direct assessment of arterial wall function, rather than measurement of the risk factors that impact upon it, has stimulated interest in the prognostic value of the technique. Indeed, recent evidence suggests that FMD may possess prognostic value in patients at high risk of CVD (Green *et al.*, 2011a) and specifically, FMD has been reported to be an accurate independent predictor of occult

coronary artery disease (Mutlu *et al.*, 2011). Moreover, the independent prognostic information provided by FMD may exceed that of traditional risk factors in clinical populations (Naghavi *et al.*, 2003a; Naghavi *et al.*, 2003b). Takase *et al.* (1998), induced endothelium dependent vasodilation in both the coronary and brachial arteries as a result of increased flow and subsequent shear stress. This group reported a robust relationship between coronary and brachial artery endothelial function and postulated that brachial artery FMD could be employed as a prognostic marker of coronary artery endothelial function. As well as being strongly and independently associated with CVD risk and mortality in coronary artery disease (Takase *et al.*, 1998; Chan *et al.*, 2003); FMD has also been reported as an accurate prognostic tool in CVD (Gokce *et al.*, 2003), peripheral vascular disease (Brevetti *et al.*, 2003; Gokce *et al.*, 2003) and chronic heart failure (Meyer *et al.*, 2005). Such studies suggest that impaired FMD response in clinical groups is potentially indicative of cardiovascular endpoints.

Interestingly, several studies have yielded conflicting results regarding the prognostic value of FMD in asymptomatic individuals; some of which support the independent prognostic value of the technique (Shechter *et al.*, 2009) while others imply no difference in the predictive competence of FMD compared with traditional risk factor assessment (Shimbo *et al.*, 2007). A noteworthy study by Yeboah and colleagues (2007) examined the prognostic value of FMD in asymptomatic older (72-98 years) participants and found FMD to be an accurate independent predictor of cardiovascular event rate. However, the prognostic value of FMD observed in this study did not outweigh that of traditional cardiovascular risk factors. This finding is in agreement with previous research works suggesting that FMD may be a less accurate predictor of CVD risk in aged cohorts (Witte *et al.*, 2005). A more recent large multi-ethnic population based study in 3026 younger asymptomatic participants by the same research

group also reported that FMD was an accurate independent predictor of incident cardiovascular events (Yeboah *et al.*, 2009). Importantly, this inverse association remained following adjustment for several major CVD risk factors, including the Framingham risk score, indicative that the prognostic value of FMD is also apparent in individuals with no overt evidence of CVD.

FMD is traditionally considered as an index of NO-mediated vasodilator function and thus a marker of NO-bioavailability, although protocols utilised to induce an FMD response in humans are not all equally NO dependent (Corretti *et al.,* 2002; Green *et al.*, 2005). Nevertheless, a recent meta-analysis conducted by Green *et al.* (2011a) revealed that the FMD response to proximal cuff occlusion, which is thought to be less NO-mediated than the response to distal cuff occlusion (Doshi *et al.*, 2001), is equally predictive of future cardiovascular events. Whilst this does not diminish the predictive power of FMD, these data suggest that NO may not be the sole contributor to the prognostic value of the technique.

2.8 Measurement of Cutaneous Microvessel Endothelial Function

As outlined above, the assessment of endothelial dysfunction as a surrogate marker of CVD has focussed on large conduit arteries. Nevertheless, in recent years a growing body of evidence has emerged suggesting that the microcirculation (network of small arteries, including cutaneous vessels) may be the initial site of endothelial damage in subjects at risk of CVD (Brodsky *et al.*, 2004). Systemic microvascular endothelial dysfunction is a key component in the inherent pathogenic complications associated with diseases such as type 2 diabetes, hypertension, coronary artery disease and hypercholesterolaemia (Joannides *et al.*, 2006). For example, there is a high prevalence of microvascular complications among type 2 diabetic patients, including neuropathy

and retinopathy. Therefore, it is imperative to independently assess the health and function of the microvasculature in clinical populations such as NAFLD. The cutaneous circulation has emerged as an accessible and representative vascular bed for investigating microvascular endothelial function and disease status. Moreover, cutaneous vasodilator endothelial function may reflect generalised microvascular function and provide a useful translational model to investigate preclinical microvascular disease (Holowatz & Kenney, 2007).

In the past, the efficacy of the cutaneous microcirculation has been assessed using techniques such as strain-gauge plethysmography to measure vasodilator capacity following various challenges that provoke a maximal dilator response. However, these techniques only offer a gross measure of whole limb blood flow and, under controlled conditions, only provide an indirect assessment of the skin blood flow (SKBF). Consequently, these techniques have been superseded by the application of non-invasive laser Doppler flowmetry (LDF). This versatile technique allows for more direct monitoring of relative blood flow changes both at rest and during maximal provocation. As well as providing an index of maximal dilator capacity of the skin (which is diminished with age and certain pathology, see; 2.10. *NAFLD and Cutaneous Microvessel Function*), LDF can also be used in conjunction with techniques such as iontophoresis and microdialysis to comprehensively interrogate the control of cutaneous microvascular endothelial function, by infusing vasoactive agonist and antagonist substances.

2.8.1 Intradermal Microdialysis

Intradermal microdialysis is a technique that allows the continuous local delivery of potent pharmacological agents into the epidermis (Figure 2.6) via a semi-permeable

membrane, whilst preventing systemic effects. Flux over this localized area of skin can be measured using LDF, therefore, any pharmacological action that evokes a change in cutaneous blood flow can be recorded (For details on intradermal microdialysis methodology, see *General Methodology, Chapter 3*). Unlike iontophoresis, which uses opposing electrical currents to deliver charged pharmacological agents to localised areas of skin, microdialysis does not exhibit any confounding effects of electrical current-induced hyperaemia. Nevertheless, the technique is limited by the potential confounding effect of the trauma caused by fibre insertion. Indeed, cutaneous drug delivery can be influenced by probe insertion depth, differences in barrier functions of the skin, lag time (duration of time before the substance enters the skin from time of administration), elimination or metabolism rate of the delivered agent and possibly the volume of distribution (Kreilgaard, 2002). Variability can be minimised through good working practice, careful instrumentation and the use of within subject experimental designs, whereby the same researcher performs all cannulations; ensuring a consistent cannulation depth is maintained across all time points.

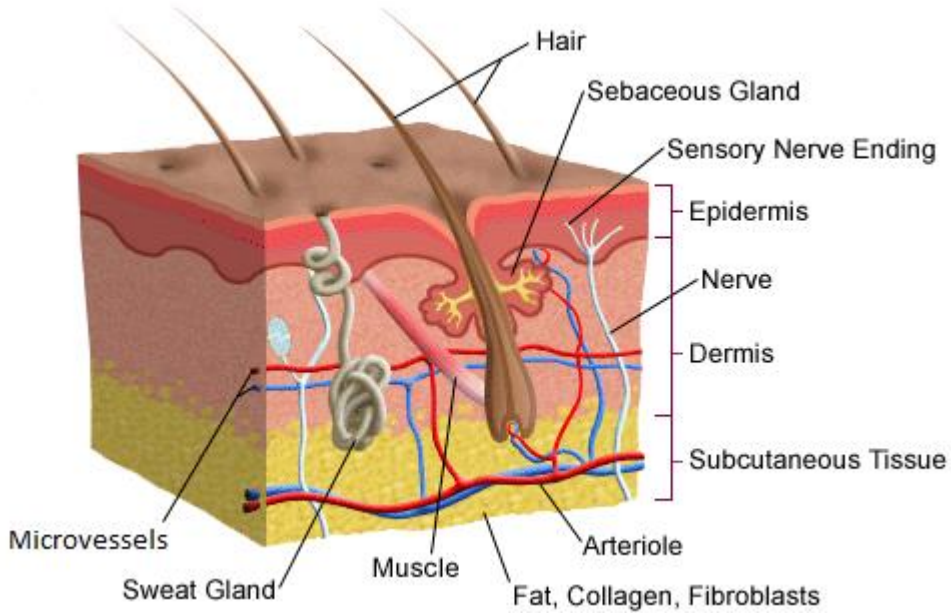


Figure 2.6 A cross sectional diagram of the skin and integrated microvessels.

2.8.2 Heat Stimulation

Particular interest has grown in the NO-mediated endothelial vasodilator function of cutaneous microvessels which has routinely been assessed using thermal provocation challenges. Studies have consistently shown NO to be a mediator of cutaneous vasodilator responses to both physiological (e.g. heating) and pharmacological stimuli (Kellogg *et al.*, 1998; Shastry *et al.*, 1998; Kellogg *et al.*, 1999; Shastry *et al.*, 2000; Minson *et al.*, 2001; Shibasaki *et al.*, 2002; Holowatz *et al.*, 2003; Kellogg *et al.*, 2005; Stewart *et al.*, 2007). Localised heating at 42°C has been reported to elicit the greatest contribution of NO to the vasodilator response (~70%, (Kellogg *et al.*, 1999; Minson *et al.*, 2001)), compared to other stimuli such as ACh (~30-60%, (Boutsiouki *et al.*, 2004; Kellogg *et al.*, 2005; Stewart *et al.*, 2007)) and whole body heating (~30-40%, (Shastry *et al.*, 1998; Wilkins *et al.*, 2003)). It is therefore the most specific methodology currently available for investigation of NO-mediated cutaneous vasodilatation.

Localised heating of the skin stimulates a temperature-dependent sustained increase in SKBF (Charkoudian, 2003), whereby maximal vasodilation of cutaneous microvessels is reached between 42°C and 44°C (Christen *et al.*, 2004). The application of a rapid heating protocol (0.5°C increase in heater temperature every 5 seconds) has been demonstrated to elicit a biphasic response (Minson *et al.*, 2001) (Figure 2.7). This maximal thermo-vasodilator response consists of an initial rapid peak in blood flow within the first 10 minutes, followed by a secondary rise to a sustained plateau after approximately 20-30 minutes of heating (Minson, 2010). It has been proposed that the initial rapid peak is primarily mediated by the axon-reflex of sensory nerves, which stimulates the release of known vasodilators; calcitonin gene related peptide and substance P which elicit an increase in SKBF. Conversely, the secondary slow rise and plateau phase of the localised heating response is reported to be largely NO-mediated

(Kellogg *et al.*, 1998; Minson *et al.*, 2001), with recent evidence suggesting that NO is generated from the endothelial NO synthase isoform (eNOS) (Kellogg *et al.*, 2008). However, eNOS inhibition by intradermal infusion of L-NMMA does not fully suppress the plateau phase response to localized heating, suggesting that other vasodilators may be involved (Minson, 2010).

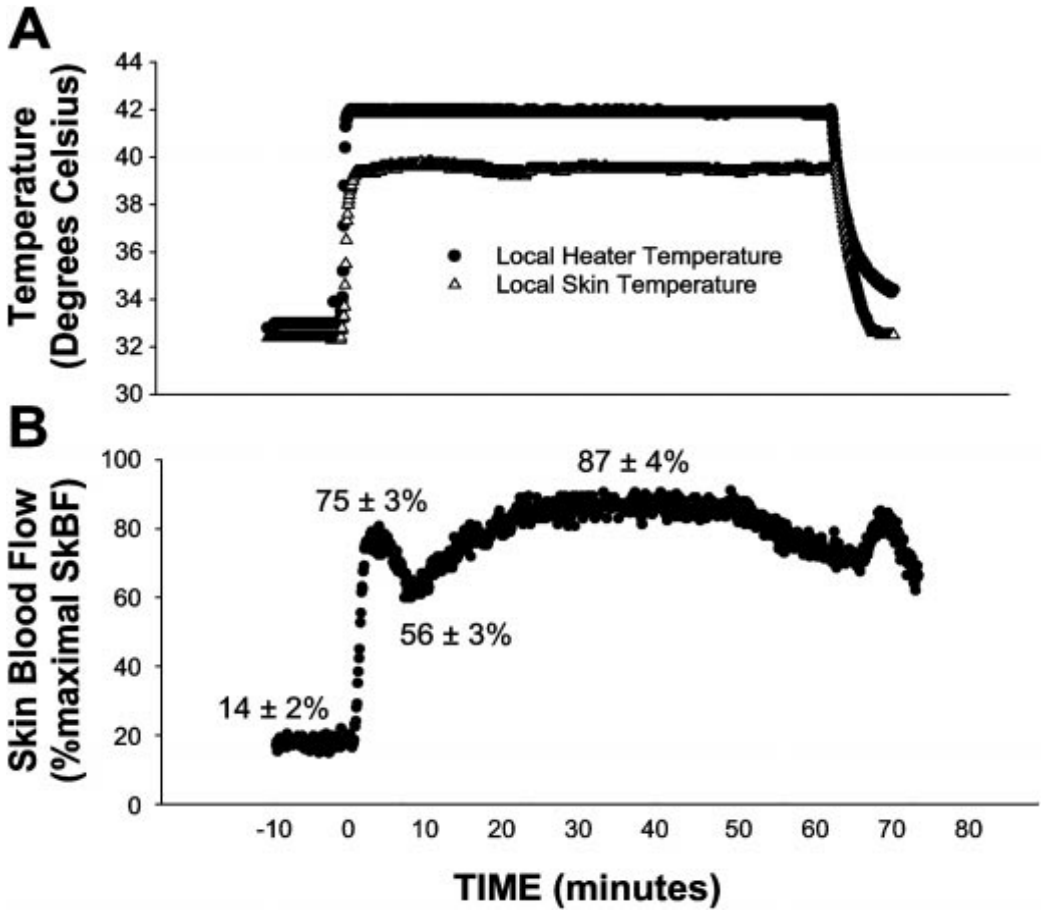


Figure 2.7 A The local heater temperature of 42°C warms the skin to ~40°C which has been shown to elicit maximal cutaneous vasodilatation. **B** Typical bi-phasic response to the application of local heating, with the initial axon reflex stimulating a response ~75% of the maximal response, and the secondary plateau phase (NO-dependent) ~87% of maximal response (Minson *et al.*, 2001).

To more thoroughly assay the cutaneous NO dilator system, a more gradual heating protocol (e.g. 0.5°C rise every 5 minutes) can be utilised that does not provoke the initial axon reflex mediated response (~30-40% of maximal response) (Figure 2.8; (Black *et al.*, 2009). Consequently, in recent years several studies have adopted this

gradual heating protocol to provide a more accurate assessment of cutaneous microvessel NO vasodilator function; (Houghton *et al.*, 2006; Black *et al.*, 2008b) and complimented this with infusion of Sodium Nitroprusside (SNP), a potent NO donor, following either peak thermal or ACh induced hyperaemia, (or a combination) to confirm the attainment of maximal cutaneous vascular conductance (Figure 2.8).

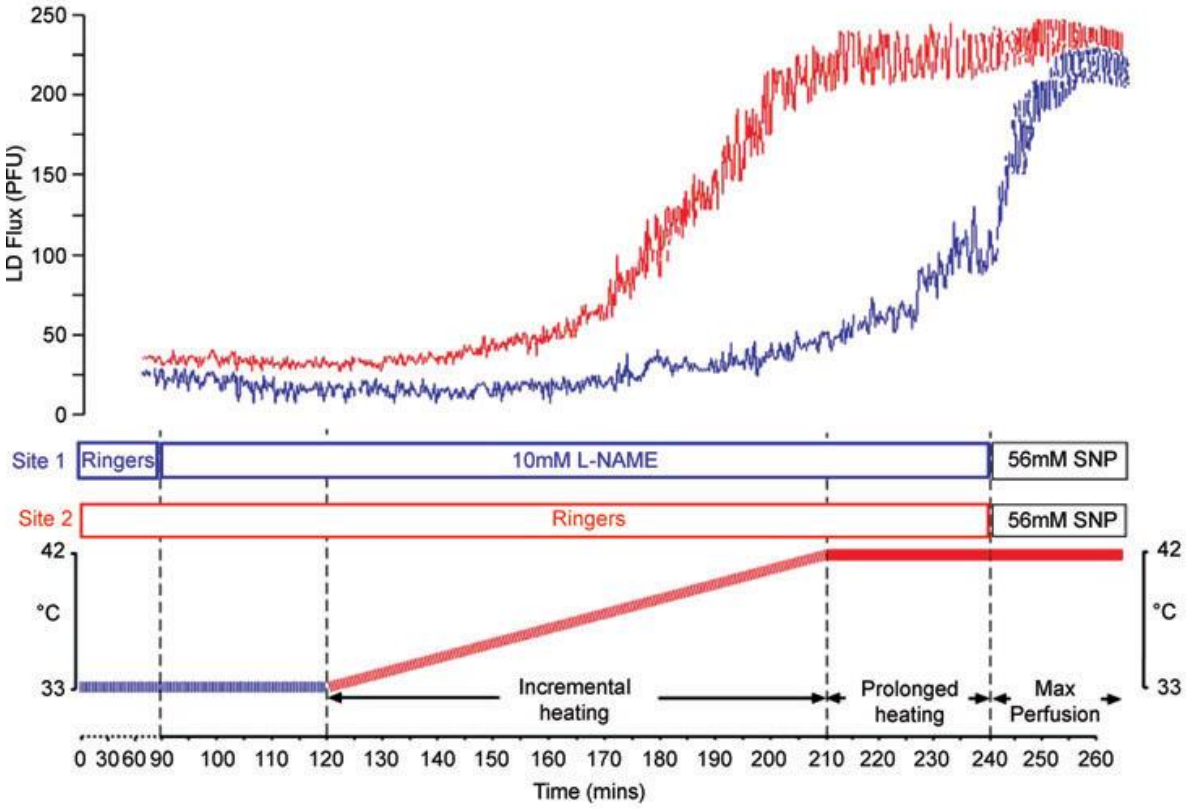


Figure 2.8 A more gradual applied heating protocol avoids provocation of the initial axon reflex mediated response (red trace). Peak thermal-induced hyperaemia can be complimented with infusion of SNP to confirm the attainment of maximal cutaneous vascular conductance (Black *et al.*, 2008b).

2.9 NAFLD and Conduit Artery Function

Evidence is beginning to accumulate indicating that NAFLD patients exhibit endothelial dysfunction. An elegant study by Villanova *et al.* (2005) reported a marked decrease in FMD in patients with NAFLD, compared to matched healthy controls. Moreover, the study also illustrated that NASH patients' exhibit more pronounced endothelial

impairment than patients with isolated simple steatosis. Indeed, the severity of liver histopathology among NAFLD patients correlated with level of endothelial impairment, independent of BMI, HOMA-insulin resistance and other classic metabolic syndrome risk factors. A strength of the aforementioned study was the utilisation of liver biopsies to provide a histological diagnosis of NAFLD, which enabled the authors to investigate the independent effects of simple steatosis and NASH on endothelial function. Nevertheless, Villanova *et al.* (2005) did not quantify intra-abdominal fat deposition, specifically abdominal visceral fat, which is known to be an important mediator of NAFLD and independently promote endothelial dysfunction (Romero-Corral *et al.*, 2010). Senturk *et al*. (2008) assessed endothelium-dependent and independent vasodilatation in 17 individuals with simple steatosis, 15 individuals with NASH and 16 matched healthy controls. Endothelium-dependent dilatation and endothelium-independent dilatation were significantly lower in NASH patients compared to those with simple steatosis alone. Furthermore, contrary to previous findings, this study also reported that individuals with isolated simple steatoisis do not exhibit endothelial dysfunction when compared with matched healthy controls.

It is well established that type 2 diabetic patients demonstrate endothelial impairment (McVeigh *et al.*, 1992; Williams *et al.*, 1996; Maiorana *et al.*, 2001). Nevertheless, there are only two studies to date that investigate the impact of NAFLD on endothelial dysfunction in type 2 diabetes. Kawashima *et al.* (2009) investigated the degree of endothelial dysfunction and insulin resistance in 25 type 2 diabetic patients, 10 of whom had biopsy-proven NASH. The study reported that patients with NASH demonstrated a significant increase in insulin resistance and a significant decrease in FMD compared to those without NASH. However, the severity of inflammation and fibrosis had no influence on FMD in type 2 diabetic subjects with NASH. Similarly, Schindhelm *et al.*

(2005) reported that in metabolically well-controlled type 2 diabetic patients, mildly elevated ALT levels, as a surrogate marker of NAFLD, were related to an attenuated endothelium-dependent dilatation, endothelium-independent dilatation and impaired peripheral insulin sensitivity. Thus, taken together it could be hypothesised that the presence of NAFLD, and in particular NASH, amplifies the degree of both insulin resistance and endothelial dysfunction in type 2 diabetic patients.

2.10 NAFLD and Cutaneous Microvessel Function

Despite it being well established that NAFLD confers an impaired hepatic microcirculatory profile (Ijaz *et al.*, 2003), no previous research has investigated the effect of NAFLD on cutaneous microvascular function as a marker of CVD risk. Previous studies have shown endothelium derived NO-mediated vasodilator function in cutaneous microvessels is related to age (Black *et al.*, 2009) and fitness (Lenasi & Strucl, 2004; Black *et al.*, 2009) in healthy individuals. Additionally, a small number of studies have investigated the impact of cardiovascular risk factors associated with NAFLD on cutaneous microvascular function. For example, Khan *et al*. (1999) demonstrated that individuals with hypercholesterolemia exhibit a diminished SKBF response following iontophoresis of ACh and SNP compared with matched controls. This finding indicates that both endothelium-dependent and -independent cutaneous microvessel vasodilator function is impaired in hypercholesterolaemic patients. Similarly, it has also been reported that obese individuals (de Jongh *et al.*, 2004) and those at increased risk of coronary heart disease (Klonizakis *et al.*, 2009) exhibit an attenuated NO-mediated response to iontophoretically applied ACh; indicative of NO-mediated microvascular dysfunction. Although these studies clearly demonstrate that individuals with cardiovascular risk factors are at high risk of cutaneous microvessel dysfunction, the administration of ACh only elicits ~30-60% of the NO contribution to

maximal SKBF (Boutsiouki *et al.*, 2004; Kellogg *et al.*, 2005; Stewart *et al.*, 2007). Consequently, information is currently lacking on the exact contribution of NO to the microvascular endothelial dysfunction exhibited by patients with cardiovascular risk factors. This is of particular relevance to the NAFLD population, as cardiovascular risk factors such as hypercholesterolemia and obesity are frequently expressed in this high risk group. However, such information can only be elucidated using the intradermal microdialysis technique which allows infusion of a specific NO blocker.

Currently, the impact of type 2 diabetes on the cutaneous microvasculature is incompletely understood. Some (Caballero *et al.*, 1999; Wick *et al.*, 2006), but not all (Veves *et al.*, 1998) studies have suggested an attenuated vasodilator response to both ACh and SNP, indicative of impaired NO-mediated vasodilatation in type 2 diabetic patients. In contrast Sokolnicki *et al*., (2007), utilised intradermal microdialysis to employ potent NO blockade to assess NO contribution during whole body heating and reported no difference in the contribution of NO to cutaneous microvascular vasodilation between type 2 diabetic patients and matched controls. However, whole body heating only elicits ~30-40% contribution of NO to the cutaneous microvessel vasodilator response (Shastry *et al.*, 1998; Wilkins *et al.*, 2003), whereas, localised heating at 42°C elicits ~70% contribution of NO (Kellogg *et al.*, 1999; Minson *et al.*, 2001). These data suggests that whole body heating does not interrogate the cutaneous microvessel NO vasodilator system as effectively as localised heating at 42°C. The current disparity in the literature could reflect the variability inherent in the disease itself, including the presence of peripheral neuropathy and the range of co-morbidities associated with type 2 diabetes. Alternatively, it could be a consequence of the inconsistency of techniques used to assess NO-mediated cutaneous microvessel function in type 2 diabetes. Most (Veves *et al.*, 1998; Caballero *et al.*, 1999; Wick *et al.*, 2006),

but not all (Sokolnicki *et al.*, 2007) of these studies have utilised iontophoresis which has well accepted limitations including the confounding effects associated with using an electrical current for drug administration into the skin, and individual variations of the electrical resistance characteristics of the skin barrier (Cracowski *et al.*, 2006). The sensitivity of intradermal microdialysis interrogates the cutaneous circulatory system with greater accuracy than iontophoresis, which might explain why the findings of Sokolnicki *et al*., (2007) contradict the findings of previous studies that utilised iontophoresis to assess NO-mediated cutaneous microvessel function in type 2 diabetes.

2.11 Current Treatment Strategies for NAFLD

There is currently no effective pharmacological treatment in reducing hepatic steatosis, therefore investigation of alternative management strategies is warranted. Moderate weight loss as well as increased physical activity are associated with improvements in insulin sensitivity (Houmard *et al.*, 2004; Petersen *et al.*, 2005) and thus are logical treatment modalities for NAFLD patients.

2.11.1 Diet & Exercise

Lifestyle modification programs comprising a structured exercise and diet intervention are recommended as a non-pharmacological treatment to reduce hepatic fat in NAFLD patients (Harrison & Day, 2007). The largest study to investigate the combined therapeutic effect of diet and exercise was conducted by Kantartzis *et al*. (2009) who reported that after 9-months, a 2.2kg reduction in total body fat gave rise to a 31% decrease in hepatic fat relative to baseline (5.4±0.5 vs. 3.7±0.3%) in 170 overweight individuals. Fifty of these subjects exhibited NAFLD, in whom a 2.4kg reduction in total body fat and a 35% relative reduction in hepatic fat (13.0±0.9 vs. 8.4±0.8) were observed following the intervention. Importantly, prior to the intervention,

cardiorespiratory fitness was an independent predictor of hepatic fat, indicative that habitual fitness and hepatic fat are causally linked. Moreover, there was a strong correlation between improvements in cardiorespiratory fitness and hepatic fat reduction following the intervention.

A modest reduction in body weight (~10% of initial body weight) following hypocaloric diet, or a combination of diet and exercise induced a 29-58% relative reduction in hepatic fat in overweight and obese individuals (Larson-Meyer *et al.*, 2006; Shah *et al.*, 2009). Nevertheless, it has also been demonstrated that even mild weight loss (≤5% of initial body weight) following a combination of diet and exercise can provoke a 20-60% relative reduction in hepatic fat (Ueno *et al.*, 1997; Tamura *et al.*, 2005; Thomas *et al.*, 2006; Thamer *et al.*, 2007; Kantartzis *et al.*, 2009). These data indicate that following lifestyle modification, significant weight loss may not be an essential prerequisite to hepatic fat reduction and suggest that exercise may confer additional benefit. Nevertheless, it is very difficult to distinguish the effect of exercise *per se* from studies incorporating both exercise and diet as part of a lifestyle intervention.

2.11.2 Exercise

Evidence from a number of cross-sectional studies have suggested a significant inverse correlation between physical activity levels and/or cardiorespiratory fitness and hepatic fat content in NAFLD patients (Suzuki *et al.*, 2005; Church *et al.*, 2006; McMillan *et al.*, 2007; Perseghin *et al.*, 2007). Nevertheless, the effect of exercise alone on reducing hepatic fat in NAFLD patients is currently unclear as the limited studies available exhibit equivocal findings (Shojaee-Moradie *et al.*, 2007; Bonekamp *et al.*, 2008; Devries *et al.*, 2008; Johnson *et al.*, 2009; Finucane *et al.*, 2010; van der Heijden *et al.*, 2010).

A randomised controlled trial conducted by Johnson *et al.* (2009) demonstrated a significant reduction in hepatic fat and VAT, independent of overall weight loss, in obese individuals following only 4-weeks of moderate/high intensity cycling exercise compared with a placebo group (sham exercise). Following this short-term exercise intervention, a mean relative reduction of 21% in hepatic fat was observed, whereas no change was seen in the placebo group. Similar findings have been demonstrated in obese adolescents, whereby 12-weeks of moderate intensity aerobic exercise stimulated ~37% relative reduction in hepatic fat as well as a reduction in VAT without provoking weight loss (van der Heijden *et al.,* 2010). Conversely, no change in hepatic fat or VAT was observed in lean adolescents following aerobic exercise training.

Bonekamp *et al*. (2008) demonstrated a reduction in hepatic fat independent of weight loss following 24-weeks of moderate intensity aerobic and resistance exercise training in type 2 diabetic patients with NAFLD compared with controls. Interestingly, unlike the aforementioned studies, this reduction in hepatic fat was not accompanied by any change to VAT and was independent of a mild reduction in total body fat and waist circumference. Similarly, a randomised controlled trail conducted by Finucane *et al.* (2010) reported that 12-weeks of moderate intensity aerobic exercise training prevents the age related increase in hepatic fat in overweight men. However, it is important to note that these overweight subjects did not exhibit NAFLD (mean 3.7% intrahepatocellular triglyceride content), therefore, the clinical relevance of this finding to the NAFLD population is limited as these individuals are not at high risk of future liver disease and CVD.

Although the aforementioned studies provide promising evidence that supports the utilisation of exercise as a treatment strategy in NAFLD, the therapeutic effect of

exercise on hepatic fat accumulation is not a unanimous finding. Indeed, studies by Shojaee-Moradie *et al.* (2007) and Devries *et al.* (2008) which adopted moderate intensity training protocols for six and twelve weeks respectively, both failed to significantly reduce hepatic fat. However, a noteworthy point regarding the Shojaee-Maradie *et al.* (2007) observation is that the subjects in this study were overweight, but did not have NAFLD (mean 3.95% intrahepatocellular triglyceride content). Additionally, Devries *et al.* (2008) determined hepatic fat content via computed tomography, whereas, all other studies utilised ^{1}H-MRS as the non-invasive gold standard technique (Cusi, 2009). Computed tomography determines hepatic fat content via highly qualitative and insensitive estimates of liver density (Stefan *et al.*, 2008), which may explain why no changes in hepatic fat accumulation were detected in this study.

The current literature provides valuable insight into the efficacy of exercise training in reducing intrahepatic triglyceride content in NAFLD patients, however, no previous study has utilised liver biopsies as the gold standard diagnostic technique to investigate the impact of exercise training on the histological severity of NAFLD. This would be of great clinical relevance as it would ascertain whether exercise training is capable of reducing hepatic inflammation and/or fibrosis and subsequently prevent the progression to cirrhosis and end stage liver disease in this high risk population.

Importantly, the current evidence suggests that increased cardiorespiratory fitness or reduced intra-abdominal obesity following exercise training does not necessarily provoke a reduction in hepatic fat accumulation (Shojaee-Moradie *et al.*, 2007; Devries *et al.*, 2008). Furthermore, it would seem that exercise has the ability to provoke a reduction in hepatic fat content, irrespective of changes in body weight, body fat

(Johnson *et al.*, 2009; Finucane *et al.*, 2010; van der Heijden *et al.*, 2010) and even VAT (Bonekamp *et al.*, 2008). This latter observation suggests that exercise has an independent therapeutic effect on hepatic fat accumulation. Nevertheless, it is a remarkable fact that no previous study has compared the therapeutic impacts of supervised exercise training with the current conventional clinical care guidelines (weight loss and increased physical activity) on hepatic fat content in NAFLD patients.

2.12 Exercise Training and FMD

Exercise has been shown to improve the structure and function of arteries, by enhancing endothelial function and thus reducing the development of atherosclerosis (Kingwell & Jennings, 1997). Regular exercise training involves recurrent exposure to dramatic changes in haemodynamics, which subsequently increases the shear stress exerted on the arterial wall. This shear stress mechanism promotes an increase in NO bioavailability by reducing the number of oxygen free radicals and up-regulating eNOS protein (Green *et al.*, 2004a; Green *et al.*, 2008). It is likely that shear stress is the predominant physiological stimulus accounting for the beneficial effect of exercise training on the vasculature. Collectively, these enhancements result in an increase in NO function which promotes efficient vasomotor function and decreases the risk of atherosclerotic development.

Structured exercise training has been shown to enhance endothelial function in healthy individuals (Kingwell & Jennings, 1997; Clarkson *et al.*, 1999; Goto *et al.*, 2003). Nevertheless, it has been postulated that healthy individuals who exhibit well preserved endothelial function may be less susceptible to exercise-induced enhancements in FMD compared with individuals who demonstrate endothelial dysfunction (Green *et al.*, 2004a). Moreover, it has also been reported that in order to enhance endothelial function

in healthy individuals, large muscle group exercise is required as localised exercise involving only small muscle groups has limited impact on NO vasodilator function (Green *et al.*, 2004b). This is likely due to the minimal haemodynamic stress associated with small muscle group exercise being insufficient to induce shear stress mediated improvements in NO function.

The therapeutic effect of exercise training on endothelial dysfunction has been observed in numerous pathological states, several of which are frequently expressed by NAFLD patients (Higashi *et al.*, 1999; Lewis *et al.*, 1999; Lavrencic *et al.*, 2000; Maiorana *et al.*, 2001; Walsh *et al.*, 2003; Watts *et al.*, 2004). A randomised crossover design study performed by Watts *et al.* (2004) reported that endothelial function, measured using FMD, was impaired in obese adolescents relative to lean controls and that eight weeks of moderate intensity circuit training normalised endothelial function. Similarly, Hambrecht and colleagues (2003) studied the effects of aerobic exercise training on endothelial function in coronary artery disease (CAD) patients, specifically in relation to the expression of eNOS. Training resulted in the up-regulation of eNOS and shear stress related eNOS phosphorylation compared with inactive controls, indicating that shear stress may be attributable for the increased NO bioavailability, and consequent enhanced FMD, observed with exercise training.

Mairorana *et al*. (2001) conducted a randomised controlled trial which reported that a combination of predominantly lower-limb resistance and aerobic exercise training for eight weeks significantly improves endothelial dysfunction exhibited in type 2 diabetes compared with inactive matched controls. Improvements in brachial artery FMD were not associated with changes in fasting blood glucose or glycated haemoglobin, suggesting that exercise-induced enhancements in endothelial function occur

independently of changes to glycaemic control in type 2 diabetic patients. This study also demonstrated that predominantly lower limb exercise is capable of inducing an enhancement in (upper limb) brachial artery NO vasodilator response, indicative that exercise has a systemic effect on endothelial function. This finding has been corroborated by several other studies (Clarkson *et al.*, 1999; Higashi *et al.*, 1999; Lewis *et al.*, 1999; Lavrencic *et al.*, 2000).

Exercise training has also been reported to improve endothelial function in individuals with the metabolic syndrome (Lavrencic *et al.*, 2000). In this well designed study, 29 male participants were randomly assigned to 12-weeks of high intensity cycle-ergometer training or to a control group, whereby participants were asked to simply maintain their habitual physical activity levels for 12-weeks. Following exercise training, FMD significantly improved, whereas no changes were observed in endothelial function in the control group. These exercise mediated improvements in FMD occurred without any changes being observed in individual components of the metabolic syndrome, indicative that exercise has a direct therapeutic effect on the vasculature in individuals with metabolic syndrome.

The consistency of the published data demonstrating exercise-induced improvements in the endothelial function of heterogeneous groups is remarkable, and contrasts with training studies involving healthy participants with normal endothelial function. This strongly suggests that subjects with impaired endothelial function may be more amenable to improvement in NO function as a result of training than healthy subjects. Interestingly, it has also been demonstrated that exercise-induced improvement in endothelial dysfunction is independent of changes in traditional markers of cardiovascular risk (Green *et al.*, 2003). This is of particular importance to the NAFLD

population as cardiovascular risk factors such as obesity, insulin resistance and hyperlipidaemia are frequently expressed in this patient group. Nevertheless, the effect of regular exercise training on endothelial function within the NAFLD population has not been previously investigated, nor has the relative impact of current clinical care.

2.13 Exercise training and NO-mediated cutaneous microvessel function

Research work investigating the effect of cardiorespiratory fitness and exercise training on cutaneous microvascular NO function generally suggests that trained healthy individuals exhibit augmented cutaneous vasodilatator responsiveness to ACh (Kvernmo *et al.*, 1998; Lenasi & Strucl, 2004; Wang, 2005). These findings are corroborated by limited evidence which suggests that the NO-mediated plateau phase of SKBF in response to localised heating is higher in trained individuals (Roche *et al.*, 2010), whereas, the predominantly axon reflex mediated initial peak is not influenced by training status (Tew *et al.*, 2011). Interestingly, it has also been reported that exercise-induced improvements in ACh-mediated vasodilatation are abolished following an eight week detraining period (Wang, 2005) and that 14-56 days of bed rest confers an attenuated NO-mediated vasodilator response in the skin of normally active healthy individuals (Crandall *et al.*, 2003; Demiot *et al.*, 2007). Collectively, the current body of evidence suggests that fitness and/or physical activity promotes increased NO bioavailability in the cutaneous microvasculature of healthy individuals.

Wang *et al.* (2005) illustrated that exercise had a therapeutic effect on cutaneous microvessel function in 10 young sedentary individuals. Plasma NO metabolites (nitrite plus nitrate) were measured by a microplate fluorometer and cutaneous microvascular perfusion responses to iontophoretically applied ACh and SNP were determined before and after 5-weeks of moderate intensity cycle-ergometer training. Following training,

plasma NO metabolite concentration and ACh-induced perfusion increased, suggestive of an improvement in cutaneous NO microvascular function. Similarly, Hodges *et al*. (2010) reported an increase in ACh-mediated SKBF following 24, 36 and 48 weeks of moderate aerobic intensity exercise in post-menopausal women. Surprisingly, this study also reported an increase in SNP-mediated SKBF after 36-weeks of exercise training, indicative that chronic exercise may induce structural changes to cutaneous microvessels. Although, the aforementioned studies provide valuable information regarding the impact of exercise training on cutaneous microvessel function, both study designs are limited as neither incorporated an inactive control group as a means of direct comparison.

Currently, research investigating the effect of exercise on the cutaneous microvascular endothelial function of diseased populations is limited and conflicting in its findings. Klonizakis and colleagues (2009) conducted a randomised controlled trial and reported that eight weeks of moderate intensity exercise training elicits an augmented cutaneous vasodilator response to ACh when compared with inactive controls in chronic venous disease patients. In contrast, it would seem that exercise has little therapeutic effect on the microvasculature of type 2 diabetic patients. Indeed, it has been recently demonstrated that there is no difference in the NO-mediated vasodilator response to localized heating in self-reported physically active type 2 diabetic patients compared to their sedentary counterparts (Colberg *et al.*, 2002). Moreover, it has also been observed that six months of aerobic exercise training does not improve the microvascular response to iontophoretically applied ACh or localised heating when compared with conventional care in type 2 diabetic patients (Middlebrooke *et al.*, 2006). However, the training stimulus of the latter study did not induce an improvement in cardiorespiratory

fitness, which suggests the training stimulus was not of a sufficient intensity to elicit improvements in cutaneous NO-mediated microvessel function.

Although elderly individuals are not regarded as a diseased population *per se*, the age related attenuation of cutaneous microvascular function is well documented (Holowatz *et al.*, 2007). Recently, Black *et al.* (2008b) utilised intradermal microdialysis to elicit NO blockade and demonstrated that moderate intensity exercise prevents the age-related attenuation of cutaneous NO-mediated vasodilator response to both localised heating and Ach. Currently, this is the only study to investigate the mechanisms of the cutaneous vasodilator function via microdialysis with selective NO inhibitor pathways following exercise training in any population.

2.14 Summary

In summary, NAFLD is the most common form of chronic liver disease in western society, affecting approximately 20-30% of general population and ~70-90% of obese and type 2 diabetic individuals (Adams & Angulo, 2005; Marchesini *et al.*, 2005; Neuschwander-Tetri, 2005). Conduit artery endothelial dysfunction, an early surrogate marker of CVD risk, is present in NAFLD, yet the mechanisms contributing to this dysfunction are incompletely understood. Excess abdominal VAT is highly prevalent in NAFLD and may contribute to endothelial dysfunction. NAFLD is a prediabetic condition, and therefore these patients are at high risk of microvascular complications that are associated with type 2 diabetes, such as neuropathy and retinopathy. Nevertheless, no research studies have been conducted on cutaneous microvessel endothelial function in this high risk group. Critically, CVD is the leading cause of death in NAFLD, however, the effect of exercise training on conduit artery and

microvessel endothelial function as a cardio-protective management strategy has not been previously investigated.

Chapter 3

GENERAL METHODOLOGY

3.1 Participants

Sedentary (<2 hours of exercise p/wk) NAFLD patients were recruited from the gastroenterology clinics at the Royal Liverpool University Hospital and the University Hospital Aintree and control individuals via local advertisement. The diagnosis of NAFLD was identified on the basis of chronically raised alanine aminotransferase (ALT) levels (>1.5 x upper normal values for 6-months or more) and confirmed upon the presence of ≥5.56% intrahepatocellular triglyceride content with no evidence of cirrhosis as determined by proton magnetic resonance spectroscopy (^{1}H-MRS) (see section 3.5.1 *Proton magnetic resonance spectroscopy (^{1}H-MRS)*). In a small number of cases the diagnosis was confirmed histologically after liver biopsy. Other causes of liver disease were excluded via a complete laboratory investigation for viral hepatitis (hepatitis B and C viral markers), autoimmune hepatitis, primary biliary cirrhosis (non-organ-specific antibodies), celiac disease (anti-gladin antibodies) and genetic diseases (α1 antitripsin, ceruloplasmin). All participants were non-smokers and non-diabetics who had no history of excessive alcohol intake as defined by an average weekly consumption of <14 units for females and <21 units for males. None of the participants had history of ischaemic heart disease or demonstrated any contraindications to exercise. None of the control individuals were taking any prescribed medication. The study conformed to the Declaration of Helsinki and was approved by the local research ethics committee (LREC) and research and development departments at all collaborating institutions. Participants were informed of the methods verbally and in writing before providing written informed consent.

3.2 Anthropometric Measurements

Height was measured in a free-standing position to the nearest 0.5 cm using a measuring device (Seca, Model 220, Germany). Body mass was measured to the nearest 0.05 kg using calibrated electronic digital scales (Seca, Model 767, Germany). From this, body mass index (BMI; mass (Kg) / (height $(m)^2$) was calculated. Waist circumference was measured at the level of the umbilicus. Resting blood pressure (mm Hg) and resting heart rate ($beats.min^{-1}$) were also determined from an average of three measures using an automated BP monitor (Dinamap, G & E Medical, Tampa, Florida).

3.3 Fasting Blood Sample

Fasting venous blood samples were drawn for the measurement of glucose. Lipid profiles including cholesterol, triglycerides, high-density lipoproteins (HDL) and low-density lipoproteins, (LDL) were enumerated and cholesterol:HDL ratio was calculated. Serum liver enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT) were also assessed. From these samples, reproductive hormone profiles were measured in pre-menopausal female participants including; follicle stimulating hormone (FSH), lutenizing hormone (LH), oestradiol, progesterone, testosterone, sex hormone binding globulin (SHBG) and free androgen index (FAI) was calculated (total testosterone / SHBG x 100). All blood samples were analysed by an experienced laboratory technician.

3.4 Vascular Function

All vascular function assessments were performed following an overnight fast, 12-hour abstinence from caffeine and a 24-hour abstinence from alcohol and strenuous exercise. Measurements were performed in a quiet, temperature controlled laboratory. Upon arrival, participants rested in the supine position for ~20 minutes before assessment

commenced. Following the rest period, HR and blood pressure were determined from an average of three measures using an automated BP monitor (Dinamap, G & E Medical, Tampa, Florida) on the left arm. Participants were then positioned with their right arm extended and immobilised with foam supports at an angle of ~80° from the torso.

3.4.1 Flow-Mediated Dilation (FMD)

A rapid inflation and deflation pneumatic device (D.E. Hokanson, Bellevue, WA) was used with an inflation cuff placed immediately distal to the olecranon process of the forearm of the imaged arm to provide a stimulus for forearm ischaemia (Corretti *et al.*, 2002). A 10-MHz multi frequency linear array probe attached to a high-resolution ultrasound machine (Siemens Acuson P50, Siemens Medical Solutions, USA) was utilised to image the brachial artery in the distal one third of the upper right arm. When an optimal image was acquired, the probe was held stable and the ultrasound parameters were set to optimise the longitudinal, B-mode images of the lumen-arterial wall interface. Continuous Doppler velocity assessment was also obtained using the high-resolution ultrasound machine and was collected at an isonation angle of 60 degrees. Ultrasound images of arterial diameter and blood flow velocity were recorded real time using specialised recording software (Camtasia). A baseline recording lasting 1-minute was acquired before the forearm cuff was inflated (~220 mmHg) for 5-minutes. Artery diameter and blood flow velocity recordings resumed 30 seconds prior to cuff deflation and continued for 3-minutes thereafter. Peak brachial artery diameter and blood flow velocity, and the time taken to reach these peaks following cuff release were recorded. The response immediately following cuff release (hyperaemia) is a reflection of brachial artery endothelial-dependent function and is expressed as FMD %.

3.4.2 Glyceryl Tri-Nitrate (GTN)

A further 15 min rest period followed, before the brachial artery was imaged once more. A 1-min baseline recording of diameter and velocity flow was conducted before the sub-lingual administration GTN, a potent NO donor. GTN forms as NO, causing a generalised vasodilatation. As such, GTN provides an endothelial-independent source of NO with which it challenges the integrity of the smooth muscle and thereby provides an insight of smooth muscle sensitivity and a subsequent short-term increase in blood flow velocity and arterial diameter. The brachial artery was imaged for 10-min following administration of GTN.

3.4.3 Brachial artery diameter and blood flow analysis

Analysis of brachial artery diameter was conducted using custom designed edge-detection and wall-tracking software, which is largely independent of investigator bias (Woodman *et al.*, 2001). Briefly, the image was taken directly from the ultrasound machine and saved as an AVI file on a PC. Subsequent software analysis of this data was performed at 30Hz using an icon-based graphical programming language and toolkit (LabVIEW 6.02, National Instruments). The initial phase of image analysis involved the identification of regions of interest (ROI) on the first frame of every individual study. These ROIs allowed automated calibration for diameters on the B-mode image (Figure 3.1) and velocities (Figure 3.2) on the Doppler strip. An ROI was then drawn around the optimal area of the B-mode image. Within this ROI a pixel-density algorithm automatically identified the angle-corrected near and far wall e-lines for every pixel column within the ROI. The algorithm begins by dividing the ROI into an upper half, containing the near wall lumen-intima interface, and a lower half containing the far wall interfaces. The near wall intimal edge is identified by a Rake routine that scans from the bottom to the top of the upper half of the ROI. The position

of the edge is established by determining the point where the pixel intensity changes most rapidly. Typical B-mode ROIs therefore, contained approximately 200 to 300 diameter measures per frame, the average of which was calculated and stored. This process transpired at 30 frames per second. A final ROI was drawn around the Doppler waveform and automatically identified the peak of the waveform. The mean diameter measures derived from within the B-mode ROI were then synchronised with the velocity measure derived from the Doppler ROI at 30 Hz. Ultimately, from this synchronised diameter and velocity data, blood flow (the product of lumen cross-sectional area and Doppler velocity and shear rate (4 times velocity divided by diameter) were calculated at 30 Hz (Black *et al.*, 2008a).

All data were written to file and retrieved for analysis in a custom-designed analysis package. It has been previously demonstrated that reproducibility of diameter measurements using this semi-automated software is significantly better than other manual methods, significantly reduces observer error, and possesses an intra-observer CV of 6.7% (Woodman *et al.*, 2001). Furthermore, our method of blood flow assessment is closely correlated with actual flow through a "phantom" arterial flow system (Green *et al.*, 2002).

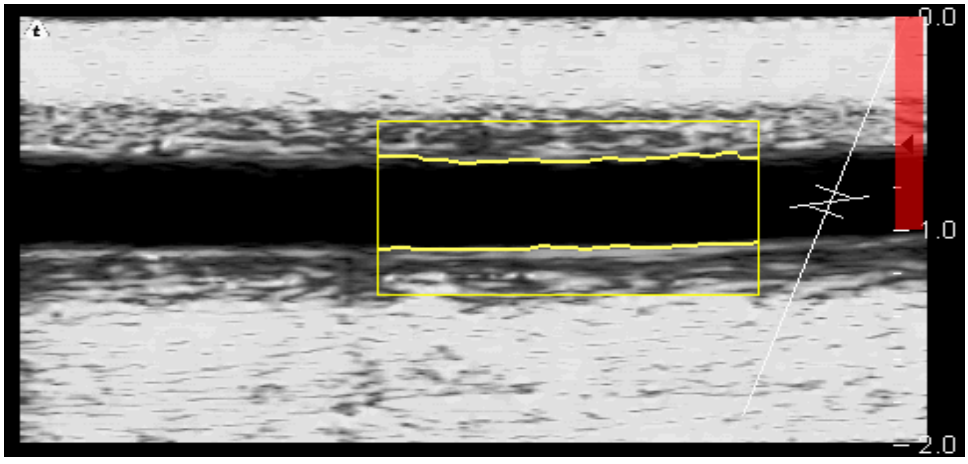


Figure 3.1 2D B-Mode Ultrasound image recording of the brachial artery in the analysis software with ROI highlighted.

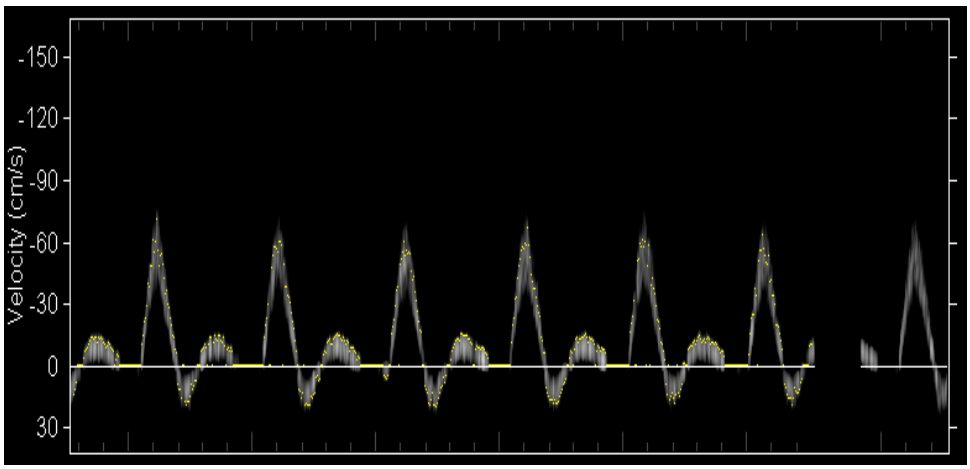


Figure 3.2 Blood flow velocity trace.

3.4.4 Vascular Data Analysis

Baseline diameter, flow, and shear rate were calculated as the mean of data acquired across the 1 minute baseline period preceding cuff inflation. Peak diameter following cuff deflation was automatically detected according to an algorithm which identified the maximum bracket of data subsequent to performance of a moving window smoothing function. This smoothing routine calculates the median value from 100 consecutive samples before the window shifts to the next bracket of data, which shares 20% overlap with the preceding bracket. The maximum value of all the calculated median values is then automatically detected and chosen to represent the peak of the post-deflation artery diameter curve. FMD was calculated as the percentage rise of this peak diameter from the preceding baseline diameter. The time to peak diameter (in seconds) was calculated from the point of cuff deflation to the maximum post-deflation diameter. Calculation of FMD and time to peak were therefore observer-independent and based on standardised algorithms applied to data which had undergone automated edge-detection and wall-tracking analysis.

In accordance with recent findings (Pyke & Tschakovsky, 2007), we calculated the shear rate stimulus responsible for endothelium-dependent FMD. The post-deflation

shear rate data, derived from simultaneously acquired velocity and diameter measures at 30 Hz, was exported to a spread sheet and the area under the shear rate curve (AUC) calculated for data up to the point of maximal post-deflation diameter (FMD) for each individual using the trapezoid rule.

3.5 Magnetic Resonance Methodology

All participants underwent magnetic resonance imaging (MRI) in a 1.5T Siemens Symphony scanner (Siemens Medical Solutions, Erlangen, Germany) in a prone position, being moved through the magnet to acquire full body coverage. Scans were anonymised prior to analysis thus ensuring the observer was blinded to all clinical details

3.5.1 Proton magnetic resonance spectroscopy (^{1}H-MRS)

In *liver*, NAFLD was defined as intrahepatocellular triglyceride content (IHTC) ≥ 5.56% (Szczepaniak *et al.*, 2005). Transverse images of the liver were used to ensure accurate positioning of the three voxels in standard sites of the liver, avoiding ducts and vasculature (Figure 3.3). Single voxel spectroscopy was conducted at each of these three sites. Voxel size was 20x20x20 mm, TE 135 ms, TR 1500 ms, with 64 acquisitions. Voxel placement in post-treatment studies was guided by reference to the pre-treatment images. ^{1}H MR spectra were quantified using the AMARES algorithm in the software package jMRUI-3.0 (Vanhamme *et al.*, 1997; Naressi *et al.*, 2001). As previously described, IHTC is expressed as % of CH_2 lipid signal amplitude relative to water signal amplitude after correcting for T_1 and T_2 (Thomas *et al.*, 2005)(Figure 3.4). T1 and T2 values for IHTC were obtained by an experienced physicist.

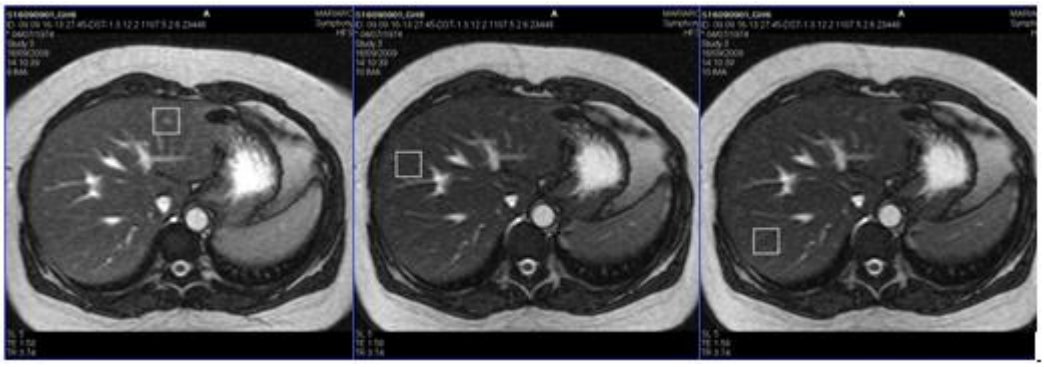


Figure 3.3 Example of the voxel positions used during liver spectroscopy.

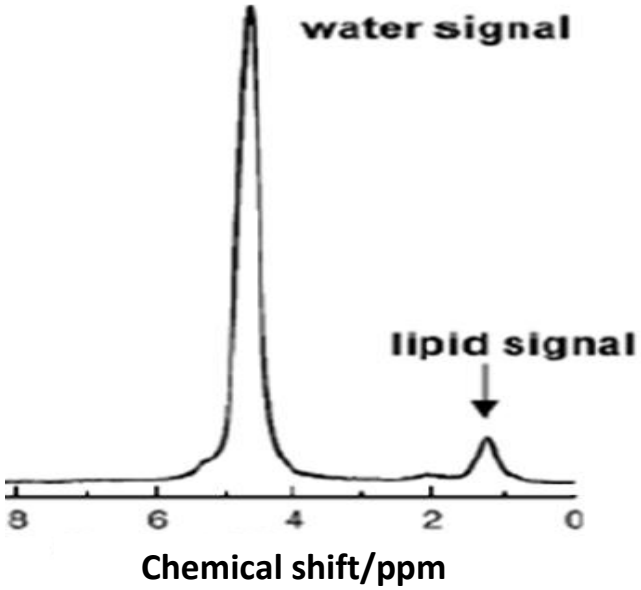


Figure 3.4 Percentage ratio of the CH^2 lipid peak area relative to the water peak area.

3.5.2 Volumetric analysis of abdominal subcutaneous and visceral fat

Abdominal subcutaneous adipose tissue (SAT) and abdominal visceral adipose tissue (VAT) was calculated from whole body axial T1-weighted fast spin echo scans (axial scans, 10 mm slice thickness followed by a 10 mm gap using the integral body coil). The abdominal region was defined as the image slices from the slice containing the femoral heads, to the slice containing the top of the liver/base of the lungs (Figure 3.5). All scans were analysed centrally using ANALYZE 4.0; CN software (Rochester, MN, USA).

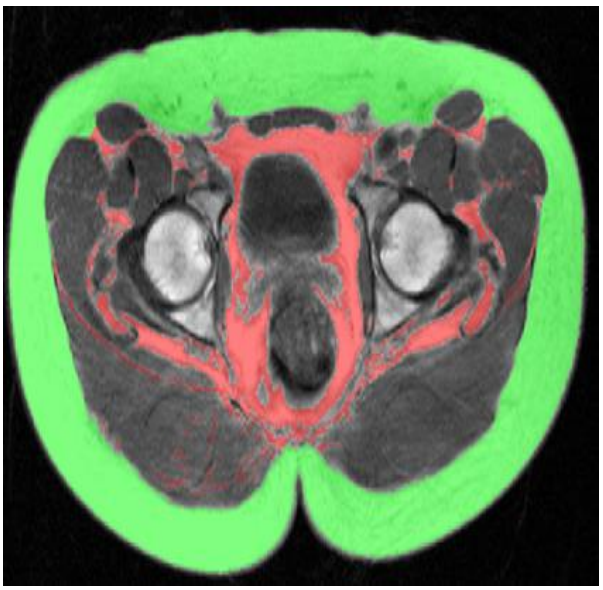


Figure 3.5 One trans-axial image of abdomen. Area highlighted in green represent abdominal subcutaneous fat and area highlight in red represent abdominal visceral fat.

3.6 Maximal Oxygen Consumption Test (VO_{2peak})

A fitness test (VO_{2peak}) was performed to quantify aerobic capacity. This comprised of a 2 minute warm up followed by an incremental exercise to volitional exhaustion performed on a treadmill ergometer (H/P/ Cosmos, Pulsar 4.0, Nussdorf-Traunstein, Germany) in a temperature controlled environment (Bruce *et al.*, 1973). Following a 2-min warm up at 2.2 $Km.h^{-1}$ on a flat gradient, the initial workload was set at 2.7 $Km.h^{-1}$ at 5° grade. Thereafter, step-wise increments in speed and grade were employed every minute (modified Bruce protocol). Heart rate was continuously measured (Polar Electro Oy, Finland) and the participants condition was monitored using the BORG scale (Burkhalter, 1996; Borg, 1998). Prior to commencing the cardiorespiratory exercise test, all patients above the age of 55 years had electrocardiogram (ECG) electrodes attached to their chest so the heart could be monitored throughout the test; this is in keeping with the American College of Sport Medicine (ACSM) exercise prescription guidelines.

VO_{2peak} during exercise was calculated from minute ventilation, measured using a pneumotach and simultaneous breath-by-breath analysis of expired gas fractions (Medgraphics CPX/D and Ultima CardiO_2 Systems, Minnesota). Gas analysers and flow probes were calibrated before each test. Oxygen consumption was recorded during the final 40 sec of each stage of the test and expressed relative to body weight ($ml.kg^{-1}.min^{-1}$). Peak oxygen consumption was calculated as the highest consecutive 10 second period of gas exchange data occurring in the last minute before volitional exhaustion, which generally occurred due to leg fatigue or breathlessness.

3.7 Supervised Exercise Training Intervention

The exercise training program consisted of 16-weeks of progressive moderate intensity aerobic exercise. Prior to commencing the exercise training, all participants attended a full familiarisation session at the University gymnasium as well as a comprehensive induction at their local recreational gymnasium. Exercise sessions were fully supervised at least once a week by a dedicated exercise physiologist (CP) within the University gymnasium. It was at this training session that participants were issued a weekly progressive exercise protocol that was specific to their basal fitness level and rate of progression. In addition, a polar heart rate monitor was used to continuously monitor heart rate at all supervised sessions.

Training comprised of a combination of treadmill and cycle ergometer based exercise with the intensity and duration of exercise sessions progressively increasing during the course of the intervention. Participants underwent 30 minutes of moderate intensity aerobic exercise 3 times a week at 30% heart rate reserve (HRR) for the initial 4 weeks. Intensity increased to 45% HRR for the following 4 weeks, until week 8, where HRR remained at 45%, but the duration of each session increased to 45 minutes. For the final

4 weeks the intensity increased to 60% HRR and each 45 minute session was performed 5 times per week (Figure 3.6). HRR was calculated using the following formula: ((Max HR – Resting HR) × Intensity) + Resting HR. The resting and maximal heart rate measures were derived from a maximal exercise test undertaken prior to, and following the baseline VO_{2peak} assessment.

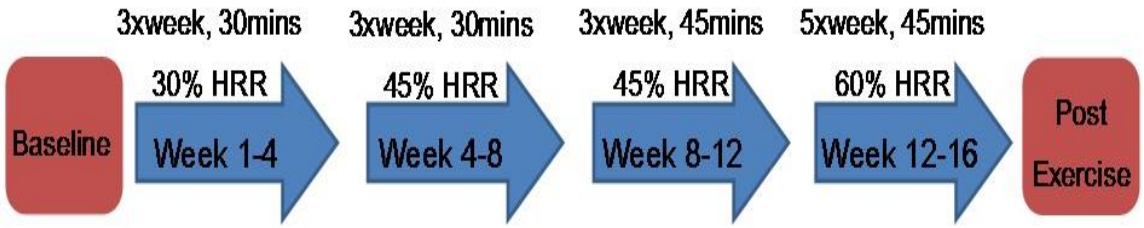


Figure 3.6 16-week moderate intensity exercise training protocol.

To facilitate maximum compliance to the exercise protocol throughout the 16-week period, all participants were closely monitored to ensure that they maintained their prescribed rate of perceived exertion (RPE) and HR during supervised sessions. All exercise sessions that were performed at the local recreational gymnasium were monitored using the *techno-gym wellness* key system. An electronic database key was allocated to each participant, onto which the exercise physiologist uploaded a weekly, tailored, exercise training protocol. Once entered into a cardiorespiratory machine, the key automatically started the machine to the uploaded intensity. Moreover, the key recorded every training session performed in the recreational gymnasiums ensuring the correct HRR was maintained and that each participant was compliant during non-supervised sessions. No dietary alterations were made by any participant throughout the exercise intervention; this was assessed using a standard food diary.

3.8 Conventional Care Intervention

Conventional care consisted of typical lifestyle advice provided at clinical consultation for a 16-week period. Participants were simply advised by their hepatologist or clinic nurse to modify their lifestyle by losing weight and increasing their physical activity. At no point was any supervision or guidance given to participants undertaking conventional care.

Chapter 4

THE IMPACT OF HEPATIC FAT AND ABDOMINAL VISCERAL ADIPOSE TISSUE ON ENDOTHELIAL FUNCTION IN NAFLD PATIENTS

4.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is regarded as the hepatic manifestation of the metabolic syndrome and is closely associated with obesity, insulin resistance, and traditional cardiovascular disease (CVD) risk factors such as hypertension and hyperlipidaemia (Rector *et al.*, 2008). CVD is the leading cause of death in NAFLD patients, exceeding that of liver related death (Ekstedt *et al.*, 2006; Ong *et al.*, 2008). Endothelial function, measured using the flow mediated dilatation (FMD) technique, is an early clinical marker of CVD. Previous studies have reported impaired FMD in NAFLD patients when compared to age and sex matched controls (Schindhelm *et al.*, 2005; Villanova *et al.*, 2005). Moreover, as the severity of NAFLD progresses from steatosis to NASH, the level of impairment in FMD has been shown to increase (Villanova *et al.*, 2005) and, ultimately, reduction in endothelium-independent dilatation becomes apparent (Senturk *et al.*, 2008). This suggests that as the disease progresses, the detrimental influence on the vasculature is greater and may extend from the endothelium to vascular smooth muscle.

It is well established that abdominal obesity is frequently expressed in NAFLD (Adams & Angulo, 2005; Angulo, 2007; Schreuder *et al.*, 2008). Specifically, excess visceral adipose tissue (VAT) is considered a pivotal feature in the pathogenesis of NAFLD and is predictive of CVD (Despres, 2007; Petta *et al.*, 2009). Several reports have demonstrated that obesity (Hashimoto *et al.*, 1998; Arcaro *et al.*, 1999; Baldeweg *et al.*, 2000; Joseph *et al.*, 2002; Williams *et al.*, 2005; Williams *et al.*, 2006), insulin resistance (Balletshofer *et al.*, 2000; Prior *et al.*, 2005) and VAT (Hashimoto *et al.*, 1998; Romero-Corral *et al.*, 2010) attenuate FMD. In addition, Villanova and colleagues (2005) demonstrated that impaired FMD in NAFLD was associated with insulin resistance and obesity. However, it is difficult to discern between the impacts of

obesity and insulin resistance in this study as BMI was the sole index of obesity and the control group was not matched for BMI. Despite widespread utilisation, BMI does not provide a comprehensive assessment of obesity status as it cannot distinguish between adipose and lean tissue (Oreopoulos *et al.*, 2011), nor the impact of obesity on intra-hepatic triglyceride content (IHTC) or abdominal VAT.

No previous research has quantified IHTC and VAT via the non-invasive gold standard ^{1}H-MRS and MRI techniques respectively to investigate the impacts of these variables on FMD in NAFLD. Therefore, the aims of this study were to (i) investigate the extent of endothelial dysfunction in NAFLD patients compared to age and BMI matched controls; and (ii) to examine the impact of IHTC and abdominal VAT on endothelial function. It was hypothesised that (i) endothelial function would be impaired in NAFLD patients when compared with BMI-matched controls and (ii) endothelial dysfunction in NAFLD patients would be mediated by IHTC and abdominal VAT.

4.2 Methods

4.2.1 Participants

Thirty two sedentary NAFLD patients (21 males, 11 females, 48±2yrs, BMI 31±1kg/m^2) and eighteen matched controls (8 males, 10 females, 48±2yrs, BMI 30±1kg/m^2) were recruited (For inclusion and exclusion criteria, please refer to *General Methodology, Chapter 3*). All NAFLD patients were normocholesterolaemic and 7 were taking anti-hypertensive medication (β-blocker *n*=3, ACE inhibitor *n*=2, Calcium channel blocker *n*=2). None of the controls were taking any prescribed medication. Pre-menopausal women (*n*=4) were tested during the early follicular phase of the menstrual cycle, defined as day 1-7.

4.2.2 Research Design and Physiological Measures

Participants were required to attend the laboratory for a number of physiological examinations, which included anthropometric assessments, a blood sample, assessment of brachial artery function, a cardiorespiratory fitness test and magnetic resonance imaging (MRI) with proton magnetic resonance spectroscopy (^{1}H-MRS) to determine abdominal fat deposition and IHTC (for details of measurement procedures, please refer to general methodology, Chapter 3). Measurements were performed following an overnight fast, 12-hour abstinence from caffeine and 24-hour abstinence from alcohol and strenuous exercise.

4.2.3 Statistical Analysis

The primary outcome variable was FMD (%) between NAFLD and control individuals. Following analysis of distribution, differences between NAFLD and controls were compared using independent t-tests or non-parametric equivalent (Wilcoxon) for all variables. Pearson's correlation coefficients (two-tailed) were calculated to evaluate relationships between FMD and all other major variables. Differences in FMD between NAFLD and controls were analysed using analysis of covariance with abdominal VAT as a covariate. Data were analysed using the SPSS 17.0 (SPSS, Chicago, Illinois) software. Data are presented in the text as mean±SE, unless otherwise stated and exact *P* values are cited (values of *P* of "0.000" provided by the statistics package are reported as "<0.0005").

4.3 Results

4.3.1 Clinical and biochemical characteristics

The characteristics of all NAFLD and control participants are listed in Table 4.1. NAFLD patients and controls were matched for age, BMI and cardiorespiratory fitness (P>0.20), however, NAFLD patients exhibited an increased waist circumference (106.9±1.8 vs. 101.1±2.3cm; P=0.04). IHTC (27.2±3.0 vs. 2.9±0.4%; P<0.0005; Figure 4.1) and serum liver enzymes were elevated in NAFLD patients (P<0.006; Table 4.1) as was abdominal VAT when compared with controls (P<0.0005; Figure 4.2). Fasting glucose did not differ between NAFLD and controls. Serum triglycerides were elevated (2.2±0.2 vs. 1.5±0.2 mmol.L^{-1}; P<0.0005) and HDL reduced (1.3±0.1 vs. 1.5±0.1 mmol.L^{-1}; P=0.03) in NAFLD patients compared with controls (P<0.03).

Table 4.1 Characteristics of NAFLD and control participants.

	NAFLD	Controls	*P* Value
Anthropometrics			
N (m/f)	21/11	8/10	n/a
Age (yrs) [+]	49 (15)	48 (13)	0.81
Weight (kg)	90.3±2.5	84.8±3.4	0.20
BMI (kg/m^2)	31±1	30±1	0.20
Waist circumference (cm) [+]	105.2 (14.9)	100.3 (14.2)	0.04*
Systolic blood pressure (mmHg)	127±2	127±2	0.91
Diastolic blood pressure (mmHg)	79±1	77±2	0.74
Hepatic and Body Fat Deposition			
IHTC (%)[++]	27.2±3.0	2.9±0.4	<0.0005*
Abdominal VAT (l)	5.4±0.3	3.4±0.2	<0.0005*
Abdominal Subcutaneous Fat (l) [++]	8.5±0.5	8.2±0.8	0.47
Liver Enzymes			
ALT (u/L) [+]	59 (60)	24 (24)	<0.0005*
AST (u/L) [+]	36 (22)	24 (6)	<0.0005*
GGT (u/L) [+]	50 (57)	23 (31)	0.006*
Glucose and Lipid Profile			
Fasting Glucose ($mmol.L^{-1}$)	5.1±0.1	5.0±0.1	0.45
Cholesterol ($mmol.L^{-1}$)	5.5±0.2	5.2±0.2	0.27
Triglyceride ($mmol.L^{-1}$) [++]	2.2±0.2	1.5±0.2	<0.0005*
HDL ($mmol.L^{-1}$)	1.3±0.1	1.5±0.1	0.03*
LDL ($mmol.L^{-1}$)	3.3±0.1	3.3±0.3	0.89
Chol:HDL ratio [+]	4.0 (1.0)	4.0 (1.0)	0.06
Cardiorespiratory Capacity			
VO_{2Peak} ($ml/kg^{-1}/min^{-1}$) [++]	28.0±1.4	27.5±2.0	0.72
Brachial Artery Vascular Function			
Flow-Mediated Dilation (%)	4.8±0.3	8.3±0.7	<0.0005*
Baseline Diameter (mm)	4.2±0.02	4.1±0.02	0.81
Peak Diameter (mm)	4.3±0.02	4.5±0.02	0.65
Shear $rate_{AUC}$ ($s^{-1}\times10^3$)	14.7±1.9	15.5±2.0	0.70
FMD- Mediated Time to Peak (s)	61.6±5.7	45.0±4.0	0.05*
GTN-Mediated Dilation (%)	16.7±1.3	16.7±1.0	0.98
GTN-Mediated Time to Peak (s)	387.5±17.9	330.0±35.6	0.27

[+] Non-parametric data presented as median (interquartile range).

[++] Logarithmically transformed data.

* Significant difference between NAFLD vs. controls ($P<0.05$).

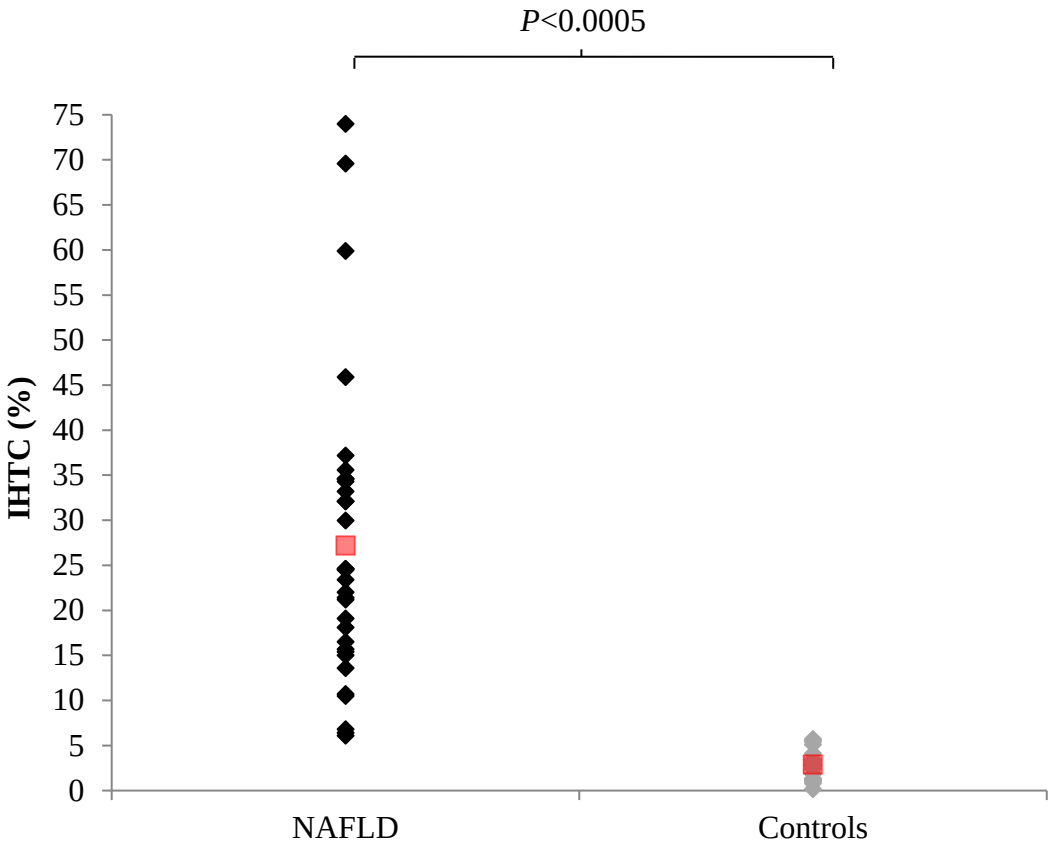


Figure 4.1 Individual IHTC data in NAFLD patients vs. BMI-matched controls (red markers represent the mean IHTC of the respective groups).

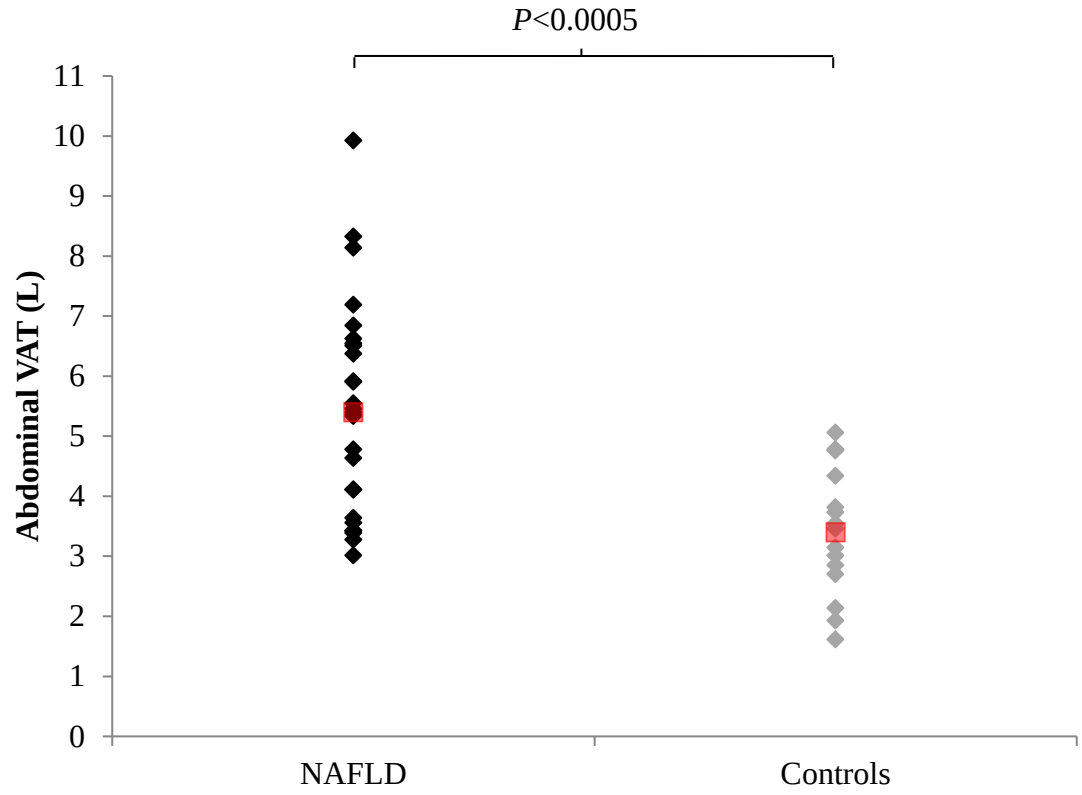


Figure 4.2 Individual abdominal VAT data in NAFLD patients vs. BMI-matched controls (red markers represent the mean abdominal VAT of the respective groups).

4.3.2 Vascular measurements

Brachial artery FMD% was significantly impaired in NAFLD patients when compared with BMI-matched controls (4.8±0.3 vs. 8.3±0.7%, P<0.0005, Figure 4.3). No differences were observed in baseline brachial artery diameter, peak diameter or shear rate between NAFLD patients and controls (*P*>0.65; Table 4.1). Nevertheless, it took NAFLD patients significantly longer to reach peak diameter (61.6±5.7 vs. 45.0±4.0s; *P*=0.05). No difference in endothelium-independent vasodilatation to GTN was observed (*P*=0.92) or endothelium independent time to peak (*P*=0.27; Table 4.1).

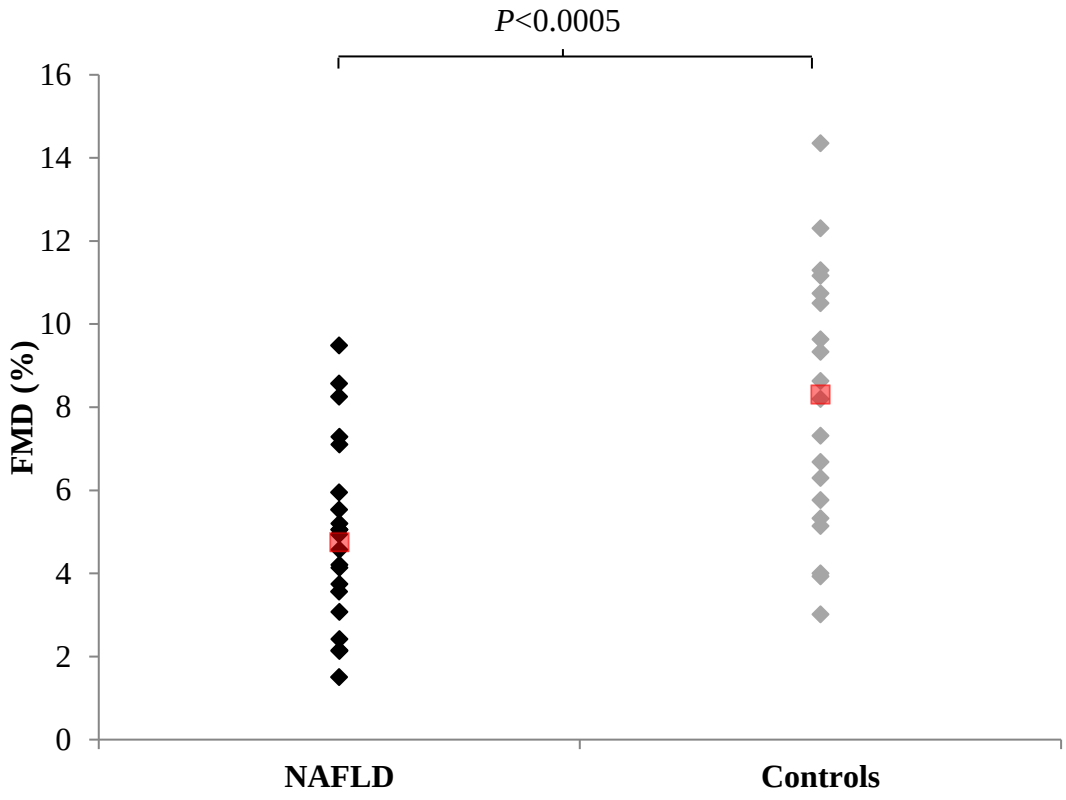


Figure 4.3 Individual FMD data in NAFLD patients vs. BMI-matched controls (red markers represent the mean FMD of the respective groups). *According to published prognostic data by Inaba et al., (2010) these data indicate that NAFLD patients are ~21% more likely to suffer a cardiovascular event than BMI-matched controls.*

4.3.3 FMD correlations

A moderate inverse correlation was observed between FMD and abdominal VAT (r = -0.48, P=0.01; Figure 4.4) in NAFLD patients, although no relationship was observed in control participants (r = -0.07, P=0.78; Figure 4.4). FMD did not correlate with any other variable in NAFLD patients or control participants (P>0.05).

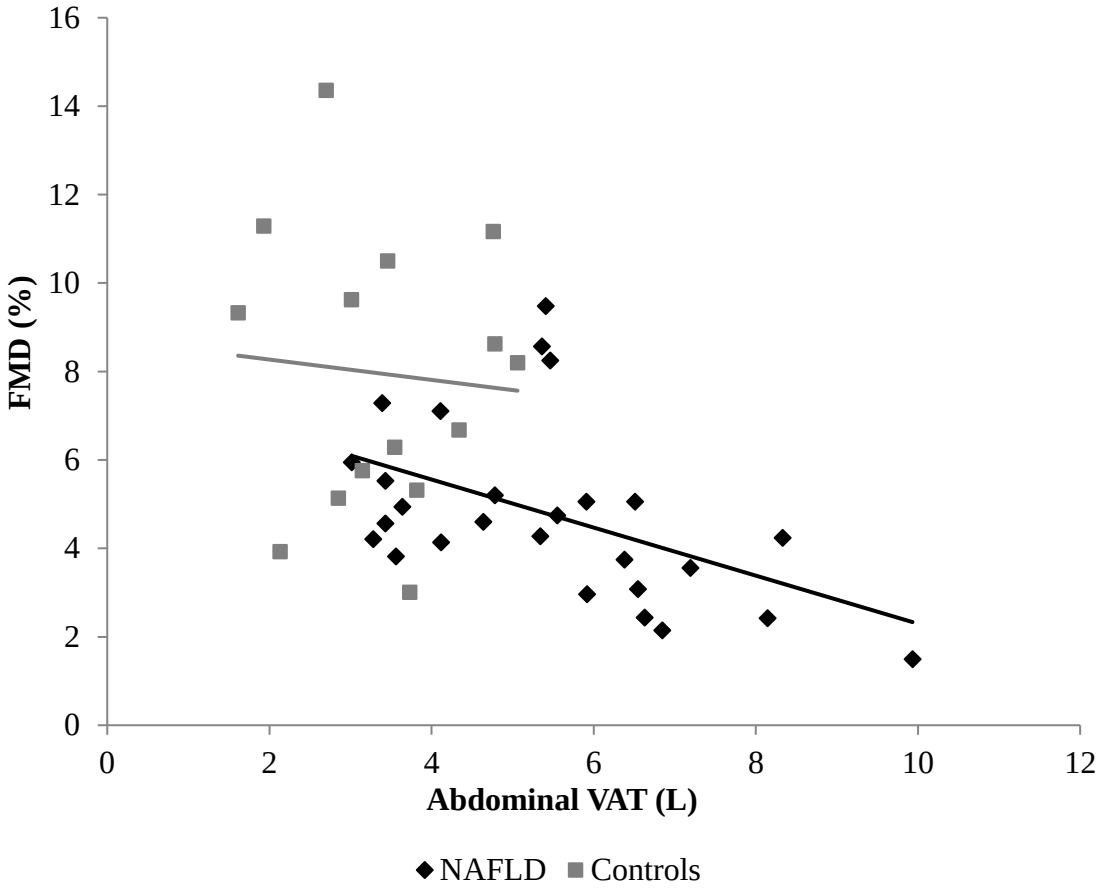


Figure 4.4 The relationship between FMD and abdominal VAT in NAFLD patients (r = -0.48, P=0.01) and BMI-matched controls (r = -0.07, P=0.78).

Impairment in FMD remained in NAFLD patients following independent covariate adjustment for abdominal VAT (5.0±0.5 vs. 7.3±0.7%, *P*=0.01; Figure 4.5).

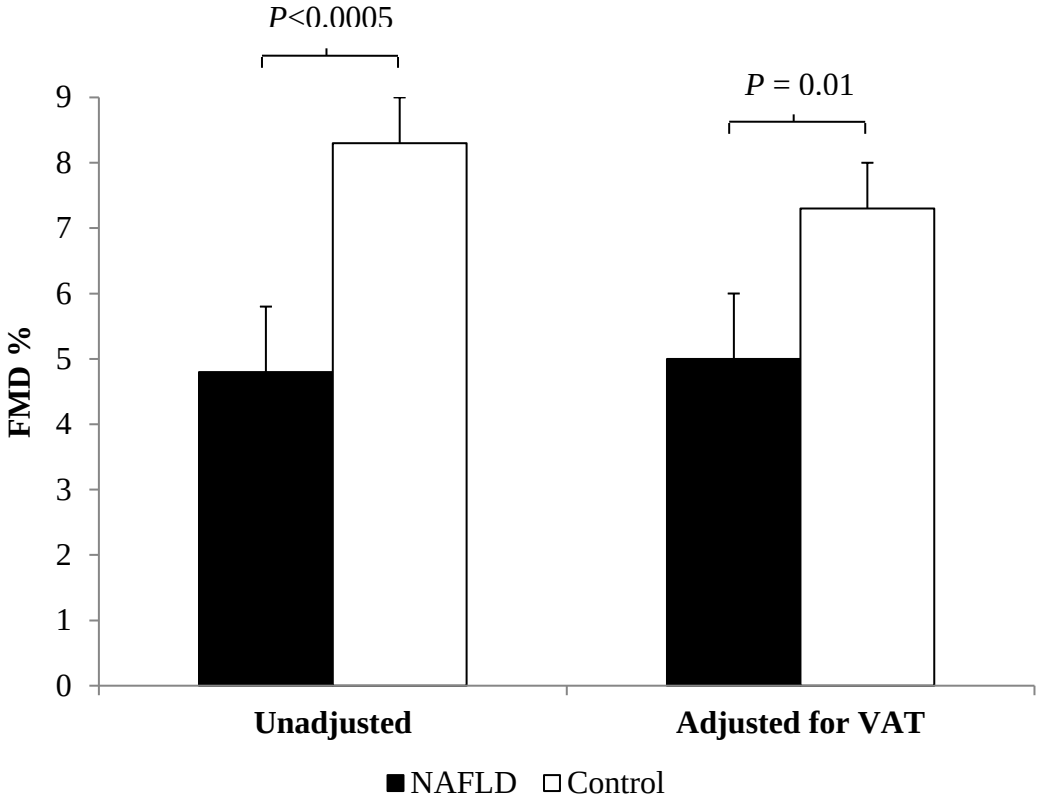


Figure 4.5 FMD response of NAFLD patients vs. BMI-matched controls after adjustment for abdominal VAT.

4.4 Discussion

The primary aim of the study was to investigate the impact of fat deposition on FMD in NAFLD and specifically, to establish whether abdominal VAT and/or IHTC had an independent detrimental impact on endothelial function. The major novel findings of this study were that (i) FMD was impaired in obese NAFLD patients compared with age and BMI matched controls; and (ii) the impairment in FMD is not explained by excess IHTC or abdominal VAT.

Prior to adjustment, a significant mean reduction in FMD of 3.5% was observed in NAFLD patients compared with control participants. This difference is clinically

relevant as recently published risk ratios indicate that the risk of a cardiovascular event increases by 21% for every 1 standard deviation (=3.5%) decrease in FMD (Inaba *et al.*, 2010), Subsequently, the findings of this chapter infer that NAFLD patients are 21% more likely to experience a cardiovascular event than individuals without NAFLD who are of a similar age, fitness and BMI. Following adjustment for abdominal VAT, the impairment in FMD exhibited by NAFLD patients reduced. Although, adjustment for abdominal VAT did not totally abolish the impairment in FMD, it appears to modestly contribute to NO-mediated endothelial dysfunction. Surprisingly, elevated IHTC in isolation had a negligible impact on FMD. Taken together, these findings suggest that neither excess IHTC nor abdominal VAT are key contributing factors to the endothelial dysfunction present in NAFLD. A previous study by Villanova *et al.* (2005) elegantly demonstrated that impaired FMD in NAFLD was associated with, but not explained by, insulin resistance. Nevertheless, VAT has been previously reported to be closely linked with the severity of NAFLD independent of insulin resistance (van der Poorten *et al.*, 2008). Given that isolated hallmark features of NAFLD such as IHTC, abdominal VAT and insulin resistance do not totally explain the decrement in FMD, other less overt pathological features may contribute to endothelial dysfunction, such as the excess secretion of inflammatory cytokines, including TNF-α and leptin.

Previous research has demonstrated that FMD is impaired in NAFLD patients when compared with overweight (Vlachopoulos *et al.*, 2010) age-matched (Villanova *et al.*, 2005; Senturk *et al.*, 2008) controls. Nevertheless, this is the first study to demonstrate that FMD is impaired in obese NAFLD patients when compared with BMI-matched obese controls (i.e. BMI>30). Moreover, this is the first study to employ the FMD technique according to the latest guidelines (Thijssen *et al.*, 2011) which include measurement of the eliciting shear rate stimulus and absolute arterial diameters. Thus, a

novel finding of the current study is that the time to reach peak diameter following forearm ischaemia was significantly greater in NAFLD patients. However, following sub-lingual administration of a potent NO donor, time to reach peak arterial diameter was similar between groups. This suggests that NAFLD patients exhibit reduced endothelial hormone production rather than delayed relaxation of the smooth muscle in response to NO (Black *et al.*, 2008a). This finding confirms that NAFLD patients demonstrate impaired endothelium-dependent vasodilator function. Another noteworthy observation is that NAFLD patients and controls were matched for cardiorespiratory fitness. Previous studies have suggested that cardiorespiratory fitness independently predicts the risk of CVD mortality (Sui *et al.*, 2007; Ekblom-Bak *et al.*, 2009). Nevertheless, fitness did not influence FMD in the current study, which supports the findings of Davison et al. (2010), who observed no association between cardiorespiratory fitness and FMD in lean and obese sedentary individuals.

NAFLD patients demonstrated a larger waist circumference and exhibited elevated abdominal VAT compared with control participants, despite being matched for BMI. VAT is considered a pivotal feature in the pathogenesis of NAFLD as it is a key source of circulating adipokines, and is also predictive of CVD (Despres, 2007; Petta *et al.*, 2009). Moreover, VAT positively correlates with steatosis (Kelley *et al.*, 2003; Nguyen-Duy *et al.*, 2003), hepatic insulin resistance (Miyazaki *et al.*, 2002) and is closely linked with the severity of NAFLD, independent of insulin resistance (van der Poorten *et al.*, 2008). One recent study reported that a modest increase in VAT (47.3±31.4 to 60.1±35.0cm^2) promoted endothelial dysfunction in healthy individuals (Romero-Corral *et al.*, 2010). Indeed, VAT has been reported to be a determinant of endothelial dysfunction, independent of traditional risk factors or liver steatosis in morbidly obese individuals (Sturm *et al.*, 2009). However, the study of Sturm *et al.* (2009) employed

ultrasound to determine VAT and hepatic steatosis; a highly operator-dependent approach which is unable to quantify the degree of steatosis (Joy *et al.*, 2003).

In summary, FMD is impaired in obese NAFLD patients when compared with BMI matched controls, however, this impairment is not explained by excess IHTC or abdominal VAT.

Chapter 5

A RANDOMISED CONTROLLED TRIAL COMPARING THE EFFECTS OF SUPERVISED EXERCISE TRAINING WITH CONVENTIONAL CLINICAL CARE ON ENDOTHELIAL FUNCTION IN NAFLD PATIENTS

5.1 Introduction

Non-alcoholic fatty liver disease (NAFLD), characterised by elevated triglycerides within the liver, is the most common form of chronic liver disease in western society, affecting approximately 20-30% of the general population (Bedogni *et al.*, 2005) and up to ~70-90% of obese and diabetic individuals (Adams & Angulo, 2005). Although NAFLD is primarily a hepatic disorder, excess liver triglyceride accumulation is an independent risk factor for cardiovascular disease (CVD), insulin resistance and type 2 diabetes (Stefan *et al.*, 2008). Moreover, epidemiological research indicates that CVD is the leading cause of mortality in NAFLD patients, responsible for 25% of deaths, a value exceeding that of liver disease *per se*, which accounts for just 6% of mortality (Ong *et al.*, 2008).

Despite the increasing prevalence of NAFLD, therapeutic options are limited, with no effective pharmacological treatment to reduce hepatic fat. Lifestyle interventions including structured exercise and diet are therefore recommended to reduce hepatic fat in NAFLD patients (Caldwell & Lazo, 2009). Evidence from a number of cross-sectional studies suggests a significant inverse correlation between physical activity levels and/or cardiorespiratory fitness and hepatic fat in NAFLD patients (Suzuki *et al.*, 2005; Church *et al.*, 2006; McMillan *et al.*, 2007; Perseghin *et al.*, 2007). Moreover, a number of studies indicate that exercise training reduces hepatic fat in NAFLD patients (Bonekamp *et al.*, 2008; Johnson *et al.*, 2009; van der Heijden *et al.*, 2010). Nevertheless, no study to date has compared the therapeutic effect of exercise with that of the general lifestyle advice provided as part of conventional clinical care on hepatic fat.

The impact of lifestyle modification or supervised exercise training on endothelial function, an early atherosclerotic marker which predicts future cardiovascular events, has not been investigated in NAFLD patients. Exercise training has been shown to improve endothelial function in healthy individuals (Green *et al.*, 2004a; Thijssen *et al.*, 2010) and in populations demonstrating risk factors for CVD (Higashi *et al.*, 1999; Lewis *et al.*, 1999; Lavrencic *et al.*, 2000; Maiorana *et al.*, 2001; Walsh *et al.*, 2003; Watts *et al.*, 2004). Importantly, exercise-induced improvements in endothelial function have been shown to occur both prior to, and independent of, changes in traditional markers of cardiovascular risk (Green *et al.*, 2003). This is of particular importance to the NAFLD population as CVD risk factors such as obesity, insulin resistance and hyperlipidaemia are frequently exhibited and contribute to the higher CV mortality in this patient group. Therefore, the aim of this study was to compare the effect of a supervised exercise intervention with conventional clinical care on endothelial function in NAFLD patients. It was hypothesised that 16-weeks of supervised exercise training would induce a greater improvement in endothelial function in NAFLD patients when compared with 16-weeks of conventional clinical care.

5.2 Methods

5.2.1 Participants

Twenty eligible NAFLD patients (11 males, 9 females, 49±3yrs, BMI 30±1kg/m^2) completed the study (Figure 5.1). All NAFLD patients were normocholesterolaemic and 6 were taking anti-hypertensive medication (β-blocker *n*=3, ACE inhibitor *n*=2, Calcium channel blocker *n*=1). Medications were not altered during the course of the study. All female participants were post-menopausal, except one, who had undergone a hysterectomy.

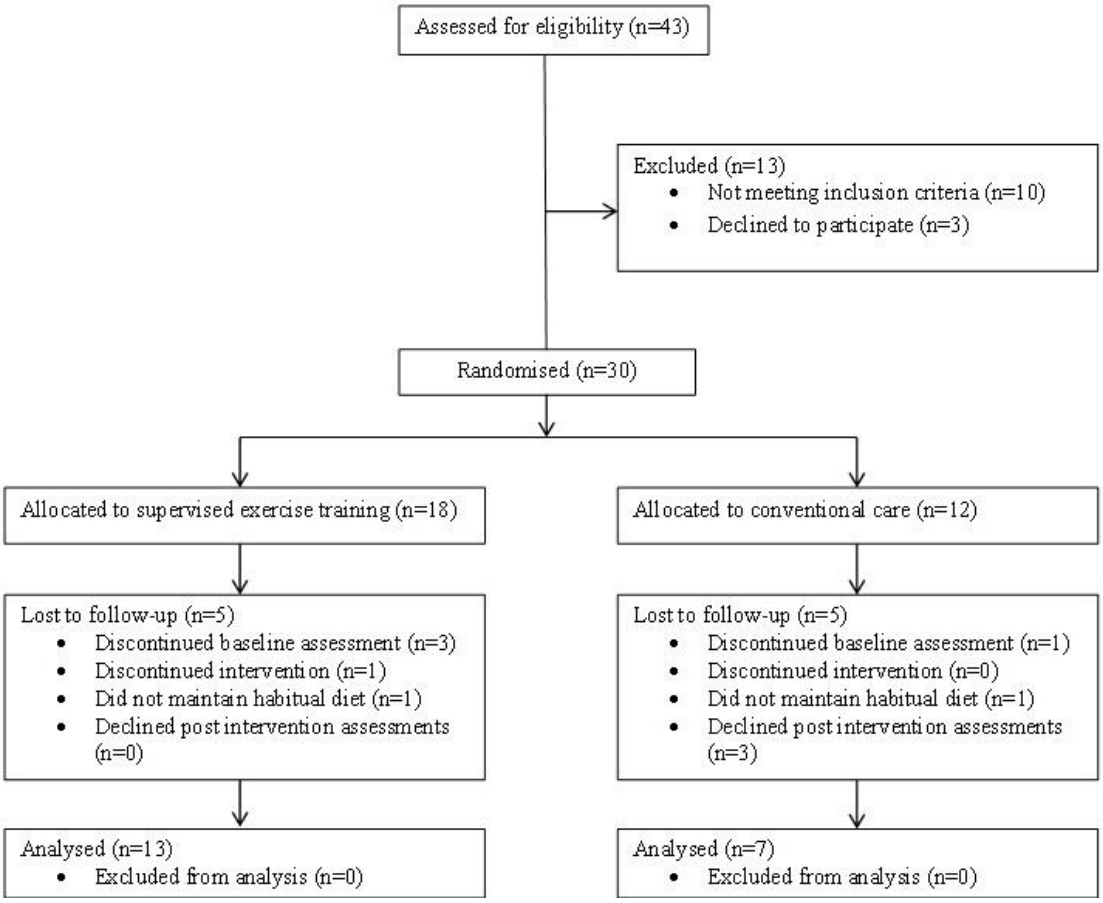


Figure 5.1 Participant flow diagram

5.2.2 Research Design

A series of physiological measurements were performed at baseline. Following this, NAFLD patients were randomly assigned to either 16-weeks of supervised and structured exercise training (*n*=13, 50±3yrs, BMI 30±1kg/m^2) or to 16-weeks of conventional care (*n*=7, 47±6yrs, BMI 31±2kg/m^2) (for details of respective interventions, please refer to *General Methodology; Chapter 3*). Upon completion of this 16-week period, all measurements were repeated.

5.2.3 Experimental Protocol

Participants reported to the laboratory to undertake the baseline measurements including anthropometric assessment, a blood sample, assessment of brachial artery endothelial function, a cardiorespiratory fitness test and magnetic resonance imaging (MRI) with proton magnetic resonance spectroscopy (^{1}H-MRS) to determine abdominal fat deposition and intrahepatocellular triglyceride content (IHTC) (for details of measurement procedures, please refer to general methodology, Chapter 3). Measurements were performed following an overnight fast, 12-hour abstinence from caffeine and 24-hour abstinence from alcohol and strenuous exercise.

5.2.4 Statistical Analysis

The primary outcome variable for this study was FMD (%) and the primary comparison was the effect of exercise vs. conventional care. For the comparison of the exercise vs. the conventional care intervention, delta (Δ) change from pre-intervention was calculated and analysed using analysis of covariance (ANCOVA), with pre-exercise data as a covariate. Pearson's correlation coefficients (two-tailed) were calculated to evaluate relationships between delta (Δ) change in FMD and the delta (Δ) change in all other major variables. Data were analysed using the SPSS 17.0 (SPSS, Chicago, Illinois) software. Data are presented in the text as mean±SE, unless otherwise stated and exact *P* values are cited (values of *P* of "0.000" provided by the statistics package are reported as "<0.0005").

5.3 Results

NAFLD patients who were randomised to supervised exercise training demonstrated 92% compliance to exercise sessions.

5.3.1 Cardiorespiratory Fitness

Cardiorespiratory fitness increased by 7.0±1.5ml.kg^{-1}.min^{-1} following exercise training, but, decreased by 2.3±2.1ml.kg^{-1}.min^{-1} following conventional care (*P*=0.002; Table 5.1).

Table 5.1 Changes in the characteristics of NAFLD patients following supervised exercise training and conventional clinical care.

		Pre Ex	Post Ex	Δ Ex	Pre CC	Post CC	Δ CC	*P* value
Anthropometrics	Weight (kg)	86.7±3.3	84.5±3.5	-2.1	91.3±8.0	90.2±8.1	-1.2	0.31
	BMI (kg/m^2)	30±1	29±1	-0.8	31±2	30±2	-0.4	0.29
	Waist circumference (cm)	103±2	99±2	-4.5	107±5	105±6	-1.9	0.17
	Systolic blood pressure (mmHg)	127±3	126±2	-0.8	125±5	124±4	-1.9	0.74
	Diastolic blood pressure (mmHg)	79±2	78±1	-0.4	76±3	74±1	-1.3	0.82
Body Fat Deposition	IHTC (%)	27.0±5.7	18.0±3.2	-8.8	25.2±3.1	20.5±3.3	-5.3	0.34
	Abdominal VAT (L)	5.8±0.6	5.7±0.5	0.1	4.3±0.4	4.2±0.4	-0.1	0.82
	Abdominal SAT (L)	8.2±0.7	7.7±0.7	-0.5	8.2±0.8	8.9±0.9	0.1	0.07
Liver Enzymes	ALT (u/L)	58±9	40±5	-22.6	85±17	64±11	-11.5	0.17
	AST (u/L)	37±5	29±2	-10.9	54±9	44±5	-2.2	0.19
	GGT (u/L)	74±23	55±15	-22.7	91±14	68±13	-20.3	0.78
Glucose and Lipid Profile	Fasting Glucose ($mmol.L^{-1}$)	5.0±0.2	4.8±0.1	-0.3*	5.4±0.3	5.5±0.3	0.5*	0.008
	Cholesterol ($mmol.L^{-1}$)	5.4±0.3	5.3±0.2	-0.1	5.4±0.3	5.3±0.4	-0.1	0.86
	Triglyceride ($mmol.L^{-1}$)	2.0±0.2	1.9±0.1	-0.1	3.0±0.7	2.1±0.4	-0.5	0.22
	HDL ($mmol.L^{-1}$)	1.3±0.1	1.4±0.1	0.03	1.2±0.1	1.2±0.0	-0.03	0.27
	LDL ($mmol.L^{-1}$)	3.2±0.2	3.1±0.2	-0.1	2.8±0.2	3.3±0.3	0.2	0.22
	Chol:HDL ratio	4.1±0.3	4.0±0.3	-0.1	4.7±0.3	4.6±0.4	-0.1	0.92
Cardiorespiratory Capacity	VO_{2peak} ($ml.kg^{-1}.min^{-1}$)	26.4±2.1	33.4±2.7	7.0*	27.5±3.7	25.0±2.6	-2.3*	0.002
Brachial Artery Vascular Function	Flow-Mediated Dilation (%)	4.8±0.6	8.6±0.7	3.6*	5.5±0.8	5.5±0.5	0.3*	0.004
	Baseline Diameter (mm)	3.8±0.3	3.9±0.2	0.1	3.8±0.3	4.0±0.2	0.2	0.92
	Peak Diameter (mm)	4.1±0.4	4.3±0.3	0.2	4.0±0.3	4.2±0.1	0.1	0.79
	Shear $rate_{AUC}$ ($s^{-1}\times10^3$)	18.4±4.0	15.0±2.3	-27.0	11.5±1.8	13.8±3.1	-9.8	0.65
	Time to Peak (s)	68.0±11.0	52.6±7.4	-11.1	52.2±10.5	40.2±8.2	-20.2	0.46
	GTN-Mediated Dilation (%)	17.1±2.5	15.9±1.5	-0.9	15.9±2.3	15.8±2.6	-0.6	0.91

Ex- Exercise group, CC- Conventional care group, SAT- Subcutaneous adipose tissue, VO_{2peak}- Peak oxygen uptake. Data are presented as mean ± SE. Delta (Δ) change from pre-intervention following adjustment for pre-intervention values. * Significant difference between ΔEx and ΔCC ($P<0.05$).

5.3.2 Vascular measurements

The change in FMD was significantly greater following supervised exercise training when compared with conventional clinical care (3.6±0.6 vs. 0.3±0.8%, *P*=0.004; Figure 5.2). There was no significant difference in baseline or peak arterial diameter, shear rate or time to peak between interventions (Table 5.1).

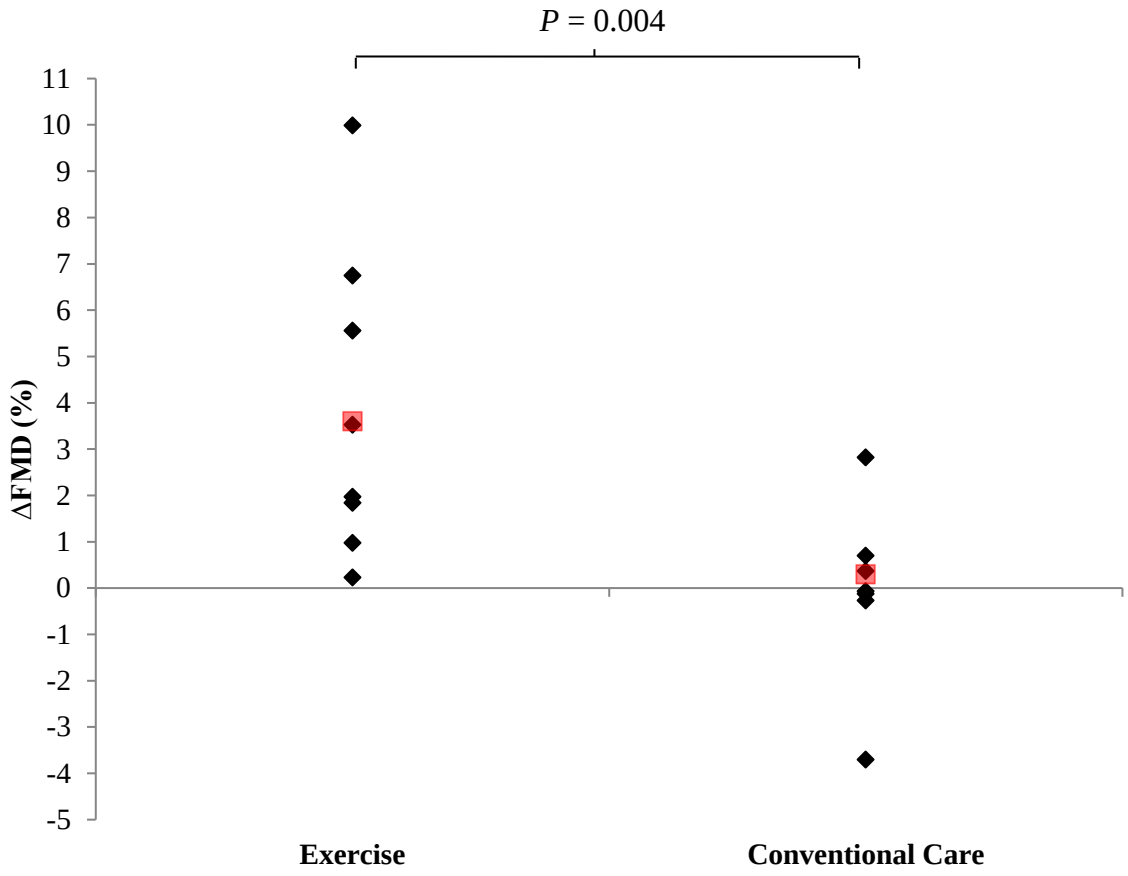


Figure 5.2 Individual delta change scores for FMD (%) following supervised exercise training and conventional care (black markers) and mean delta change following adjustment for pre-intervention data (red markers)**.** *According to published prognostic data by Inaba et al.,(2010) these data indicate that supervised exercise training may have reduced the risk of a future cardiovascular event by ~21%, whereas conventional care has a negligible impact on the future risk of cardiovascular events in NAFLD patients.*

5.3.3 Biochemical characteristics

Fasting plasma glucose reduced by 0.3±0.1mmol.L^{-1} following exercise training, but, increased by 0.5±0.2mmol.L^{-1} following conventional care (*P*=0.008; Table 5.1). There was no significant difference in serum liver enzymes, total, high-density lipoprotein or low-density lipoprotein cholesterol, HDL: cholesterol ratio or triglyceride concentration between interventions (P>0.05; Table 5.1).

5.3.4 Hepatic and abdominal fat deposition

There was no significant difference between the effect of exercise and conventional care on IHTC (P>0.05; Figure 5.3). Abdominal SAT reduced by 0.5±0.1L following exercise training and increased by 0.1±0.2L following conventional care (*P*=0.07). There was no significant difference in weight, waist circumference, abdominal VAT or BMI between interventions (Table 5.1).

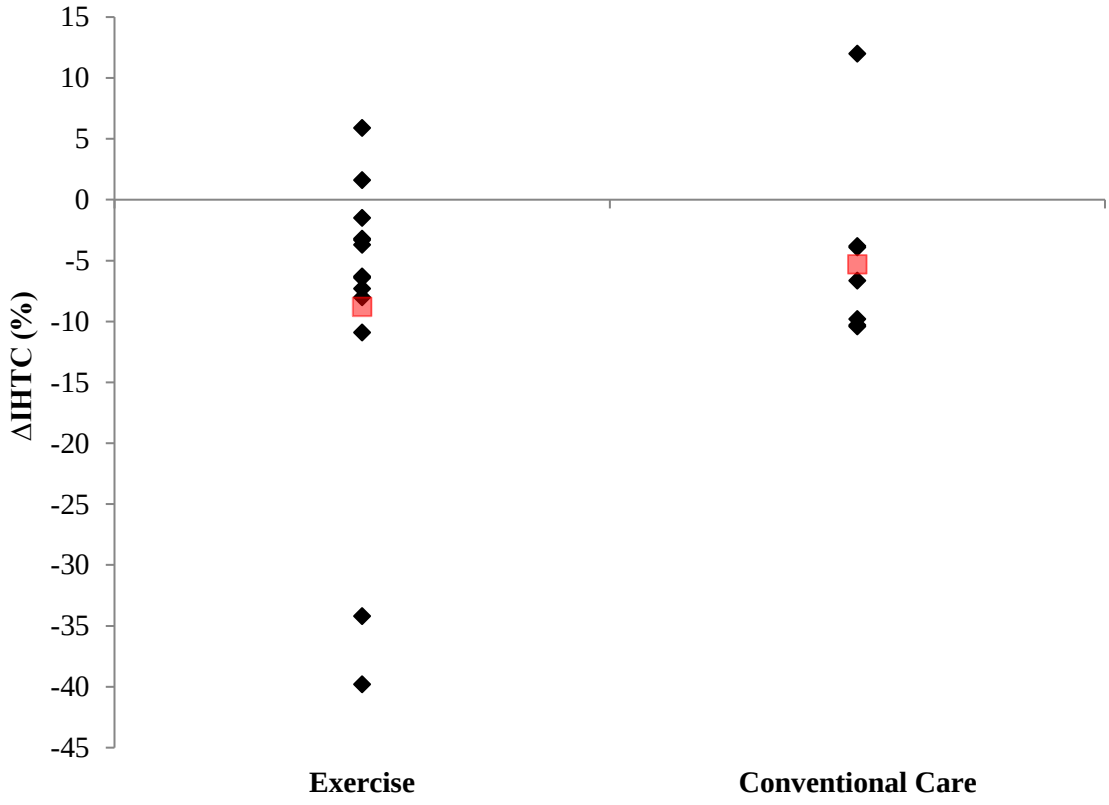


Figure 5.3 Individual delta change scores for IHTC (%) following supervised exercise training and conventional care (black markers) and mean delta change following adjustment for pre-intervention data (red markers)**.**

5.3.5 Change score correlations

The increase in FMD following exercise training was not correlated with changes in cardiorespiratory fitness (r=-0.02, *P*=0.96), abdominal SAT (r=-0.01, *P*=0.81) or fasting plasma glucose (r=-0.003, *P*=0.99). There was a moderate inverse correlation between the change in FMD following conventional care and the change in cardiorespiratory fitness (r=-0.68, *P*=0.18) and fasting plasma glucose (r=-0.59, *P*=0.22), but neither of these correlations reached statistical significance. There was no correlation between the change in FMD and the change in abdominal SAT (r=0.26, *P*=0.74) following conventional care.

5.4 Discussion

The aim of the present study was to compare the effect of a supervised moderate intensity exercise training intervention with conventional clinical care on endothelial function in NAFLD patients. The major finding was that supervised exercise training induced a greater increase in brachial artery FMD when compared with conventional care. These improvements were independent of reductions in IHTC and abdominal VAT. These data suggest that supervised exercise training is an effective management strategy, capable of improving endothelial function in NAFLD patients and hence the prevalence of heart disease and stroke in these high risk patients.

This is the first study to examine the effect of exercise training on endothelial function in NAFLD. Previous studies in these patients have shown exercise training to have a therapeutic effect on traditional CVD risk factors such as waist circumference (Baba *et al.*, 2006) and insulin resistance (van der Heijden *et al.*, 2010). Nevertheless, endothelial dysfunction is regarded as the earliest manifestation of atherosclerotic disease, which is evident prior to overt clinical manifestations of disease. Furthermore, endothelial

function is a strong and independent prognostic marker of future cardiovascular events (Green *et al.*, 2011a). Indeed, supervised exercise training mediated a mean improvement in FMD of 3.6% in NAFLD patients, which according to the prognostic data recently published by Inaba and colleagues (2010) is clinically relevant as this may subsequently reduce the risk of a cardiovascular event by ~21% in these high risk patients. Consequently, this study illustrates that regular exercise has a cardioprotective impact on the vasculature of NAFLD patients, which is superior to that of current conventional care in the UK. This exercise-mediated reduction in CVD risk is of particular clinical importance given that CVD is the leading cause of mortality in NAFLD patients, exceeding that of liver disease (Ong *et al.*, 2008).

Providing the most up-to-date guidelines for the measurement of FMD are utilised (Thijssen *et al.*, 2011), it is accepted that the FMD response in conduit vessels such as the brachial artery is largely dependent on endothelial release of nitric oxide (NO) (Green *et al.*, 2011a). Chapter 4 of this thesis along with other previous research (Villanova *et al.*, 2005) has demonstrated that NAFLD patients exhibit impaired FMD and thus express a reduced production of the anti-atherogenic molecule, NO. The observation that exercise training enhanced FMD in the present study, although novel in NAFLD subjects, is in keeping with previous research that demonstrates exercise training improves NO-mediated endothelial function in comparable diseased populations such as type 2 diabetes (Maiorana *et al.*, 2001), hypercholesterolaemia (Walsh *et al.*, 2003) and metabolic syndrome (Lavrencic *et al.*, 2000). Although exercise training is associated with improvements in traditional cardiovascular risk factors, these are typically quite modest in magnitude and unlikely to fully explain the benefits of exercise in terms of cardiovascular risk reduction (Green *et al.*, 2008; Joyner & Green, 2009). Regular exercise training has been shown to promote an increase in

NO bioavailability by reducing the number of oxygen free radicals and up-regulating endothelial NO synthase protein (Green *et al.*, 2004a; Green *et al.*, 2008) and these effects may be independent of improvement in risk factors (Green *et al.*, 2003). Increased NO bioavailability is thought to be caused by direct impacts of recurrent shear stress as a result of repeated exercise bouts (Green *et al.*, 2010). Consequently, these chronic benefits observed in NAFLD patients are indicative of an increase in NO production, which enhances vasomotor function and also decreases the risk of atherosclerotic development.

Despite a significant increase in endothelium dependent FMD with exercise training, no difference in brachial artery endothelial independent vasodilatation in response to sublingual GTN administration were observed following the respective interventions. This finding is broadly consistent with those of previous studies investigating similar metabolic diseases (Maiorana *et al.*, 2001), and further suggests that the exercise-mediated changes in endothelial function are due to an increase in NO production rather than an increase in sensitivity of vascular smooth muscle. Moreover, exercise training did not induce any change in baseline brachial artery diameter, suggesting that, if structural changes in the arterial wall occurred as a result of exercise, they were not apparent at rest. This is also in keeping with previous research (Green *et al.*, 2011b).

Somewhat surprisingly, the differences in FMD we observed were not accompanied by significant changes in IHTC or abdominal VAT. Several previous studies have demonstrated moderate intensity exercise can significantly reduce IHTC in NAFLD patients (Bonekamp *et al.*, 2008; Johnson *et al.*, 2009; van der Heijden *et al.*, 2010), However, this is the first study to compare an exercise intervention to conventional care of weight loss and increased physical activity. The current data clearly indicates that

supervised exercise training reduces IHTC, yet the conventional care group also reduced IHTC, albeit to a lesser degree. Intriguingly, a difference in fasting glucose was evident post intervention, with a reduction in glucose observed following exercise but an increase in glucose following conventional care. Although this data is limited to the measurement of plasma glucose alone, this could indicate that despite a modest reduction in IHTC, conventional care does not aid in glucose control. Given that NAFLD is a pre-diabetic condition, this finding implies that exercise-induced improvements in cardiorespiratory fitness are necessary for effective maintenance of glycaemic control in patients at high risk of type 2 diabetes. This is corroborated by the findings of the Diabetes Prevention Program Research Group (2002), which demonstrated that a lifestyle intervention consisting of increased physical activity and weight loss is more effective than metformin in reducing the incidence of type 2 diabetes. Specifically, lifestyle intervention induced a 58% reduction, whereas metformin only elicited a 31% reduced incidence of type 2 diabetes.

Another noteworthy observation was that neither intervention caused significant reductions in abdominal VAT despite a trend for a reduction in abdominal SAT. This is in contrast to one previous study which observed a 12% relative reduction in abdominal VAT as well as a 21% relative reduction in IHTC in NAFLD patients following four weeks of high intensity cycle ergometer exercise training (Johnson *et al.*, 2009). It is probable that a higher intensity exercise training intervention may have mediated greater reductions in both IHTC and abdominal VAT in the present study. Nonetheless, improvements in endothelial function following supervised exercise training in the current study were independent of concomitant changes in fat levels, implying that exercise has a direct therapeutic impact on endothelial function in NAFLD.

In summary, these novel findings suggest that supervised exercise training is an effective management strategy capable of improving endothelial function in NAFLD patients. Improvements in endothelial function occurred without significant reductions in IHTC or abdominal VAT and were not mediated by improvements in cardiorespiratory fitness or glycaemic control. Consequently, these data indicate that exercise has an independent and direct therapeutic impact on endothelial function in NAFLD, which may decrease the risk of heart disease and stroke in these high risk patients. Thus, exercise prescription should be recommended as a cardioprotective management strategy in NAFLD.

Chapter 6

ENDOTHELIAL FUNCTION IN NAFLD PATIENTS 12 MONTHS FOLLOWING THE CESSATION OF SUPERVISED EXERCISE TRAINING

6.1 Introduction

Prevention of highly prevalent diseases such as type 2 diabetes and cardiovascular disease (CVD) is a major public health challenge. In the UK alone, 2.9 million people (4.9% of the population) are affected by diabetes (Shaw *et al.*, 2010). Furthermore, 2.7 million people are living with CVD and alarmingly, CVD is the main cause of mortality in the UK accounting for approximately 191,000 deaths each year (Allender *et al.*, 2011). Non-alcoholic fatty liver disease (NAFLD), a recently identified phenomenon, which is related to obesity, confers an independent risk of insulin resistance, type 2 diabetes and CVD (Stefan *et al.*, 2008). Epidemiological research indicates that CVD accounts for a greater number of deaths than that of liver disease in NAFLD patients (Ekstedt *et al.*, 2006; Ong *et al.*, 2008; Soderberg *et al.*, 2010) and some report CVD to be the leading cause of mortality (Ekstedt *et al.*, 2006; Ong *et al.*, 2008).

Obesity and type 2 diabetes are now extremely common in Westernised societies and NAFLD is also becoming increasingly prevalent (20-30% of general population, ~70-90% of obese and type 2 diabetic individuals (Adams & Angulo, 2005; Marchesini *et al.*, 2005; Neuschwander-Tetri, 2005). Therefore, effective and sustainable interventions to reduce hepatic fat and the risk of type 2 diabetes and CVD are vital. Currently, there are no recommended pharmacological treatments to reduce hepatic fat therefore current clinical guidelines recommend lifestyle interventions comprising of physical activity and diet. Critically, Chapter 5 demonstrated for the first time that supervised exercise training improves endothelial function, an early marker in the development of atherosclerosis and predictor of future cardiovascular events, in NAFLD patients. Moreover, exercise training also provoked a 9% absolute reduction in intrahepatocellular triglyceride content (IHTC), a finding that is corroborated by several research groups (Bonekamp *et al.*, 2008; Johnson *et al.*, 2009; van der Heijden *et al.*,

2010). Importantly, these beneficial effects were evident over and above that of general lifestyle advice, provided as part of clinical care, suggesting that supervised exercise training is an effective non-pharmacological management strategy.

Lifestyle modification is difficult to maintain without supervision, yet, for exercise training to be endorsed as a credible therapeutic intervention to reduce the risk of type 2 diabetes and CVD, it is essential that the beneficial effects are evident long-term. For example, the diabetes prevention program research group (Knowler *et al.*, 2009) conducted a 10-year follow up study in type 2 diabetic patients. The authors reported a diminished incidence of type 2 diabetes following a lifestyle intervention compared with metformin and placebo interventions, indicative that lifestyle intervention, in the form of exercise and weight loss, delayed or even prevented the onset of diabetes. Therefore, the aim of the present study was to investigate whether exercise-induced improvements in endothelial function in NAFLD patients are sustained 12-months following the cessation of supervised exercise training. It was hypothesised that exercise-induced improvements in endothelial function in NAFLD patients would abolish 12-months following the cessation of supervised exercise training.

6.2 Methods

6.2.1 Participants

Nine NAFLD patients (5 males, 4 females, 50±5yrs, BMI 30±1kg/m^2) were recruited. All NAFLD patients were normocholesterolaemic; one participant was hypertensive and was treated with a β-blocker. Medications were not altered during the course of the study. All female participants were post-menopausal, except one, who had undergone a hysterectomy.

6.2.2 Research Design

Physiological measurements were performed in all NAFLD patients at baseline, following a 16-week supervised moderate intensity exercise training program and 12-months following the completion of the exercise training program.

6.2.3 Experimental Protocol

Participants reported to the laboratory to undertake a number of physiological examinations, which included anthropometric assessments, a blood sample, assessment of brachial artery function, a cardiorespiratory fitness test and proton magnetic resonance spectroscopy (^{1}H-MRS) to determine intrahepatocellular triglyceride content (IHTC) (for details of measurement procedures, please refer to *General Methodology; Chapter 3*). Measurements were performed following an overnight fast, 12-hour abstinence from caffeine and 24-hour abstinence from alcohol and strenuous exercise.

6.2.4 Statistical Analysis

A within subjects repeated measures analysis of variance (ANOVA) was used to evaluate differences between baseline data, post training data and 12-months post training data. Data were analyzed using the SPSS 17.0 (SPSS, Chicago, Illinois) software. Data are presented in the text as mean±SE and exact *P* values are cited (values of *P* of "0.000" provided by the statistics package are reported as "<0.0005").

6.3 Results

6.3.1 Cardiorespiratory Fitness

There was a significant difference in cardiorespiratory fitness across the three time points (*P*=0.004; Figure 6.1). Specifically, peak oxygen uptake increased from 26.4±2.3ml.kg^{-1}.min^{-1} at baseline to 33.0±3.5ml.kg^{-1}.min^{-1} following supervised exercise training (*P*=0.009). Additionally, peak oxygen uptake reduced (33.0±3.5 vs. 26.7±2.8ml.kg^{-1}.min^{-1}) 12 months following the completion of exercise training (*P*=0.004). There was no difference between cardiorespiratory fitness at baseline and 12 months following the completion of supervised exercise training (26.4±2.3 vs. 26.7±2.8ml.kg^{-1}.min^{-1}; *P*=0.86).

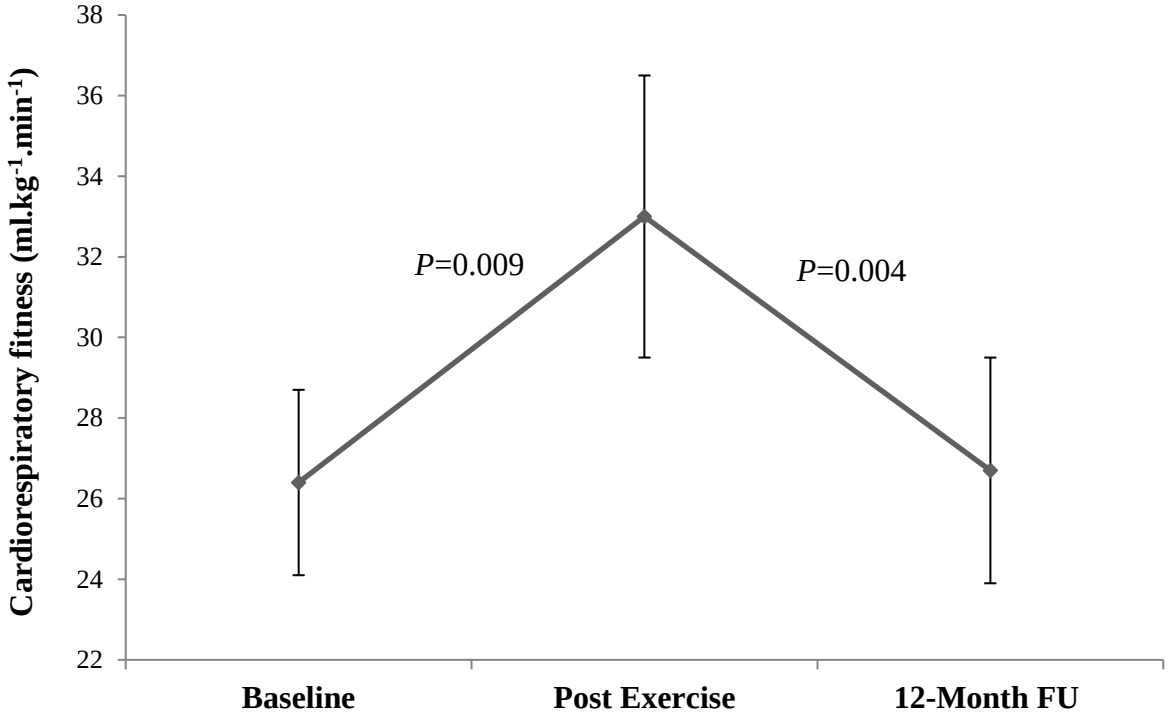


Figure 6.1 Cardiorespiratory fitness of NAFLD patients at baseline, following 16-weeks of structured and supervised exercise training (Post Exercise) and 12 months following (12-Month FU) the cessation of supervised exercise (*P*=0.004).

Table 6.1 Characteristics of NAFLD patients at baseline, following 16-weeks of structured and supervised exercise training (Post Exercise) and 12 months following (12-Month FU) the cessation of supervised exercise.

	Baseline	Post Exercise	12-Month FU	*P* value
Anthropometrics				
Weight (kg)	85.0±4.3	82.5±4.6	84.5±6.5	0.41
BMI (kg/m^2)	30±1	29±1	30±1	0.38
Waist circumference (cm)	101.8±2.7	97.6±3.1	100.6±5.0	0.15
Systolic blood pressure (mmHg)	126±2	124±3	130±5	0.54
Diastolic blood pressure (mmHg)	78±2	78±2	78±4	0.89
Liver Function				
IHTC (%)	23.3±5.9	16.6±4.1	28.4±7.2	0.13
ALT (u/L)	54±12	36±6 †	65±13 ‡	0.02
AST (u/L)	35±6	27±2 †	42±5 ‡	0.01
GGT (u/L)	87±33	63±21†	71±15	0.05
Glucose and Lipid Profile				
Fasting Glucose ($mmol.L^{-1}$)	5.0±0.2	4.8±0.2	5.1±0.2	0.38
Cholesterol ($mmol.L^{-1}$)	5.3±0.4	5.3±0.3	5.7±0.3	0.12
Triglyceride ($mmol.L^{-1}$)	2.0±0.2	1.9±0.2	2.0±0.2	0.87
HDL ($mmol.L^{-1}$)	1.4±0.1	1.4±0.4	1.4±0.1	0.44
LDL ($mmol.L^{-1}$)	3.0±0.3	3.0±0.3	3.2±0.3	0.33
Chol:HDL ratio	3.8±0.3	3.8±0.3	4.0±0.3	0.56
Cardiorespiratory Capacity				
VO_{2peak} ($ml.kg^{-1}.min^{-1}$)	26.4±2.3	33.0±3.5 †	26.7±2.8 ‡	0.004
Brachial Artery Vascular Function				
Flow-Mediated Dilation (%)	5.1±0.8	7.9±0.8 †	5.0±0.5 ‡	0.007
Baseline Diameter (mm)	4.0±0.3	4.0±0.3	4.2±0.3	0.36
Peak Diameter (mm)	4.2±0.4	4.4±0.3	4.4±0.3	0.48
Shear $rate_{AUC}$ ($s^{-1}\times10^3$)	17.4±4.8	16.6±3.3	16.7±3.1	0.83
Time to Peak (s)	64.0±13.6	55.4±10.1	79.4±16.0	0.23
GTN-Mediated Dilation (%)	13.5±1.9	14.6±1.9	14.9±0.7	0.74

† Significantly different from baseline data ($P<0.05$).
‡ Significantly different from post exercise data ($P<0.05$).

6.3.2 Vascular measurements

There were significant changes in FMD across the three time points ($P=0.007$; Figure 6.2). FMD improved from 5.1±0.8% to 7.9±0.8% in response to supervised exercise training ($P=0.004$) and reduced (5.0±0.5%) 12 months following the completion of supervision ($P=0.02$) to a similar level to that observed at baseline (5.1±0.8 vs. 5.0±0.5%; $P=0.95$). There was no difference in GTN mediated dilation between the three time points ($P=0.60$; Table 6.1). In addition, there was no significant difference in

baseline or peak arterial diameter, shear rate or time to peak between assessments (*P*>0.05; Table 6.1).

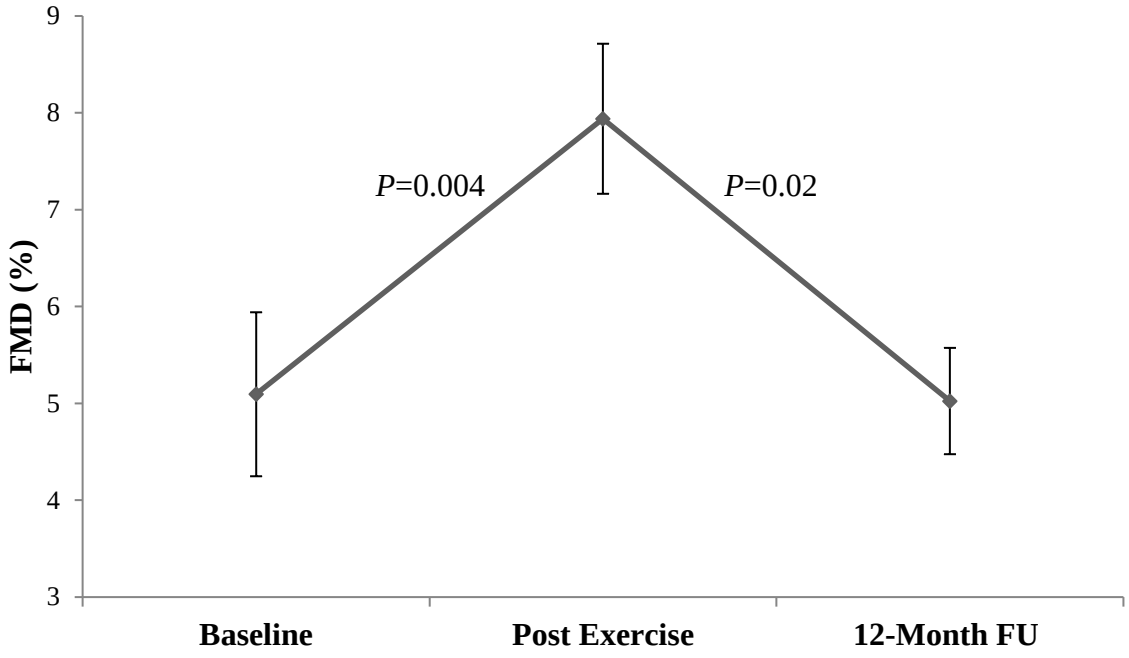


Figure 6.2 FMD in NAFLD patients at baseline, following 16-weeks of structured and supervised exercise training (Post Exercise) and 12 months following (12-Month FU) the cessation of supervised exercise (*P*=0.007).

6.3.3 Biochemical characteristics

Serum liver enzymes all differed across the three time points (*P*<0.05; Table 6.1). ALT (54±12 vs. 36±6u/L; *P*=0.01), AST (35±6 vs. 27±2u/L; *P*=0.06) and GGT (87±33 vs. 63±21u/L; *P*=0.005) all decreased in response to supervised exercise training. Twelve months following the completion of supervised exercise, all serum liver enzymes increased to similar levels observed at baseline (*P*>0.05). Specifically, ALT increased from 36±6 to 65±13u/L (*P*=0.02), AST increased from 27±2 to 42±5u/L (*P*=0.01) and GGT increased from 63±21 to 71±15u/L (*P*=0.08). No significant changes in fasting glucose or blood lipid profiles were evident across the three time points (*P*>0.05; Table 6.1).

6.3.4 Hepatic fat content

No difference in IHTC was evident across the three time points (*P*=0.13; Figure 6.3).

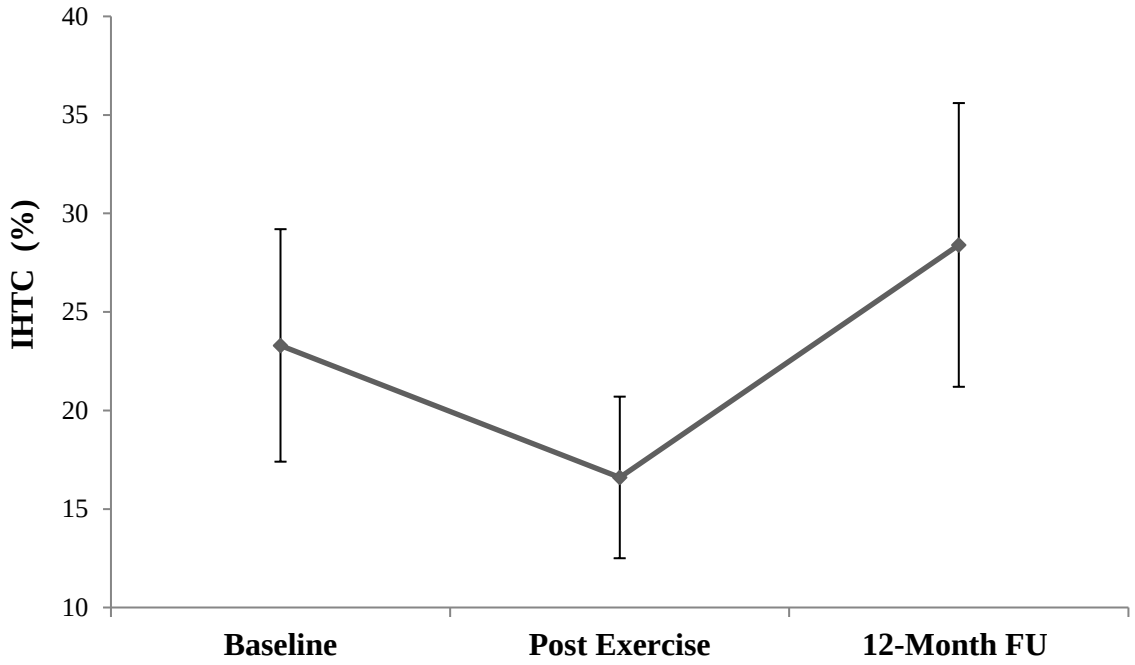


Figure 6.3 IHTC in NAFLD patients at baseline, following 16-weeks of structured and supervised exercise training (Post Exercise) and 12 months following (12-Month FU) the cessation of supervised exercise (*P*=0.13).

6.4 Discussion

The aim of the present study was to investigate whether exercise-induced improvements in endothelial function in NAFLD patients are sustained 12-months following the cessation of supervised exercise. The major finding was that exercise-induced improvements in brachial artery FMD were abolished 12-months following the cessation of supervised exercise. These data suggest that in order to chronically sustain the cardio-protective impact of supervised exercise training on the vasculature of NAFLD patients and subsequently reduce the risk of future cardiovascular events in this high risk group, long term exercise supervision and guidance is required.

This is the first study to examine the effect of exercise training on endothelial function in NAFLD and to conduct a long term follow up assessment to establish whether the

exercise-induce improvements are sustained. Following 16-weeks of supervised moderate intensity exercise training FMD increased by 2.8% in NAFLD patients, however, this improvement was abolished 12-months following the cessation of supervised exercise. Endothelial dysfunction is characterised by a diminished FMD response and is regarded as the earliest manifestation of atherosclerotic disease, which is evident prior to overt clinical manifestations of CVD. Furthermore, FMD is a strong independent prognostic marker of future cardiovascular events (Green *et al.,* 2011a). Therefore, these findings suggest that the cardio-protective benefit and consequential reduced risk of future cardiovascular events observed following supervised exercise training in NAFLD patients is eradicated 12-months after the cessation of supervised exercise.

Although an exercise training follow-up study of this nature on endothelial function has not been previously conducted in any population, Vona *et al.* (2009) demonstrated that exercise-induced improvements in endothelial function are abolished after one month of detraining in CVD patients. This finding elegantly demonstrated that improvements in endothelial function are not chronically sustained following cessation of exercise. The reduction in cardiorespiratory fitness observed 12 month post-intervention suggests that this patient group did not successfully maintain the exercise training regimen and/or did not achieve the optimal exercise dose required to sustain cardiovascular benefits without guided supervision. It is clear from the current data that in order for exercise to induce a sustained therapeutic benefit in NAFLD patients, guided supervision is essential.

Unlike several previous studies (Bonekamp *et al.*, 2008; Johnson *et al.*, 2009; van der Heijden *et al.*, 2010), a statistically significant reduction in IHTC following exercise

training was not observed in the current study. However, it is important to note that the exercise-induced absolute reduction in IHTC of 9% was clinically relevant and, that this improvement was abolished 12-months following cessation of supervised exercise training. Indeed, this trend is supported by the serum liver enzymes data, which demonstrate a significant reduction following exercise that is abolished 12-months after the cessation of supervised exercise. Moreover, this data also supports the notion that supervised exercise is crucial in order to promote sustained long term improvements to cardiovascular and metabolic health in NAFLD patients.

In summary, these novel findings suggest that whilst supervised exercise training is an acute non-pharmacological management strategy capable of improving endothelial function in NAFLD, the cardio-protective impact of supervised exercise training is not chronically sustained following the cessation of supervised exercise. In order to sustain the exercise-induced improvements in the endothelial function of NAFLD patients and subsequently reduce the prevalence of heart disease and stroke in this high risk group, long term exercise supervision and guidance is required.

Chapter 7

THE IMPACT OF NAFLD ON NITRIC OXIDE MEDIATED CUTANEOUS MICROVASCULAR FUNCTION AND THE EFFECTS OF SUPERVISED EXERCISE TRAINING COMPARED WITH CONVENTIONAL CLINICAL CARE

7.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is primarily a hepatic disorder, characterised by excess triglyceride accumulation in the liver and is associated with an increased risk of cardiovascular disease (CVD) (Stefan *et al.*, 2008). Endothelial dysfunction is an early and integral event in CVD and a strong predictor of future cardiovascular risk in both symptomatic and asymptomatic individuals (Green *et al.*, 2011a). Recent cross sectional studies have shown that NAFLD patients exhibit impaired endothelial function in conduit arteries (Villanova *et al.*, 2005; Senturk *et al.*, 2008). In addition, Chapter 5 illustrated that a structured exercise intervention improves conduit artery endothelial function in this group who are at high risk of cardiovascular events (Targher *et al.*, 2010), suggesting that exercise enhances cardiovascular health in NAFLD patients.

Whilst conduit artery endothelial function reflects macrovascular atherosclerotic risk, cutaneous vasodilator function reflects generalised microvascular function and provides a translational model to investigate pre-clinical disease (Holowatz *et al.*, 2008). Cutaneous microvessel dysfunction correlates with coronary artery endothelial dysfunction (Shamim-Uzzaman *et al.*, 2002; Bonetti *et al.*, 2004; Khan *et al.*, 2008) and several cardiovascular risk factors including obesity (de Jongh *et al.*, 2004), hypertension (Carberry *et al.*, 1992; Rizzoni *et al.*, 2003), hypercholesterolaemia (Khan *et al.*, 1999), and type 2 diabetes (Sokolnicki *et al.*, 2007); all of which frequently manifest in NAFLD patients (Ekstedt *et al.*, 2006) . Although, hepatic microvascular dysfunction is well documented in NAFLD (Ijaz *et al.*, 2003), no previous research has investigated the cutaneous microcirculation as a model of preclinical microvascular disease in NAFLD patients.

Previous research has demonstrated that endothelium-derived nitric oxide (NO)-mediated vasodilator function in cutaneous microvessels is related to age and fitness in healthy individuals and that exercise training enhances microvascular function by up-regulating NO (Black *et al.*, 2008b) in older subjects. Nevertheless, research investigating the impact of exercise training on cutaneous NO-mediated microvascular function in diseased populations is scant. The aims of the present study were therefore to (i) describe the NO-mediated cutaneous vasodilator response to local heating in NAFLD, versus matched controls; and (ii) utilise a randomised controlled intervention to investigate NO-mediated cutaneous microvascular responses to supervised exercise training compared with conventional clinical care. It was hypothesised that (i) the NO-mediated cutaneous vasodilator response to local heating would be impaired in NAFLD patients when compared with matched controls and (ii) supervised exercise training would induce a greater improvement in the NO-mediated cutaneous vasodilator response to local heating in NAFLD when compared with conventional clinical care.

7.2 Methods

7.2.1 Participants

Thirteen sedentary NAFLD patients (7 males, 6 females, 50±3yrs, BMI 31±1kg/m^2) and seven matched controls (3 males, 4 females, 48±4yrs, BMI 30±2 kg/m^2) were recruited. All NAFLD patients were normocholesterolaemic and 3 were taking anti-hypertensive medication (β-blocker *n*=1, Calcium channel blocker *n*=2). Medications were not altered during the course of the study. None of the controls were taking any prescribed medication. Pre-menopausal women (*n*=2) were tested during the early follicular phase of the menstrual cycle, defined as day 1-7.

7.2.2 Research Design

A series of physiological measurements were performed at baseline in all participants. Following this, all NAFLD patients were randomly assigned to either 16-weeks of supervised and structured exercise training (*n*=6, 45±5yrs, BMI 31±1kg/m^2) or to 16-weeks of conventional care (*n*=5, 51±3yrs, BMI 30±2kg/m^2) (for details of respective interventions, please refer to *General Methodology, Chapter 3*). Eleven NAFLD patients completed the 16-week intervention period, following which baseline measurements were repeated (Figure 7.1).

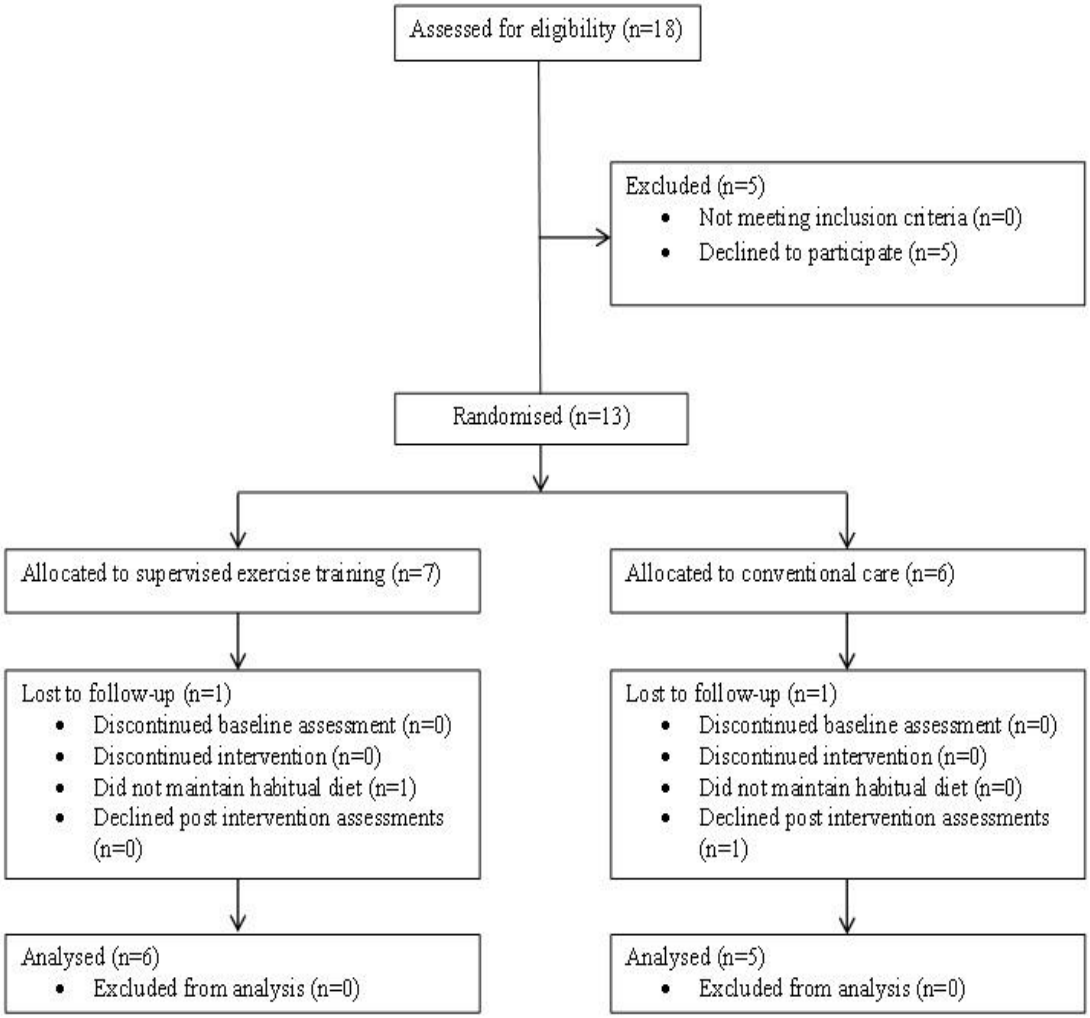


Figure 7.1 Participant flow diagram

7.2.3 Experimental Protocol

All participants reported to the laboratory to undertake a series of baseline measurements including anthropometric assessment, a blood sample, an assessment of cutaneous NO vasodilator function, a cardiorespiratory fitness test and magnetic resonance imaging (MRI) with proton magnetic resonance spectroscopy (^{1}H-MRS) to determine abdominal fat deposition and intrahepatocellular triglyceride content (IHTC). All participants were studied at the same time of day to control for the impact of circadian variation. Measurements were performed following an overnight fast, 12-hour abstinence from caffeine and 24-hour abstinence from alcohol and strenuous exercise.

7.2.4 Assessment of cutaneous NO vasodilator function

7.2.4.1 Microdialysis fibre instrumentation

All intradermal microdialysis assessments were performed in a quiet, temperature controlled laboratory. Upon arrival, participants were instrumented and cannulation for microdialysis probe insertion was undertaken (~15-min). Once seated comfortably in a custom-designed bed, the right arm was supinated and supported for insertion of microdialysis fibres. The insertion sites were marked on the skin and cold packs were applied as a form of local anaesthesia. Two 21-gauge needles were inserted ~5cm apart from one another and ~0.3-1.0mm beneath the epidermal surface so to enable threading and placement of two microdialysis fibres (Linear 30, CMA Microdialysis Ltd, Stockholm, Sweden), containing 10mm long 6 kDa membranes. The needles were then removed and the embedded fibres were perfused with saline solution at a rate of 5 μl.min^{-1} using a microinfusion pump (Model 11 plus, Harvard Apparatus, MA, USA) (Figure 7.2).

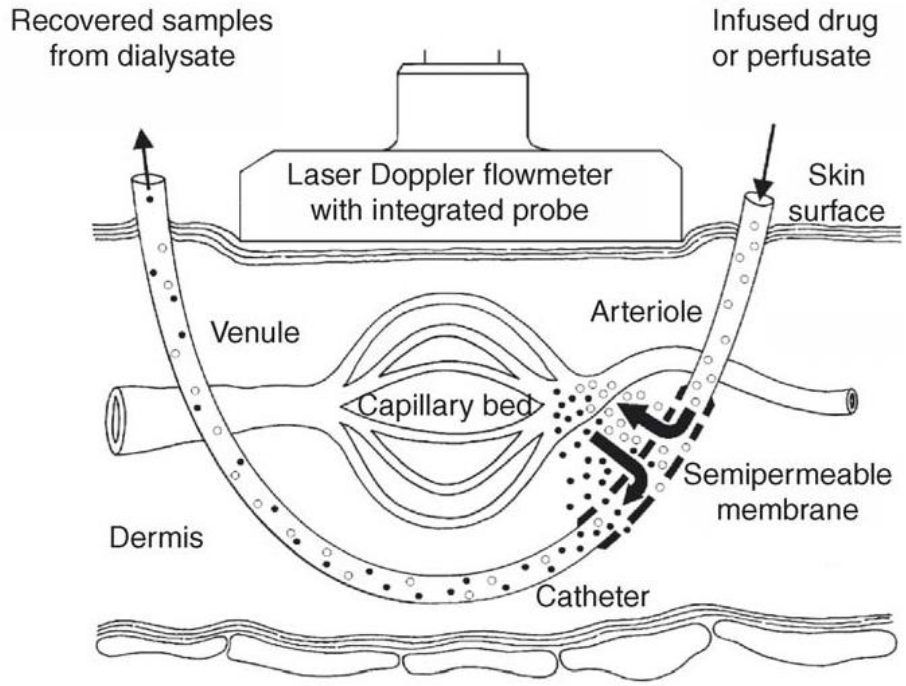


Figure 7.2 The microdialysis fibre is placed ~0.3-1.0mm beneath the epidermal surface so that it is positioned within the dermal space. The 10mm semi-permeable membrane allows infusion of agonists directly into the vascular bed to be investigated. A laser Doppler probe is positioned above it to monitor skin blood flow (SKBF) (Cracowski *et al.*, 2006).

Following this, integrated laser Doppler probes (Model 413, Periflux 5001 System, Perimed AB, Sweden) combined with local heating disks (Perimed 455, Stockholm, Sweden) set at 33°C were placed above both embedded microdialysis fibres (Figure 7.2). Laser Doppler flowmetry uses a laser diode to emit a monochrome light at a penetrative depth of approximately $1mm^3$. The incident monochrome light reflects off moving blood cells causing a shift in the returning wavelength (Doppler effect) from which estimates of cutaneous blood flux (the concentration of red blood cells x their velocity) can be made. Consequently, LDF enables sensitive and quantifiable detection of relative changes in skin blood flow (SKBF) in response to a given stimulus.

7.2.4.2 Physiological NO-mediated vasodilatation

Following a ~90-min equilibration period, the skin surrounding both microdialysis probes was gradually heated, using local heating disks, from 33 to 42°C at a rate of

0.5 °C per 2.5-min (45-min). Thereafter, both sites were continuously heated at 42°C for a further 30-min. This gradual heating protocol was used to minimise the impact of heating on axon reflexes, which are less NO-mediated than slow heating component responses (Minson *et al.*, 2001; Houghton *et al.*, 2006). Saline solution was infused throughout the protocol in one probe and L-N^G-monomethyl arginine (L-NMMA; 10mM, 5 μl min^{-1}, Clinalfa, CalBiochem) infused through the second probe, from 30-min prior to the onset of heating. Sodium nitroprusside (SNP, 56mM, Mayne Pharma, Warwickshire, UK), a potent NO donor, was infused at the end of the protocol for 30-min (Minson *et al.*, 2002; Cracowski *et al.*, 2006) to initiate peak vasodilatation (Figure 7.3).

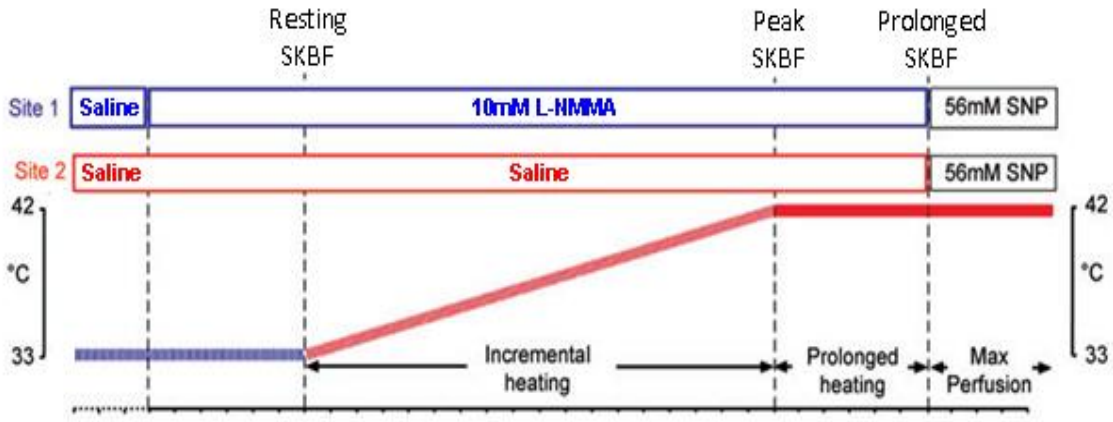


Figure 7.3 Schematic of the physiological (localised heating) NO-mediated vasodilatation protocol.

7.2.4.3 Assessment of forearm skin blood flow

To obtain an index of SKBF, cutaneous red cell flux was measured by placing integrated laser-Doppler probes, each consisting of a seven-laser array, above each microdialysis fibre. The laser-Doppler probe signals were continuously monitored via an online software chart recorder (PSW, Perimed, Sweden). At each designated study time point (2.5 minute intervals), SKBF was assessed by averaging laser-Doppler flux (LDf), measured in perfusion units (PU), over a stable 30-second period. These data

were subsequently converted to cutaneous vascular conductance (CVC), calculated as LDf/MAP (PU $mmHg^{-1}$), where MAP was derived from contemporaneous automated blood pressure measures (Dinamap; GE Pro 300V2) in the contralateral arm. Values were then expressed relative to the maximal CVC achieved during infusion of 56mM SNP at 42 °C, as %CVCmax, the preferred method of data expression adopted in the literature (Cracowski *et al.*, 2006).

7.2.4.4 Data Reduction

Data during the incremental heating were calculated and presented at each temperature (every 0.5°C from 33°C to 42°C) for both the saline and L-NMMA microdilaysis sites. The contribution of NO was calculated by subtracting individual L-NMMA data from saline data collected contemporaneously. Data are therefore presented as the NO contribution to microvascular red cell flux or SKBF. Summary data were calculated for resting SKBF (measured at 33°C following trauma cessation of ~90 min); Peak SKBF (measured at 42°C following the incremental heating protocol); and Prolonged SKBF (measured following 30 min of continuous heating at 42°C).

7.2.5 Statistical Analysis

All differences in baseline characteristics between groups (NAFLD and controls) were compared using independent *t*-tests. Data that was not normally distributed was logarithmically transformed. Data are presented in the text as mean±SE and exact *P* values are cited (values of *P* of "0.000" provided by the statistics package are reported as "<0.0005"). All data were analysed using the SPSS 17.0 (SPSS, Chicago, Illinois) software.

Cross-sectional comparison between NAFLD patients and matched controls: To ensure successful increase in NO production with the local heat stimulus and successful blockade of NO production, saline and L-NMMA data were individually compared using a two-way repeated measures analysis of variance (ANOVA) (site vs. temperature). For the comparison of NAFLD versus controls the incremental heating data was analysed using a two-way factor (group vs. temperature) repeated measures ANOVA. Statistically significant interactions between these two factors were followed-up with subsequent pairwise comparisons. The summary data for the comparison between NAFLD and controls was analysed using a between groups ANOVA.

Effect of exercise training vs. conventional care: A two-way repeated measures ANOVA (site vs. temperature), as described above, was employed to ensure successful increase in NO production and blockade respectively. For the comparison of the exercise versus the conventional care intervention delta (Δ) change from pre-intervention was calculated and analysed using analysis of covariance (ANCOVA) with pre-exercise data as a covariate. Similarly, the summary data for the effect of exercise versus conventional care data was analysed using ANCOVA with pre-exercise data as a covariate.

7.3 Results

7.3.1 Cross-sectional comparison between NAFLD and matched controls

NAFLD patients and controls were well matched for age, BMI and fitness, however, NAFLD patients exhibited an increased waist circumference (106±2 vs. 99±2cm; *P*=0.05; Table 7.1). There was a ~7 fold greater deposition of IHTC (*P*=0.0004) in NAFLD patients and a suggestion of increased abdominal VAT (*P*=0.05). Serum liver enzymes were elevated in NAFLD patients (*P*<0.01) and HDL reduced (1.3±0.1 vs. 1.5±0.1mmol.L^{-1}; *P*=0.05) when compared with controls.

Table 7.1 Baseline characteristics of NAFLD and control participants.

	NAFLD	**Controls**	***P* Value**
Anthropometrics			
N (m/f)	13	7	N/A
Age (yrs) $^{+}$	48(13)	48(15)	0.60
Weight (kg)	88.6±3.5	84.4±4.0	0.46
BMI (kg/m^2)	31±1	30±2	0.64
Waist circumference (cm)	106±2	99±2	0.05*
Systolic blood pressure (mmHg)	128±3	128±4	0.98
Diastolic blood pressure (mmHg)	78±2	80±3	0.54
Hepatic and Body Fat Deposition			
IHTC (%)$^{++}$	28.8±5.3	3.2±0.7	0.0004*
Abdominal VAT (l)	4.8±0.4	3.8±0.7	0.05*
Abdominal SAT (l) $^{++}$	8.9±0.8	8.5±1.4	0.81
Liver Enzymes			
ALT (u/L) $^{++}$	72±12	29±5	0.002*
AST (u/L) $^{++}$	44±6	25±3	0.02*
GGT (u/L)	75±11	32±6	0.01*
Glucose and Lipid Profile			
Fasting Glucose (mmol.L^{-1})	5.0(0.9)	4.9(0.8)	0.60
Cholesterol (mmol.L^{-1})	5.6±0.2	5.3±0.3	0.36
Triglyceride (mmol.L^{-1})	2.5±0.4	1.7±0.3	0.23
HDL (mmol.L^{-1})	1.3±0.1	1.5±0.1	0.05*
LDL (mmol.L^{-1})	3.3±0.2	3.6±0.6	0.53
Chol:HDL ratio $^{+}$	5.0(1.0)	4.0(1.0)	0.10
Cardiorespiratory Capacity			
VO_{2Peak} (ml/kg^{-1}/min^{-1}) $^{+}$	22.8(15.4)	27.8(19.2)	0.22

SAT- Subcutaneous adipose tissue, VO_{2peak}- Peak oxygen uptake
$^{+}$ Non-parametric data presented as median (interquartile range).
$^{++}$ Logarithmically transformed data.
* Significant difference between NAFLD vs. controls (*P*<0.05).

7.3.2 Incremental Heating

In response to local heating, %CVCmax steadily and significantly increased at the microdialysis site perfused with saline and the site perfused with L-NMMA in all participants (P<0.0005) (Figure 7.4). However, at the L-NMMA site %CVCmax was significantly decreased in both NAFLD patients and controls (P<0.0005; Figure 7.4), suggesting that the response to local heating is partially mediated by the NO dilator system in both groups. A significant microdialysis site x temperature interaction was evident (*P*<0.001, Figure 7.4); Pairwise comparisons revealed significant differences between the saline and L-NMMA site from 39.5-42ºC and subsequently when the heating stimulus remained at 42°C for 5 minutes (peak) in NAFLD patients and from 40-42ºC and at peak SKBF in controls (*P*<0.05).

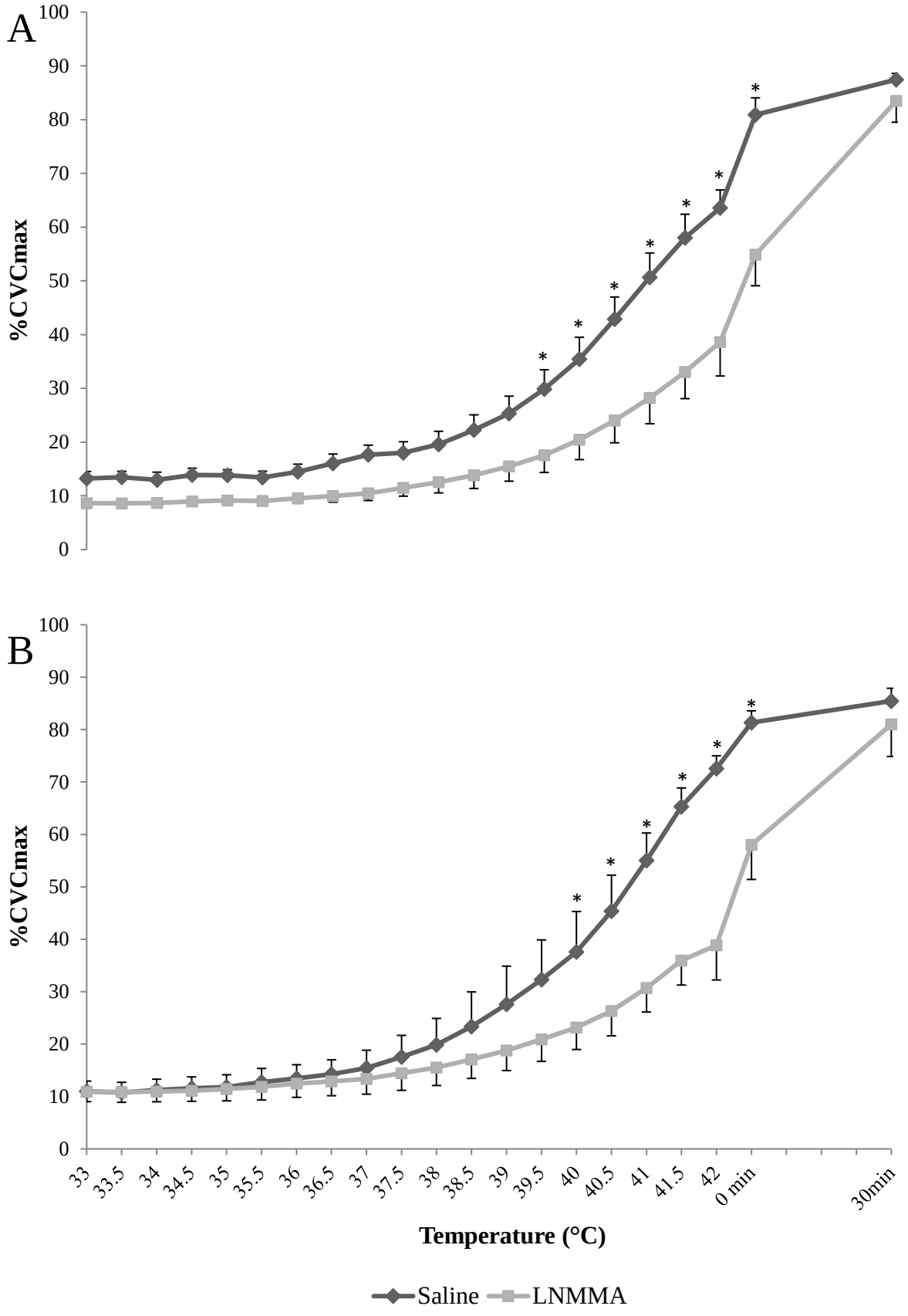


Figure 7.4 The effect of incremental and prolonged heating on SKBF in the saline and L-NMMA microdialysis sites in NAFLD patients (A) and matched controls (B).
0 min-peak SKBF, 30 min-prolonged SKBF.

7.3.3 Nitric Oxide contribution to incremental heating

There was no significant interaction between NAFLD and controls in the saline or L-NMMA microdialysis sites (P>0.13), and similarly, NO contribution (saline %CVCmax minus L-NMMA %CVCmax) did not differ between NAFLD and controls (P=0.47; Figure 7.5).

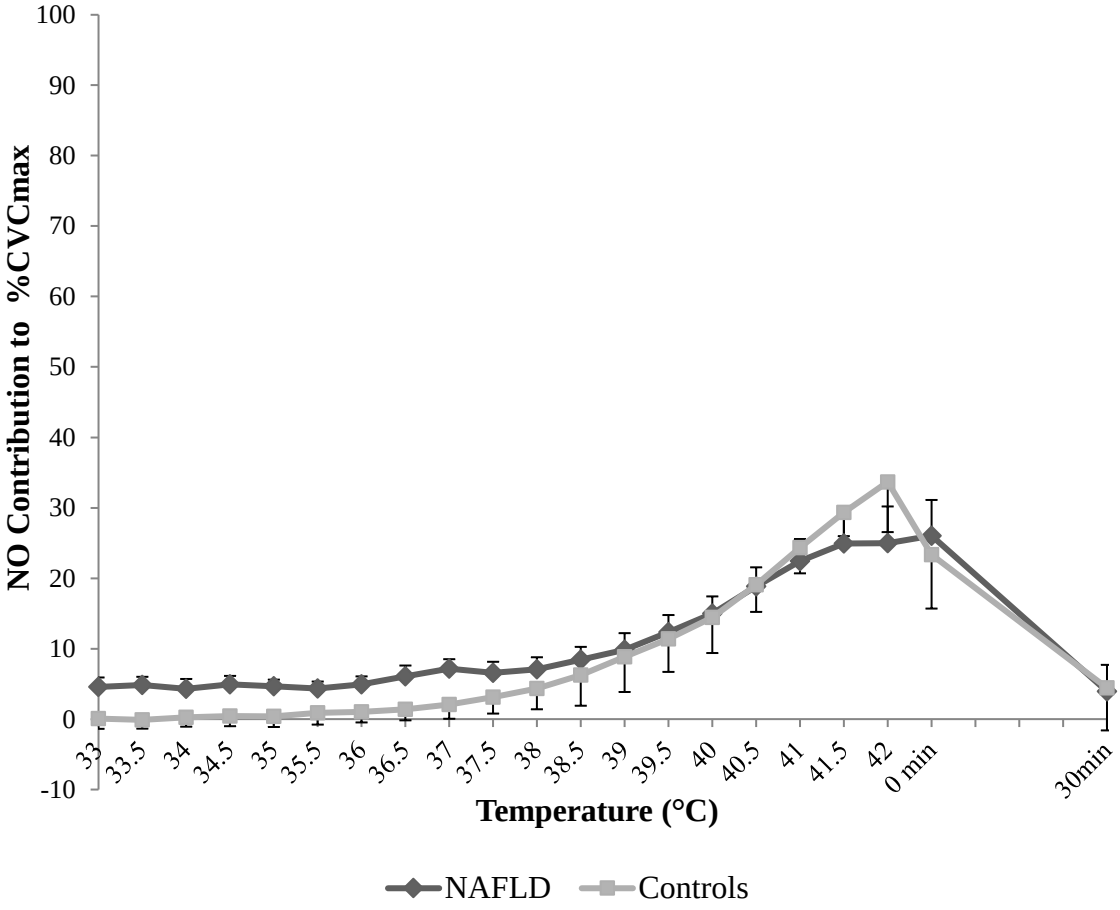


Figure 7.5 The effect of incremental and prolonged heating on the contribution of NO (saline %CVCmax minus L-NMMA %CVCmax) in NAFLD patients and matched controls. 0 min-peak SKBF, 30 min-prolonged SKBF.

When the data is expressed in summary fashion, it remains evident that there were no significant differences in the contribution of NO to resting, peak or prolonged SKBF in NAFLD patients compared to controls (P>0.05; Figure 7.6), and that the % contribution of NO to peak vasodilatory response was similar between groups at ~30%.

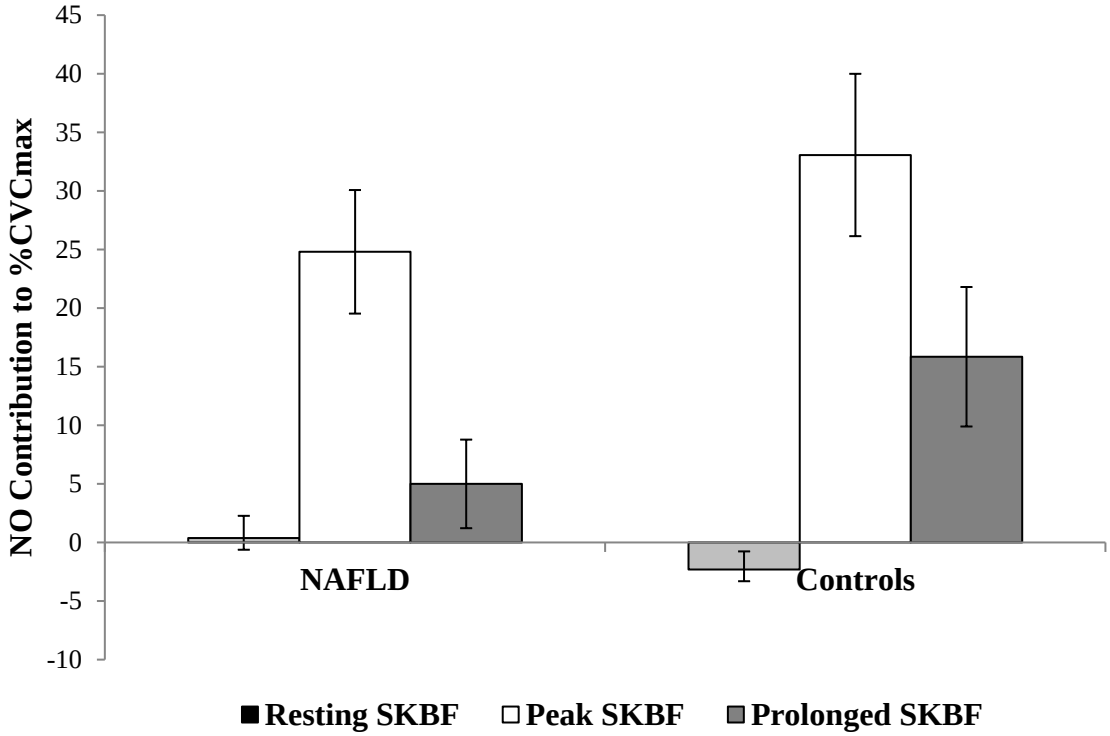


Figure 7.6 Mean NO contribution (saline %CVCmax minus L-NMMA %CVCmax) to resting, peak (42°C) and prolonged (30 mins at 42°C) SKBF in the NAFLD and matched controls.

7.3.4 The effect of supervised exercise training vs. conventional care

The characteristics of all NAFLD patients allocated to each group are presented in Table 7.2. NAFLD patients who were randomised to supervised exercise training demonstrated 94% compliance to exercise sessions. Following the intervention, cardiorespiratory fitness (10.1±2.2 vs. -0.9±2.5; *P*=0.01) improved and ALT (-34±6 vs. -12±7; *P*=0.04) and AST (-16±2 vs. -4±3; *P*=0.01) reduced in the exercise-trained group compared to the conventional care group. However, there was no difference between the effect of exercise training and conventional care on BMI, waist circumference, IHTC, abdominal VAT, plasma lipid profile or plasma glucose concentration.

Table 7.2 Changes in the characteristics of NAFLD patients following supervised exercise training (*n*=6) and conventional clinical care (*n*=5).

	Pre Ex	Post Ex	Δ Ex	Pre CC	Post CC	Δ CC	*P* value
Anthropometrics							
Weight (kg)	92.9±4.4	90.8±4.5	-2.3±0.9	84.1±7.4	83.2±7.9	-0.7±0.9	0.27
BMI (kg/m^2)	31±1	30±1	-0.8±0.3	30±2	30±2	-0.8±0.4	0.98
Waist circumference (cm)	109±3	103±3	-5.5±2.5	104±4	101±4	-3.4±2.7	0.61
Systolic blood pressure (mmHg)	132±2	127±3	2±4	124±7	122±5	-3±4	0.43
Diastolic blood pressure (mmHg)	81±2	78±2	-2±2	75±4	72±1	-6±2	0.11
Body Fat Deposition							
IHTC (%)	34.4±11.0	18.7±4.7	-13.0±3.0	24.4±4.6	20.5±4.7	-6.5±3.3	0.18
Abdominal VAT (L)	5.8±0.6	5.4±0.8	-0.5±0.3	4.0±0.5	4.2±0.4	0.3±0.4	0.09
Abdominal SAT (L)	9.2±1.3	8.9±1.4	-0.4±0.3	8.2±0.9	8.4±1.1	0.2±0.4	0.25
Liver Enzymes							
ALT (u/L)	67±16	36±4	-34±6*	80±23	64±16	-12±7*	0.04
AST (u/L)	42±10	29±3	-16±2*	50±11	44±6	-4±3*	0.01
GGT (u/L)	59±11	41±6	-29±11	109±13	80±16	-16±12	0.51
Glucose and Lipid Profile							
Fasting Glucose ($mmol.L^{-1}$)	4.9±0.1	4.9±0.2	-0.1±0.2	5.5±0.5	5.7±0.4	0.3±0.2	0.25
Cholesterol ($mmol.L^{-1}$)	5.5±0.3	5.2±0.3	-0.3±0.2	5.7±0.3	5.6±0.5	-0.1±0.2	0.51
Triglyceride ($mmol.L^{-1}$)	2.0±0.2	1.8±0.2	-0.6±0.2	3.5±0.9	2.5±0.4	-0.6±0.2	0.81
HDL ($mmol.L^{-1}$)	1.3±0.1	1.3±0.1	0.03±0.04	1.2±0.1	1.2±0.1	-0.04±0.04	0.26
LDL ($mmol.L^{-1}$)	3.3±0.3	3.1±0.2	-0.1±0.1	2.8±0.3	3.5±0.4	0.3±0.2	0.10
Chol:HDL ratio	4.2±0.2	3.8±0.2	-0.6±0.3	5.0±0.3	5.0±0.3	0.3±0.3	0.09
Cardiorespiratory Capacity							
VO_{2peak} ($ml.kg^{-1}.min^{-1}$)	26.8±3.2	36.5±3.6	10.1±2.2*	22.4±1.1	25.0±2.6	-0.9±2.5*	0.01

Ex- Exercise group, CC- Conventional care group, SAT- Subcutaneous adipose tissue, VO_{2peak}- Peak oxygen uptake. Data are presented as mean ± SE. Delta (Δ) change from pre-intervention following adjustment for pre-intervention values. * Significant difference between ΔEx and ΔCC ($P<0.05$).

7.3.5 Post Intervention response to incremental heating

Pre and post-exercise training, SKBF significantly increased in response to local heating at both the saline and L-NMMA sites (*P*>0.0005; Figure 7.7). There was however, a significant interaction (site x temperature) between the saline and L-NMMA microdialysis sites both pre and post exercise training (*P*>0.0005; Figure 7.7), with significant differences between sites evident from 39-42ºC pre-exercise and from 36.5-42ºC and at peak SKBF post-exercise. Similarly, there was a significant interaction (site x temperature) between the saline and L-NMMA microdialysis sites both pre and post conventional care (*P*>0.0005; Figure 7.8). Pairwise comparisons revealed differences from 39-42°C and at peak SKBF pre-conventional care and 40-42°C and at peak SKBF post-conventional care.

7.3.6 Post intervention nitric oxide contribution to incremental heating

When data is expressed as NO contribution to %CVCmax, there was a significant interaction (intervention vs. temperature) in NO contribution during incremental heating from 37.5°C to 42°C in the exercise trained group (P=0.01; Figure 7.9), but no interaction in the conventional care group (*P*=0.99; Figure 7.9).

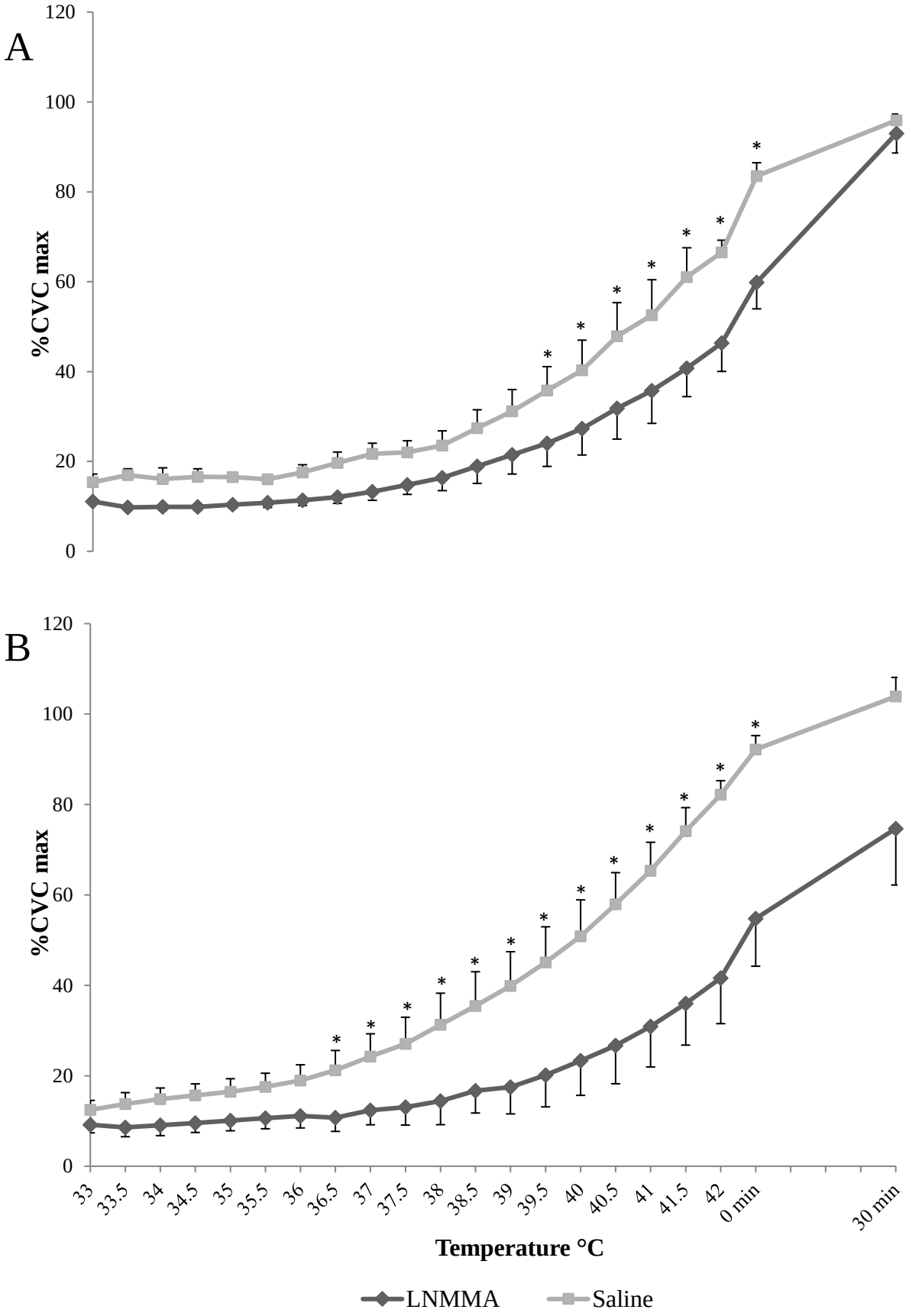


Figure 7.7 The effect of incremental and prolonged heating on SKBF in the saline and L-NMMA microdialysis sites pre (A) and post exercise (B) *Significant difference between L-NMMA and saline sites (P<0.05). 0 min-peak SKBF, 30 min-prolonged SKBF.

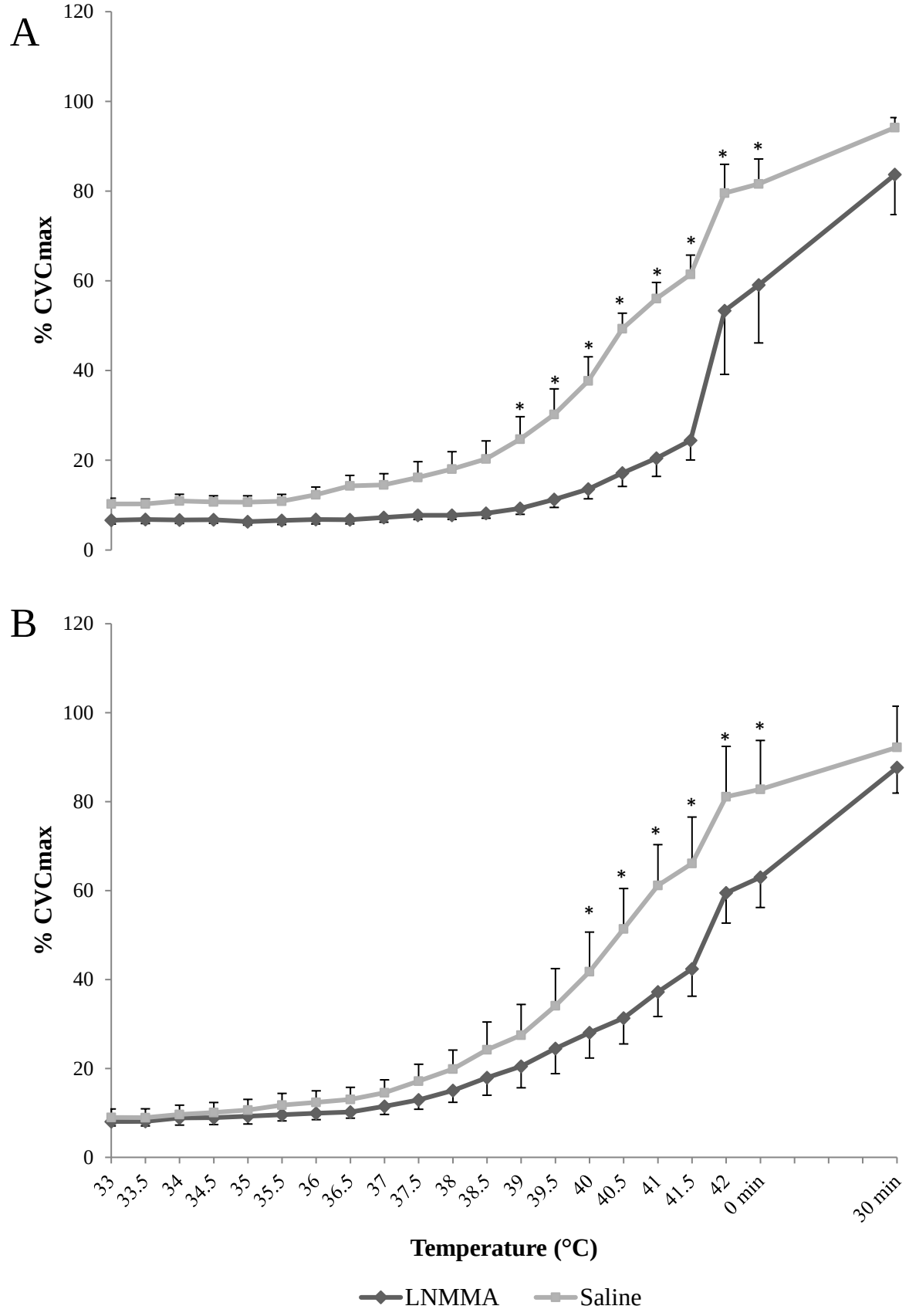
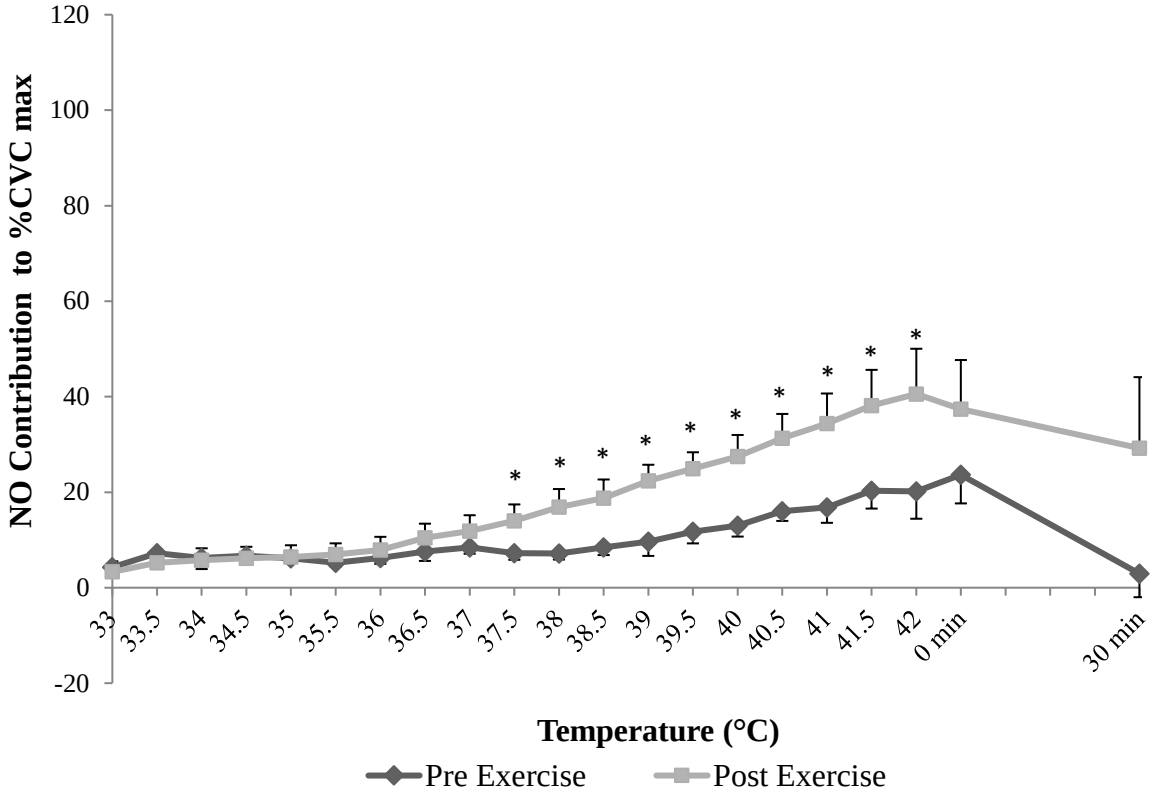


Figure 7.8 The effect of incremental and prolonged heating on SKBF in the saline and L-NMMA microdialysis sites pre (A) and post conventional care (B) *Significant difference between L-NMMA and saline sites (P<0.05). 0 min-peak SKBF, 30 min-prolonged SKBF.



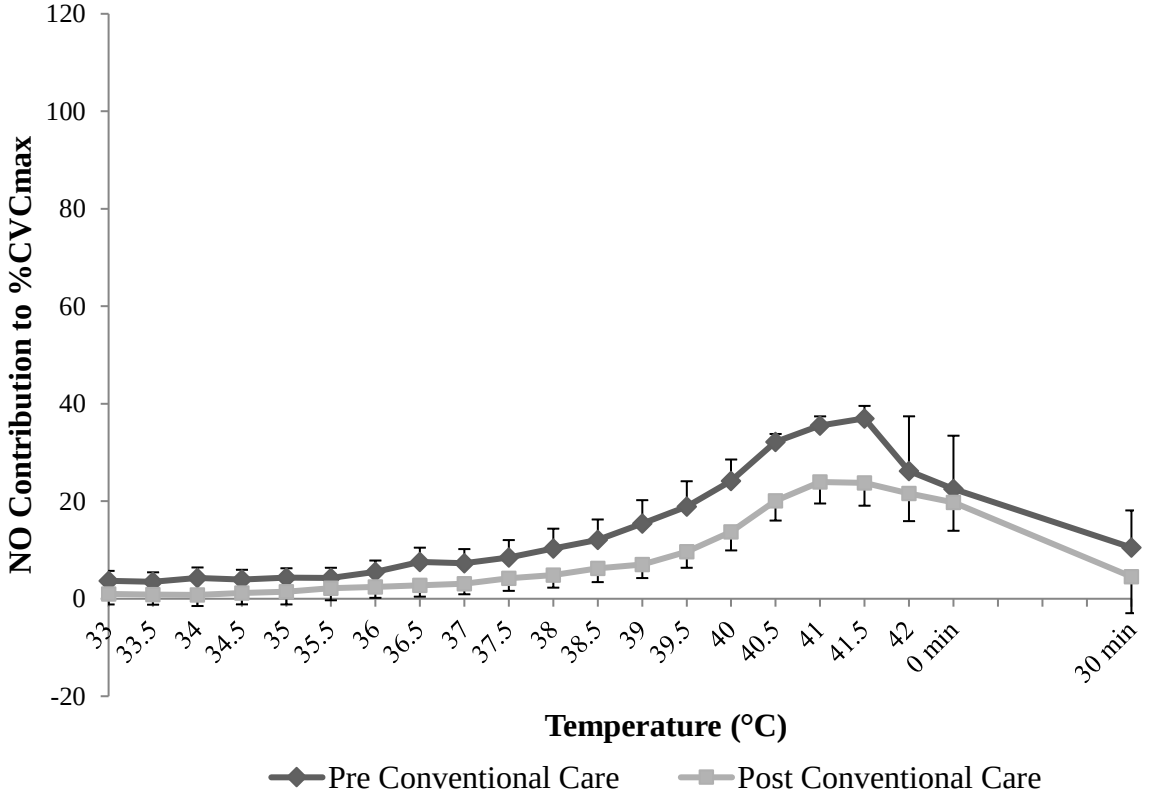


Figure 7.9 The effect of exercise and conventional care on the NO contribution (saline %CVCmax minus L-NMMA %CVCmax) to SKBF in response to incremental heating. *Significant difference between pre and post exercise (P<0.05). 0 min-peak SKBF, 30 min-prolonged SKBF.

7.3.7 Δ Change in response to incremental heating

There was no significant interaction between exercise and convention care in the saline or L-NMMA microdialysis sites (P>0.13). Nevertheless, a significant interaction was evident for NO contribution (P=0.01). Pairwise comparisons revealed that the exercise group demonstrated a significantly greater NO response to incremental heating from 37.5°C (11.0 %CVCmax) to 42°C (33.6 %CVCmax; P<0.05; Figure 7.10).

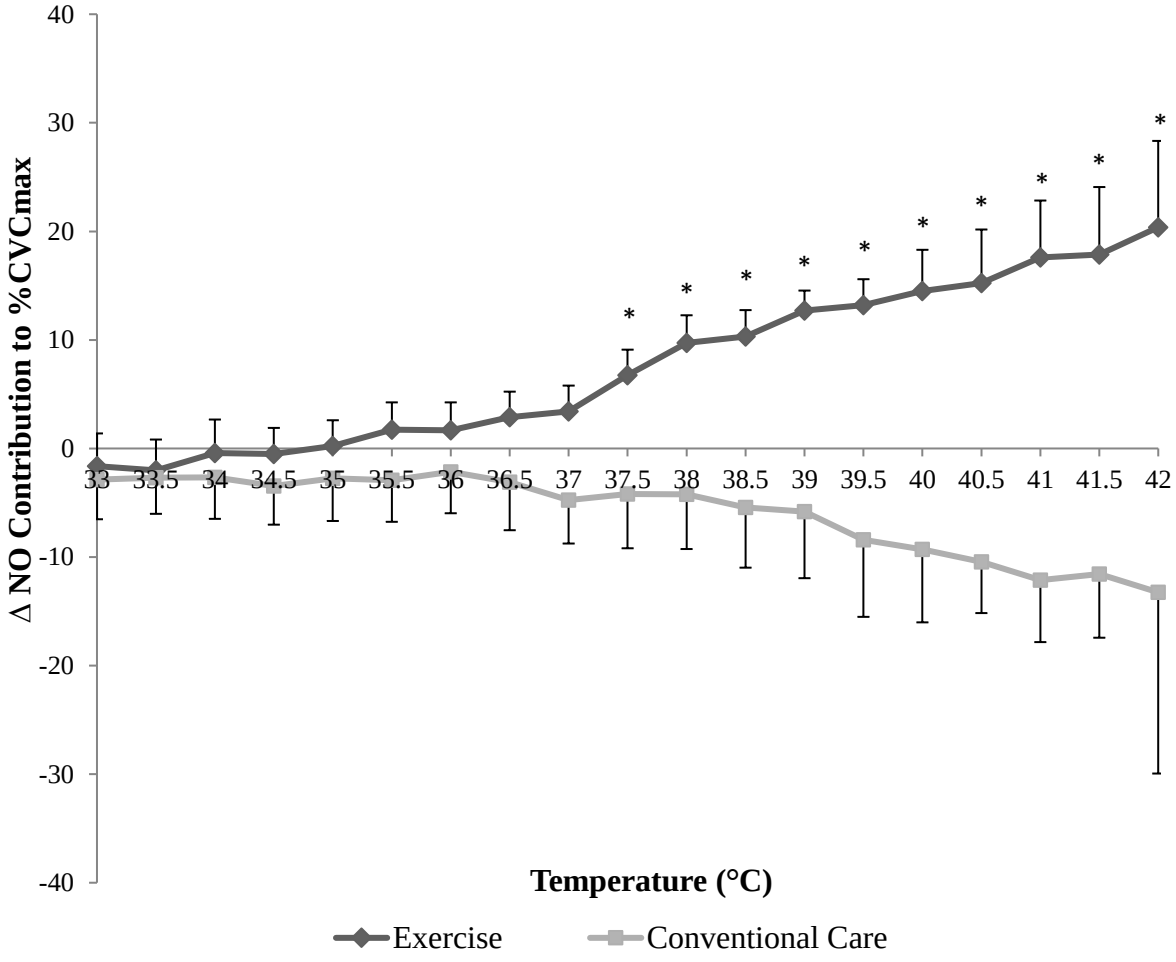


Figure 7.10 The change in the contribution of NO (saline %CVCmax minus L-NMMA %CVCmax) to SKBF in response to incremental heating following the exercise and conventional care intervention. *Significant difference between exercise and conventional care (P<0.05). 0 min-peak SKBF, 30 min-prolonged SKBF.

The contribution of NO to SKBF at rest (3.3±2.5 vs. -5.8±2.7 %CVCmax; *P*=0.04; Figure 7.11) and at peak heating (42°C) (22.5±9.7 vs. -18.0±8.1 %CVCmax; *P*=0.05; Figure 7.11) was significantly greater following exercise-training when compared with conventional care. There were no significant differences between groups in the contribution of NO to SKBF following prolonged heating at 42°C (Figure 7.11).

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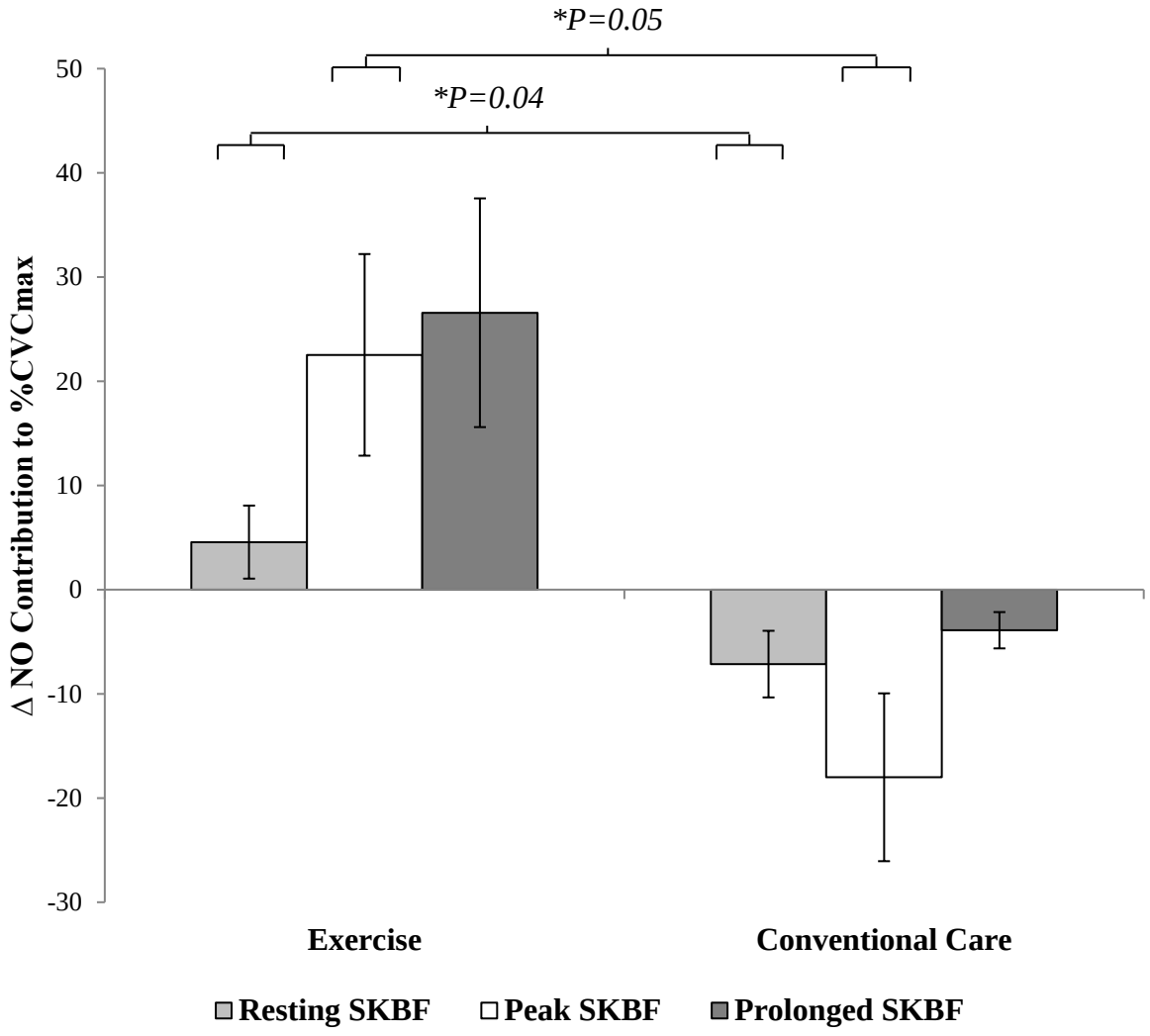

Figure 7.11 The change in mean NO contribution to resting, peak (42°C) and prolonged (30 mins at 42°C) SKBF in the NAFLD patients following the exercise and conventional care intervention.

7.4 Discussion

The aim of this study was to investigate cutaneous microvascular function in NAFLD patients. The major findings were that (i) cutaneous NO-mediated microvascular function was similar in NAFLD when compared with age, BMI and fitness matched controls; and (ii) supervised exercise training induced an increase in NO contribution, indicative of greater NO bioavailability, during incremental heating when compared with conventional care. These findings suggest, for the first time that supervised exercise training is an effective management strategy capable of improving NO-mediated cutaneous microvascular function in NAFLD patients.

This is the only study to explore cutaneous microvessel function in NAFLD. The current results report no differences in the cutaneous microvasculature between NAFLD patients (prior to randomised intervention) and matched control individuals. A likely explanation for this is that NAFLD patients were compared with sedentary and obese controls. Previous studies have suggested that cutaneous microvessel dysfunction in healthy individuals is explained by age (Black *et al.*, 2008b) and/or fitness (Black *et al.*, 2008b). Similarly, obesity has also been shown to impair cutaneous microvessel function in healthy individuals (de Jongh *et al.*, 2004). Therefore, as NAFLD and controls were obese sedentary individuals, it is plausible that microvascular dysfunction was present in both NAFLD and controls due to similar lifestyle practice. This finding is corroborated by Sokolnicki *et al.* (2007) who reported no difference in the relative contribution of NO to SKBF during local heating between type 2 diabetics (a patient group that shares the common pathology of insulin resistance) and matched controls.

Previous research suggests that exercise training is an effective treatment strategy to improve cutaneous microvessel function in young (Wang, 2005) and older sedentary

individuals (Black et al., 2008) as well as postmenopausal women (Hodges *et al.*, 2010). The current study utilised a randomised control intervention, whereby NAFLD patients either underwent a supervised moderate intensity exercise training program or a conventional care intervention which adhered to the current clinical treatment guidelines for patients diagnosed with NAFLD (weight loss and increased physical activity). The supervised exercise intervention mediated a greater NO response to the incremental heating protocol than conventional care. Specifically, the exercise intervention mediated an up-regulation of NO during heating, characterised by a greater contribution of NO and subsequent increase in SKBF; which were only apparent as temperature increased above 37.5°C. This finding of increased NO contribution with exercise training corroborates previous data in sedentary elderly individuals (Black *et al.*, 2008b) and suggests that up-regulation of NO in response to incremental local heating in the microvasculature may be a function of enhanced cardiorespiratory fitness. Furthermore, the NO contribution to SKBF at rest was greater following supervised exercise training when compared with conventional care. To our knowledge this also is a novel finding, which indicates exercise training may increase the tonic release of NO and thus promote an improved basal microvascular function. In the current study, improved NO-mediated microvascular function was associated with enhanced endurance capacity, a finding consistent with that of Black *et al*. (2008b). The importance of changes in fitness status are also evidenced by the observation that no changes were found in microvascular function following 6-months of aerobic exercise training in type 2 diabetic patients that exhibited no improvement in cardiorespiratory fitness (Middlebrooke *et al.*, 2006).

Whilst the current data suggest that exercise training up-regulates the NO contribution to local heating, peak vasodilator capacity remains similar following the different

treatment regimes. Following conventional care, peak vasodilator capacity was maintained despite an apparent reduction in the contribution of NO. Therefore, it is likely that compensatory vasodilator mechanisms (such as prostacyclins) exist which preserve heat-induced vasodilator responses in the presence of impaired NO-mediated microvascular function. In contrast, exercise training increased NO bioavailability which may modulate the balance between NO and alternative vasodilatory pathways, causing compensatory vasodilator mechanisms to become redundant. Indeed, the presence of redundancy in vasomotor control is well recognised in other arterial beds (Joyner & Wilkins, 2007).

Direct therapeutic effects of exercise on the cutaneous microvasculature are evidenced by the present study, via improvements in NO-mediated cutaneous microvessel function following exercise training occurring without reductions in weight, IHTC or abdominal VAT. Moreover, as predominantly lower limb exercise training induced an enhancement in cutaneous microvessel NO vasodilator function in the forearm, these data suggest that exercise training has a systemic therapeutic effect on cutaneous microvascular function. This finding is corroborated by Black *et al.* (2008b) who reported a similar systemic impact of exercise on cutaneous microvascular function in old sedentary healthy individuals.

There are a number of noteworthy methodological issues concerning this study which warrant consideration. An advantage of the current study is the utilisation of intradermal microdialysis as the optimal technique (Cracowski *et al.*, 2006) to elicit NO blockade allowing specific evaluation of NO contribution to cutaneous microvessels during incremental heating. Previous training studies have demonstrated that exercise improves acetylcholine-mediated vasodilatation, which infers an increase in NO production.

Whilst acetylcholine is a precursor of vasodilatation, it is not exclusive to NO, as it only accounts for ~30-60% of the NO-mediated vasodilator response (Kellogg *et al.*, 2005). Furthermore, these studies utilised the iontophoresis approach, which has well accepted limitations (Cracowski *et al.*, 2006). Intradermal microdialysis facilitates the infusion of a potent NO blockade enabling the exclusive evaluation of NO contribution to cutaneous microvessel vasodilator responses. One possible limitation of this study is that, unlike previous intradermal microdialysis studies which infused *N*-nitro-L-arginine methyl ester (L-NAME) to elicit NO blockade during incremental heating (Kellogg *et al.*, 1999; Black *et al.*, 2008b), the current study utilised a less potent NO blocker, L-NMMA. Although L-NMMA is a less potent NO blocker than L-NAME (Goldsmith *et al.*, 1996), it has been reported to elicit similar specific inhibitory effects on NO-mediated vasodilatation (Dietz *et al.*, 1994).

In summary, the findings from this study suggest that cutaneous microvascular function can be improved in NAFLD patients; via an up regulation of the anti-atherogenic molecule NO. Furthermore, the therapeutic effects of supervised exercise training are superior to that of current conventional care guidelines and infer a reduction in CVD risk independent of weight loss and diminished IHTC and abdominal VAT.

Chapter 8

SYNTHESIS OF FINDINGS

8.1 Aims and Objectives

The research work undertaken in this thesis was designed to investigate cardiovascular and metabolic health in non-alcoholic fatty liver disease (NAFLD) and to establish whether supervised exercise training could ameliorate the cardio-metabolic status of NAFLD patients. Specifically, the studies described in this thesis investigated the impact of intrahepatocellular triglyceride content (IHTC) and abdominal visceral adipose tissue (VAT) on conduit artery endothelial function and, for the first time, examined cutaneous microvessel endothelial function in NAFLD patients. Additionally, a major focus of this thesis was to investigate whether a 16-week supervised exercise training program of moderate intensity had a therapeutic impact on conduit artery and cutaneous microvessel endothelial function that exceeded the effect of the current conventional care guidelines in the UK. Finally, a 12-month follow up assessment was performed after the cessation of supervised exercise training in an attempt to explore the longevity of any exercise-induced improvements in conduit artery endothelial function and IHTC in NAFLD.

8.2 General Discussion

CVD is the leading cause of mortality in NAFLD patients, exceeding that of liver disease (Ekstedt *et al.*, 2006; Ong *et al.*, 2008). Endothelial dysfunction is an early marker in the development of atherosclerosis and predictor of future cardiovascular events, and therefore was the primary outcome variable within this thesis. Previous research has demonstrated that brachial artery FMD is impaired in NAFLD patients when compared with age (Villanova *et al.*, 2005; Senturk *et al.*, 2008) and overweight (Vlachopoulos *et al.*, 2010) matched controls. Insulin resistance is a key pathological feature of NAFLD and has been associated with impaired FMD in these patients (Villanova *et al.*, 2005). Importantly, there is a strong association between insulin

resistance, abdominal VAT and hepatic steatosis in NAFLD (Chitturi *et al.,* 2002; Park *et al.,* 2007). Notably, VAT is positively correlated with steatosis (Kelley *et al.*, 2003; Nguyen-Duy *et al.,* 2003) independently of insulin resistance (van der Poorten *et al.*, 2008) and has been found to be a predictor of CVD (Despres, 2007; Petta *et al.,* 2009). Specifically, VAT has been reported to be a determinant of endothelial dysfunction, independent of traditional risk factors, in morbidly obese individuals (Sturm *et al.*, 2009). Taken together, these data indicate that VAT is a pivotal feature in the pathogenesis of NAFLD, which may infer additional risk of future cardiovascular events. Nevertheless, no previous research has quantified IHTC and abdominal VAT in NAFLD in order to examine the impact of these fat depots on endothelial function.

Chapter 4 confirmed that obese NAFLD patients exhibit impaired FMD when compared with age and BMI matched controls, and thus express a reduced production of the anti-atherogenic molecule, NO, in response to increases in blood flow and associated shear stress. However, when co-varied, the impairment in endothelial function was not explained by IHTC or abdominal VAT. Following 16-weeks of supervised moderate intensity exercise training brachial artery endothelial function in NAFLD patients improved (Chapter 5). Importantly, supervised exercise training induced a significantly greater improvement in endothelial function than current conventional care guidelines. Chapter 6 demonstrated that exercise-induced improvements in endothelial function were abolished 12-months following the cessation of supervised exercise. Chapter 7 illustrated that cutaneous NO-mediated microvascular function was not impaired in NAFLD when compared with age, BMI and cardiorespiratory fitness matched controls. Nevertheless, supervised exercise training enhanced NO contribution to skin blood flow (SKBF) in the microvessels of NAFLD patients to a greater degree than that of conventional care.

Flow mediated dilatation (FMD) is an early clinical marker of CVD, which was found to be impaired in NAFLD when compared with matched controls, indicative of diminished NO bioavailability and subsequent endothelial dysfunction. Previous research has reported that FMD is an independent predictor of future cardiovascular events in high risk individuals, whereby risk of an event is approximately nine-fold higher in individuals with a FMD response below 8.1% (Gokce *et al.*, 2003). Chapter 4 demonstrated that despite being obese and sedentary, characteristics known to independently predict endothelial dysfunction (Black *et al.*, 2009; Davison *et al.*, 2010) the control participants elicited a relatively healthy FMD response (8.3±0.7%), compared with NAFLD (4.3±0.3%). These data indicate that NAFLD may provoke a detrimental impact on conduit artery endothelial function, which exceeds the adverse effects of global obesity and sedentary behaviour (Figure 8.1), and exposes these patients to significantly heightened risk of CVD.

In contrast to the observed impairment in conduit artery endothelial function, no differences were seen in cutaneous vasodilator function between NAFLD and matched controls at baseline, which may infer that NO-mediated cutaneous microvascular function is not impaired in NAFLD. However, when compared with the findings of Black *et al*. (2008b), who reported that the NO contribution to SKBF in response to peak heating was ~40% in young sedentary individuals and ~50% in older fit individuals, the data presented in Chapter 7 indicates that both NAFLD and controls demonstrated impaired cutaneous endothelial function, as both groups only elicit a ~30% NO contribution in response to the same peak heating stimulus. This finding is corroborated by Sokolnicki *et al.*, (2007) who reported a similar NO contribution to SKBF during peak body heating (~30%) in overweight type 2 diabetics and BMI matched control individuals. These data suggest that NO-mediated cutaneous

microvascular function may be more susceptible to the adverse impact of obesity and sedentary behaviour than conduit arteries. Indeed as depicted in Figure 8.1, it is plausible that microvascular dysfunction precedes any dysfunction found in the larger conduit arteries, implying that the cutaneous microcirculation could be used as a marker to investigate endothelial dysfunction at an early stage in the care pathways of patients. Despite this observation, the findings of this thesis indicate that NAFLD provokes an additional detrimental impact on conduit artery endothelial function, over and above that caused by lifestyle factors (Figure 8.1).

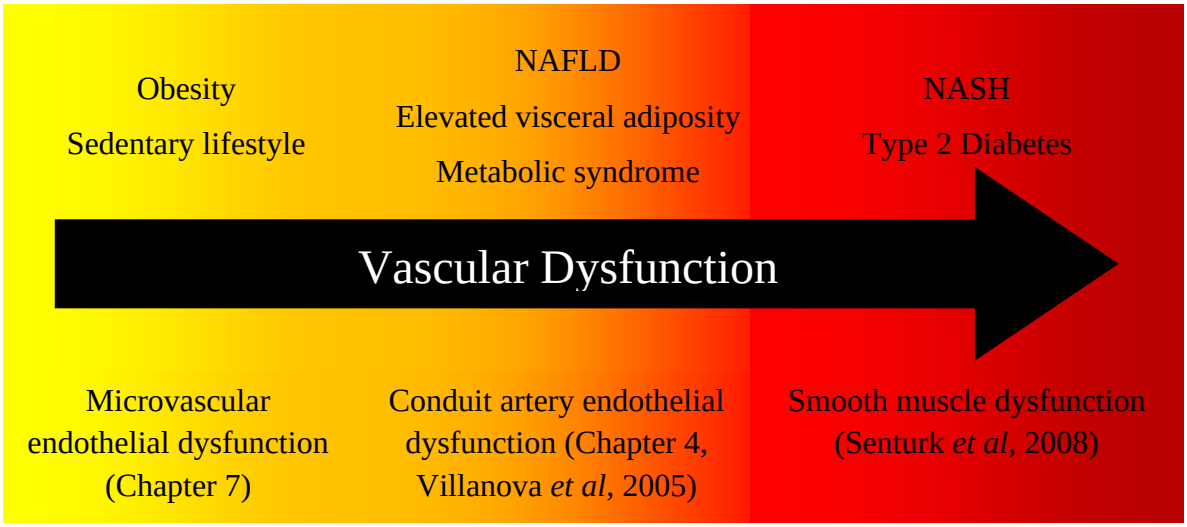

Figure 8.1 A schematic illustrating that as the severity of NAFLD and its associated comorbidities increases, vascular dysfunction becomes more aggressive.

As cutaneous NO-mediated microvessel function reflects generalised microvascular function and provides a translational model to investigate pre-clinical disease (Holowatz *et al.*, 2008), the deficit described in Chapter 7 supports the previous research that suggests hepatic microvessel function is impaired in NAFLD (Ijaz *et al.*, 2003). However, this raises the question of whether NAFLD directly provokes microvessel dysfunction, or whether generalised microvascular dysfunction precedes NAFLD and impacts on hepatic metabolism, which subsequently promotes triglyceride

accumulation within the hepatocytes. Indeed, this is a research area that warrants further investigation in the future.

Chapters 5 and 7 indicate that there is no statistical difference between the therapeutic impact of supervised exercise training and conventional care on IHTC. Nevertheless, these Chapters did demonstrate that supervised exercise training induced clinically relevant absolute reductions (9-13%) in IHTC in NAFLD patients which are comparable with previous findings (Bonekamp *et al.*, 2008; Johnson *et al.*, 2009; van der Heijden *et al.*, 2010). In contrast, supervised moderate intensity exercise did not induce a reduction in abdominal VAT. A previous study observed a 12% relative reduction in abdominal VAT in NAFLD patients following four weeks of high intensity cycle ergometer exercise training (Johnson *et al.*, 2009). Therefore, it is plausible that a higher intensity exercise training intervention may have mediated a reduction in abdominal VAT.

Supervised exercise training induced a clinically significant reduction in IHTC, enhanced cardiorespiratory fitness and improved endothelial function across different levels of the vascular tree in sedentary and obese NAFLD patients. The exercise-induced improvement in FMD and NO contribution to SKBF in cutaneous microvessels compare with other diseased or aging populations (Maiorana *et al.*, 2000; Maiorana *et al.*, 2001; Black *et al.*, 2008b). It is interesting to note that the exercise-induced improvement in FMD relative to baseline was 73%, whereas a 100% improvement was observed in the NO contribution to peak cutaneous microvessel blood flow. Although these improvements are broadly similar, these data suggest that NO-mediated cutaneous microvessel function is more amenable to exercise-mediated enhancement than conduit

arteries in NAFLD, again implying that the microcirculation is more plastic and susceptible to change.

The results in Chapters 5 and 7 imply that the exercise-induced improvement in endothelial function was due to an increase in NO production, rather than an increase in sensitivity of the smooth muscles cells. Regular exercise training promotes an increase in NO bioavailability by reducing the number of oxygen free radicals and up-regulating endothelial NO synthase protein (Green *et al.*, 2004a; Green *et al.*, 2008). Increased NO bioavailability is thought to be caused by recurrent increases in shear stress as a result of repeated exercise bouts (Green *et al.*, 2010). The chronic enhancements observed in NAFLD patients are indicative of an increase in NO production, which promotes efficient vasomotor function and decreases the risk of atherosclerotic development. Critically, previous evidence demonstrates that interventions which improve NO dependent endothelial function are associated with improved mortality and morbidity in similar diseased populations (O'Driscoll *et al.*, 1997; O'Driscoll *et al.*, 1999). This is of vital clinical importance given that CVD is the leading cause of mortality in NAFLD (Ong *et al.*, 2008).

Enhanced FMD and cutaneous microvessel function was evident independent of statistically significant reductions in IHTC and abdominal VAT. These findings suggest that exercise has an independent and direct therapeutic impact on both conduit artery and cutaneous microvessel endothelial function in these patients who are at high risk of CVD and future cardiovascular events. Moreover, since *lower limb* exercise training induced the enhancements seen in both conduit artery and cutaneous microvessel NO vasodilator function of the *upper limb*, these data suggest that exercise training has a systemic therapeutic effect on endothelial function across the vasculature in NAFLD.

This finding is corroborated by Black and colleagues (2009), who reported that lower limb exercise had a similar systemic impact on conduit artery and cutaneous microvascular function of the upper limb, in old sedentary individuals.

A noteworthy observation of this thesis and a powerful public health message is that moderate intensity exercise training is capable of improving conduit artery and cutaneous microvessel endothelial function in NAFLD. It would be interesting to investigate whether exercise training of a higher intensity induced any additional benefits to cardiovascular and metabolic health in NAFLD, particularly given the observation that high intensity training reduces abdominal VAT by 12% (Johnson *et al.*, 2009) in these patients. However, given that Chapter 6 demonstrated that the cardio-protective impact of supervised *moderate* intensity exercise training was abolished by patients returning to sedentary habits, 12-months following the cessation of supervision; it is unlikely that long term compliance to high intensity exercise training would be maintained.

8.3 Methodological considerations and limitations

This thesis has several noteworthy advantages in terms of methodology. Firstly, the use of whole body magnetic resonance imaging (MRI) to quantify fat deposition and the non-invasive gold standard proton magnetic resonance spectroscopy (^{1}H-MRS) allowed very precise quantification of hepatic fat in all of the studies. Secondly, utilisation of the most recent peer reviewed guidelines and technology to assess FMD (Thijssen *et al.*, 2011) coupled with the utilisation of custom designed edge detection and wall tracking analysis software, brought maximal precision to these measurements. Thirdly, employment of a randomised control trial in Chapters 5 and 7 minimised allocation bias in the assignment of care pathways. Finally, use of intradermal microdialysis allowed

the interrogation of the control mechanisms of the microcirculation by using both agonist and antagonist vasoactive substances to challenge cutaneous NO-mediated microvascular function.

Despite these advantages, the studies within this thesis have a number of limitations. Although rigorous inclusion and exclusion criteria and in-depth diagnosis were employed, participants did not undergo a liver biopsy to histologically confirm the diagnosis of NAFLD. Consequently, it was not possible to stratify between simple steatosis and NASH. Furthermore, a measurement of peripheral insulin resistance was not incorporated within the thesis and is clearly an area for future study, since this would have provided a powerful covariate to the analysis.

8.4 Future Directions

There are several potential areas of future research which have emerged from the data presented in this thesis. Primarily, further examination of the relationship between, insulin resistance and endothelial dysfunction in NAFLD patients is warranted. Specifically, future studies should employ the euglycaemic clamp technique as a more robust measure of peripheral insulin resistance. Moreover, as isolated hallmark features of NAFLD such as IHTC, abdominal VAT and insulin resistance do not totally explain the decrement in FMD, investigation into the relationship between endothelial dysfunction and other less overt pathological features, such as the excess secretion of inflammatory cytokines, including TNF-α and leptin may be warranted.

Although no difference in cutaneous NO-mediated microvascular function was observed in NAFLD when compared with age, BMI and cardiorespiratory fitness matched controls, future research should compare microvessel endothelial function of

NAFLD patients with lean controls, sub-divided by cardiorespiratory fitness, in order to further interrogate the impact of obesity and sedentary behaviour on cutaneous NO-mediated microvascular function. Furthermore, as the peak vasodilator capacity of cutaneous microvessels was maintained following conventional care despite an apparent reduction in the contribution of NO, future studies should investigate other compensatory vasodilator mechanisms (such as prostacyclins) that may exist to preserve heat-induced vasodilator responses in the presence of diminished microvascular NO bioavailability.

Future research investigating the impact of exercise training on endothelial function in biopsy-proven NASH patients would be of great clinical relevance. Villanova *et al.* (2005) illustrated that NASH patients exhibit more pronounced endothelial impairment than patients with isolated simple steatosis. However, it is currently unknown whether exercise training has a similar therapeutic impact on endothelial function as the severity and scale of NAFLD worsens. Similarly, the therapeutic impact of high intensity exercise on fat deposition and endothelial function warrants further consideration. Finally, in order to further evaluate the longevity of supervised exercise training, future follow up studies should provide intermittent guidance counselling (perhaps using mobile technologies) throughout the cessation period to establish whether minimal communication can aid the chronic sustainability of exercise-induced improvements to cardiovascular health in NAFLD.

8.5 Summary and Conclusions

The primary aim of this thesis was to explore NO-mediated endothelial function at different levels of the vascular tree in NAFLD patients and to establish whether supervised exercise training had a sustained therapeutic impact on endothelial function.

The findings of this thesis provide novel data for the literature, and significantly advance our understanding of endothelial function in NAFLD patients and the cardioprotective role of supervised exercise training in this high risk group.

The findings of this thesis indicate that NO-mediated endothelial dysfunction is present in the brachial artery of NAFLD patients. Moreover, impaired conduit artery endothelial function in NAFLD is not explained by elevated IHTC or abdominal VAT. Supervised exercise training improved brachial artery and cutaneous microvessel NO-mediated endothelial function in sedentary and obese NAFLD patients. Improvements in endothelial function at different levels of the vascular tree occurred without statistically significant reductions in IHTC or abdominal VAT. However, the improvement observed in brachial artery NO-mediated endothelial function was abolished 12-months following the cessation of supervised exercise training.

8.6 Clinical Relevance and Recommendations

The research work undertaken in this thesis supports previous evidence indicating that NAFLD, *per se*, infers an adverse CVD risk profile, but importantly, also provides information pertaining to the extent of CVD risk evident in this clinical population. Recently published robust risk ratios indicate that the risk of a cardiovascular event increases by 21% for every 1 standard deviation (=3.5%) decrease in FMD (Inaba *et al.*, 2010). Chapter 4 demonstrated a mean reduction in FMD of 3.5% in NAFLD patients compared with control participants, inferring that NAFLD patients are 21% more likely to experience a cardiovascular event than individuals without NAFLD who are of a similar age, fitness and BMI. Additionally, chapters 5 and 6 are the first studies to demonstrate the therapeutic effects of supervised exercise training on conduit artery and cutaneous microvessel health in NAFLD patients. Indeed, supervised exercise training

mediated a mean improvement in FMD of 3.6% in NAFLD patients, which according to the prognostic data published by Inaba and colleagues (2010) may have reduced the risk of a cardiovascular event by ~21% in this high risk group. Given that CVD is the leading cause of death in NAFLD patients, exceeding that of liver related mortality (Ekstedt *et al.*, 2006; Ong *et al.*, 2008), the findings of this thesis have the potential to impact clinical practice and subsequently improve the CVD related prognosis of this high risk group.

The findings of this thesis indicate that the therapeutic effects of supervised exercise training are superior to that of current conventional care guidelines and confer a reduction in CVD risk independent of weight loss and diminished IHTC and abdominal VAT. Consequently, these data indicate that exercise has an independent and direct therapeutic impact on endothelial function in NAFLD, which may decrease the risk of heart disease and stroke in these high risk patients. Nevertheless, in order to chronically sustain exercise-induced improvements in the endothelial function, long term exercise supervision and guidance is required. Since higher levels of NO confer anti-atherogenic benefit, the findings of this thesis have potentially important implications for the prevention of macro and micro-vascular dysfunction in NAFLD and imply that supervised exercise prescription should be recommended as a cardioprotective management strategy.

Chapter 9

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