UNIVERSITY OF LIVERPOOL

Ectopic lipid storage in Non-Alcoholic Fatty Liver Disease is not mediated by impaired mitochondrial oxidative capacity in skeletal muscle

A thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Master in Philosophy

Andrew John Irwin

October 2014

Background: Non-alcoholic fatty liver disease (NAFLD) is a condition characterised by a synergistic association between insulin resistance, obesity and ectopic fat. This disease state has an increased prevalence of type Π diabetes (T2DM) and cardiovascular disease (CVD), thought to be mediated by the insulin resistance. There is much evidence to support the hypothesis that mitochondrial function in skeletal muscle may be implicated in the development of insulin resistance and obesity. We aim to determine if mitochondrial dysfunction contributes to ectopic fat accumulation and the associated metabolic syndrome by altering the post-prandial energy storage.

Methods: To test this hypothesis, we performed a cross sectional study of 17 patients with NAFLD and 18 controls. Baseline biochemistry, lipid profile, liver function tests (LFT's) glucose and insulin levels were assessed. Measurement of insulin resistance was made using HOMA-IR. Assessment of body composition was measured using Tanita bio-impedance analysis (Tanita BC 420, Dolby Medical Stirling, UK). Magnetic Resonance Imaging (MRI) and ^{1}H-Magnetic Resonance Spectroscopy (MRS) determined abdominal, visceral, subcutaneous, skeletal muscle and intra hepatic fat respectively. We measured mitochondrial function within the quadriceps muscle during the study by measuring Phosphocreatine (PCr) recovery kinetics using 31P MRS. This analysis was carried out in the NAFLD group and compared to healthy controls

Results: Patients with NAFLD matched with control subjects for age, BMI and VO_{2max} had evidence of ectopic fat (higher LF and VAT). Despite this no difference in mitochondrial function was apparent.

Conclusions: Altered skeletal muscle mitochondrial function does not explain ectopic fat deposition in patients with NAFLD.

Acknowledgements

I wish to acknowledge the following people who have given me support and advice during the period of this research project and the writing of this thesis.

I am indebted to my supervisors, firstly Dr Daniel Cuthbertson, who as the principle investigator for the study has given his support and advice as well as sharing his knowledge of NAFLD.

To Professor Graham Kemp, firstly, for his support and advice, but also for his technical skills in interpreting the multiple data sets and for having designed the original exercise protocol.

I would like to thank Professor John Wilding for his support in completing this study, and also for affording the opportunity to present this work at departmental meetings.

Many thanks to Dr Victoria Sprung for taking the time to proof read this thesis, and for her help and advice on the final presentation.

I also wish to thank my advisory panel, Dr Maria Anderson and Dr Christina Daousi, for their help and advice in completion of this work.

I also wish to thank my colleague Shirley Cooper for her unique computer skills 'try this and see if it works'; which it always did!

To the research staff at MARIARC, Val Adams, Research Radiographer, and Bill Bimson Senior Technical Officer, not just for their technical skills but for their enthusiasm and accommodation of research scans. Without their help and support this work would not have been possible.

Most importantly I would like to thank the patients and control volunteers who took part in this at times demanding study. Without their commitment it would not have been possible.

The work presented in this thesis is mine, and has not yet been presented, nor is currently being presented for any other degree qualification. The research for this thesis was carried out in the University Hospital Aintree, the Magnetic Resonance and Image Analysis Research Centre at the University of Liverpool, and at Liverpool John Moores University.

This research study was supported by an initial grant from the *Novo Nordisk Corporation* but mostly funded by the *Weight Matters Charity*, headed by Dr Daniel Cuthbertson at Aintree Hospital, Liverpool, for which I am most grateful.

Last but not least, to my wife Karen for her support enthusiasm and a listening ear, also to my sons Elliot and Joseph, as I would like to dedicate this work to you.
Try and you may succeed; if you don't try you will never know what may have been achievable in your lives.

Declaration

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

Submitted manuscripts based on the work contained within this thesis

DJ Cuthbertson, **A Irwin**, CJA Pugh, H Jones, VS Sprung, C Daousi, VL Adams, WE Bimson, F Shojaee-Moradie, AM Umpleby, JP Wilding & GJ Kemp (2014). Ectopic lipid storage in Non-Alcoholic Fatty Liver Disease is not mediated by impaired mitochondrial oxidative phosphorylation in skeletal muscle. *Clinical Science,* 127(12), 655-663.

Poster Communication

Cuthbertson DJ, **Irwin A**, Jones H, Daosui C, Adams VL, Bimson WE, Shoajee-MoradieF,Umpleby M, Wilding JP, & Kemp GJ. Skeletal muscle mitochondrial function is not impaired in patients with nafld compared with age and bmi-matched healthy controls. *5th International Congress on Prediabetes and the Metabolic Syndrome.* Vienna, Austria, April 18-20, 2013.

List of Contents

List of abbreviations / key words

Adiponectin	A protein involved in regulating glucose and fatty acid breakdown
ALT	Alanine Aminotransferase
ANCOVA	Analysis of covariance
AST	Aspartate transaminase
ATP	Adenosine Triphosphate
BMI	Body mass index
BP	Blood pressure
ChREBP	Carbohydrate response element binding protein
CI	Confidence interval
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
FFA	Free fatty acid
HDL	High density lipoprotein
HOMA-IR	Homeostatic model assessment
IHCL	Intrahepatocellular Lipids
IMCL	Intramyocellular Lipids
Leptin	A hormone which regulates fat storage
LDL	Low density lipoprotein
LFT	Liver function tests
MAP	Mean arterial pressure

MR	Magnetic Resonance
MRI	Magnetic resonance imaging
MRS	Magnetic Resonance Spectroscopy
MVC	Maximal voluntary contraction
NAFLD	Non-alcoholic fatty liver disease
Pi	Inorganic Phosphate
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
SAR	Specific Absorption Rate
SD	Standard deviation
SE	Standard error
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error
SREBP1c	Sterol regulatory element-binding protein 1c
TNF	Tumour necrosis factor
VLDL	Very low density lipoprotein
VO_{2peak}	Maximal oxygen consumption
^{1}H-MRS	Proton magnetic resonance spectroscopy

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

1.1 Non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the general population and is said to be present when fatty liver infiltration affects >5% of hepatocytes, in individuals with a history of alcohol consumption <14 units a week for females and <21 units for males, without evidence of other causes of liver disease such as hepatitis and Wilsons disease. NAFLD is a progressive condition and represents a spectrum of varying severity of liver disease, ranging from simple steatosis to co-existent inflammation with hepatocyte ballooning and necrosis, variable grades of fibrosis, and ultimately cirrhosis and increased risk of hepatocellular carcinoma. Non-alcoholic steatohepatitis (NASH) represents the more advanced stages of the disease, i.e. the inflammatory component in addition to steatosis, which carries a higher risk of cardiovascular disease (CVD) and mortality. Approximately 10-25% of patients with NAFLD are said to develop NASH. The factors responsible for this change from steatosis to steatohepatitis has been the subject of extensive investigation and speculation, but currently the pathphysiology of this switch remains elusive (Day & James, 1998b).

Although the pathogenesis of NAFLD is not yet fully understood, much progress has been made in recent years in understanding the mechanisms of progression from steatosis to more advanced liver inflammation and fibrosis. The disease is mostly silent and is often discovered through incidentally elevated liver enzyme levels. It is strongly associated with obesity and insulin resistance and is currently considered by many as the hepatic component of the metabolic syndrome (Cortez-Pinto & Camilo, 2004; Niaz *et al.*, 2011). Because NAFLD resembles alcoholic liver disease but occurs in people who drink little or no alcohol, excessive daily alcohol consumption

must be ruled out before making the diagnosis. Numerous other conditions leading to fatty liver must be excluded by history, physical examination, and appropriate testing. The most common symptoms that brings NAFLD to medical attention are malaise, fatigue, and right upper quadrant or diffuse abdominal discomfort, however fatty liver disease rarely results in symptoms until the disease is far advanced so hepatomegaly, abnormal enlargement of the liver is also a frequent finding. Serum asparate transaminase (AST) and alanine transaminase (ALT) are elevated in almost 90% of patients (Lavine & Schwimmer, 2004). The AST/ALT ratio is usually less than 1; this is much lower than the ratio in alcoholic hepatitis, which is usually above 2 (Sorbi *et al.*, 1999). NAFLD is more likely to develop in individuals who are obese or overweight, and also in type two diabetes (T2D) due to an increased uptake of fat in the liver (Mirza, 2011).

Non-alcoholic fatty liver disease affects up to a third of the population worldwide and is thought to carry an increased risk of cardio metabolic risk with altered cardiovascular outcomes independent of traditional cardiovascular risk factors and the metabolic syndrome. It is almost universally characterized by insulin resistance and is strongly associated with obesity and T2D (Petersen *et al.*, 2003).

Day and James proposed a “two hit” model in an attempt to provide a pathophysiological rationale for the progression of damage to the liver. It is thought that the reversible intracellular deposition of triacylglycerols, first hit, leads to metabolic and molecular alterations that sensitize the liver to the second hit, this is in general referred to as due to oxidative damage and cytokine induced liver injury. This two hit theory provides a framework to revise the main mechanisms involved in

the pathogenesis of hepatic steatosis and its progression to steatohepatitis (Burkhalter, 1996).

A number of radiological modalities can be used in order to detect the presence of fat in the liver, however there is no method available that can differentiate between the histological subtypes from non-alcoholic hepatic steatosis which is relatively benign to the more aggressive NASH (Schwenzer *et al.*, 2009). The use of ultrasound scanning can reveal an echo bright liver due to diffuse fatty liver infiltration, but this finding can be nonspecific and therefore cannot be used to aid the diagnosis of NAFLD. The use of Fibroscan ultrasound (transient elastography) however is more specific as this gives a number representing the stiffness of the liver and is therefore used in some centres as a diagnostic tool. The use of CT and MRI whilst able to identify steatosis lack the sensitivity to detect liver inflammation or more advanced fibrosis. Magnetic resonance spectroscopy (MRS) has advantages over other imaging methods such as CT, MRI and ultrasound as this method facilitates the acquisition of quantitative data rather than qualitative or semi quantitative (Rofsky & Fleishaker, 1995). In suspected NAFLD, the indications for the use of liver biopsy whilst remaining controversial, is the only way to distinguish between simple steatosis steatohepatitis and fibrosis. However as there is no specific treatment other than weight management for NAFLD such an invasive procedure as liver biopsy may not be of clinical benefit, and performing this may not outweigh the associated risks. For this reason MRS was used within this study to enable quantification of liver fat as a percentage of intrahepatocellular lipids in both the controls and subjects with NAFLD.

1.2 Obesity

Obesity is generally defined as a condition in which an individual has a body mass index (BMI) $\geq 30 kg/m^2$ (Evans & Colls, 2009). The BMI being a measure of the relationship between an individual's weight and height. The medical significance in determining BMI is that this describes the body weight relative to height and BMI has been found to correlate with total body fat content in adults (Bhaskaran *et al.*, 2013). A limitation of this measure is that within certain groups for example body builders who have larger muscle masses; a higher BMI does not correlate with increased fat content. Worldwide obesity is approaching epidemic proportions in the US and many other industrialized countries. In the US the prevalence of obesity now exceeds 30% of the population and represents the highest rate of obesity worldwide (Flegal *et al.*, 2012). Obesity and insulin resistance are both key features of the metabolic syndrome and are strongly associated with NAFLD (Gallagher *et al.*, 2010).

1.3 Ectopic Fat

The distribution of adipose tissue appears to affect its role in metabolic syndrome. Fat that is visceral or intra-abdominal correlates with inflammation, whereas subcutaneous fat does not. There are a number of potential explanations for this, including experimental observations that omental fat is more resistant to insulin and may result in a higher concentration of toxic free fatty acids in the portal circulation (Turkoglu *et al.*, 2003). Abdominal fat is known to produce potentially harmful levels of cytokines, such as tumour necrosis factor, adiponectin, leptin, resistin, and plasminogen activator inhibitor (Despres *et al.*, 2008).

NAFLD is a marker of ectopic fat accumulation combined with a low-grade chronic inflammatory state. This results in a number of processes including abnormal glucose, fatty acid and lipoprotein metabolism, increased oxidative stress, deranged adipokine profile, increased coaguloability of blood, endothelial dysfunction, and an accelerated progression of atherosclerosis (Solomon *et al.*, 2008).

A high proportion of NAFLD patients display features of the metabolic syndrome and there is evidence linking NAFLD to increased cardiovascular disease risk over and above that associated with the metabolic syndrome. This suggests that NAFLD may contribute to accelerated atherogenesis (Solomon *et al.*, 2008), cardiac risk being increased as non-alcoholic fatty liver disease becomes more severe. This also leads to an increased prevalence of type two diabetes mediated by insulin resistance and cardiovascular mortality, which represents the main cause of premature death in NAFLD.

1.4 The metabolic syndrome

The metabolic derangements that characterize the metabolic syndrome (MS) have been implicated in the development of NAFLD. Of all of the individual clinical components of the metabolic syndrome, obesity has the strongest association with NAFLD and generally speaking, the more obese the patient the higher the risk of having a fatty liver (Luyckx *et al.*, 1998). Thirty percent of patients who are obese have fatty liver, and up to 80% of morbidly obese patients (BMI > 35) have NAFLD (Menshikova *et al.*, 2006), regardless of BMI, patients with truncal/central obesity are also at greater risk of fatty liver disease.

The metabolic syndrome has been defined by the American Heart association as an individual exhibiting three or more of the characteristics listed table 1 (Grundy *et al.*, 2005).

Table 1.1 Criteria for metabolic syndrome.

Risk factor	**Defining level**
Abdominal obesity (waist measurement)	Men: Greater than 40 in. (102 cm) Asian men: Greater than 36 in. (90 cm) Women: Greater than 35 in. (88 cm) Asian women: Greater than 32 in. (81 cm)
Triglycerides	1.7 mmol/l or higher, or taking medicine for high triglycerides
High-density lipoprotein (HDL) cholesterol	Men: Less than 1.03mmol/l Women: Less than 1.3 mmol/l Or taking medicine for low HDL cholesterol
Blood pressure	130/85 mm Hg or higher, or taking medicine for high blood pressure
Fasting blood sugar	5.6 mmol/l or higher, or taking medicine for high blood sugar

It has been suggested that patients meeting any of three points highlighted in the above diagnostic criteria have increased risk of developing heart disease and diabetes. According to (Giovannucci, 2007) the metabolic syndrome also increases the risk of stroke, NAFLD and increased risk of liver and colon cancers. Risk factors associated with metabolic syndrome include a family history, poor diet and limited exercise; it is thought to be caused by adipose tissue dysfunction and insulin resistance.

Although the metabolic syndrome has been linked with NAFLD, the origin and nature of the metabolic stressors that are involved in the progress from simple steatosis to NASH have as yet to be identified. Epidemiological research studies that

have focused on this link between the metabolic syndrome and NAFLD have only led to limited information as to potential interventional targets for treatments including nutritional and pharmacological interventions. A recent study has found supporting evidence that NAFLD is not invariably associated with the presence of the metabolic syndrome and that there may be mechanisms other than insulin resistance contributing to the chronic inflammation that is known to underpin liver fat accumulation. It has been postulated that in the absence of the metabolic syndrome increased haemoglobin may be a risk factor in the development of NAFLD (Yilmaz, 2012).

1.5 Insulin Resistance

Insulin resistance is also thought to be linked to the pathogenesis of NAFLD. Insulin resistance can be identified in different sites of the body. The liver where it causes an over production of glucose in the presence of fasting hyperinsulinaemia, the muscle causing a decrease of glucose uptake and utilisation and also the adipose tissue where the release of fatty acids is not adequately suppressed by insulin which are released into the blood stream (Gustafson *et al.*, 2007).

Any carbohydrate ingestion prompts a release of insulin by the pancreas in order to maximize storage and utilization of the glucose that will shortly be entering the circulation. Insulin is a hormone that is known to act through a transmembrane receptor on the surface of most cells. However when insulin is present in the circulation in elevated concentrations or for long periods of time due to dietary excess, these insulin receptors are down regulated, this produces the condition of insulin resistance within the body (Malavolti *et al.*, 2012). It is known that a higher

concentration of insulin is therefore required to affect insulin signalling in the cells due to this insulin resistance. In the short term, insulin resistance is known to be reversible, in that if the blood insulin is lowered for several hours or days eventually the usual number of insulin receptors will return to the cell surface. However, when insulin has been kept chronically high for years, the resilience of the system to recover goes away (Gaggini *et al.*, 2013).

Eventually insulin resistance therefore becomes a constant feature in the individual, the liver resists turning off gluconeogenesis, muscles also resist taking up glucose. In most people, fat cells remain insulin responsive, but eventually they too become resistant and release free fatty acids from fat deposits into the circulatory system. It is not certain that chronic high insulin alone produces insulin resistance, for instance genetic susceptibility and inflammatory processes may play a role. However, once the symptoms of insulin resistance are observed clinically, taking steps to reduce chronic high insulin will permit at least a partial recovery of insulin sensitivity (Campbell, 2000). Not coincidentally, these actions will also cause the loss of fat while sparing the loss of lean muscle mass.

As prevalence of disease continues to increase in conjunction with sedentary lifestyles and nutrient-poor calorie rich diets, and given the recognised link of insulin resistance with NAFLD, diet and improving insulin sensitivity by increased physical activity as may be of therapeutic value.

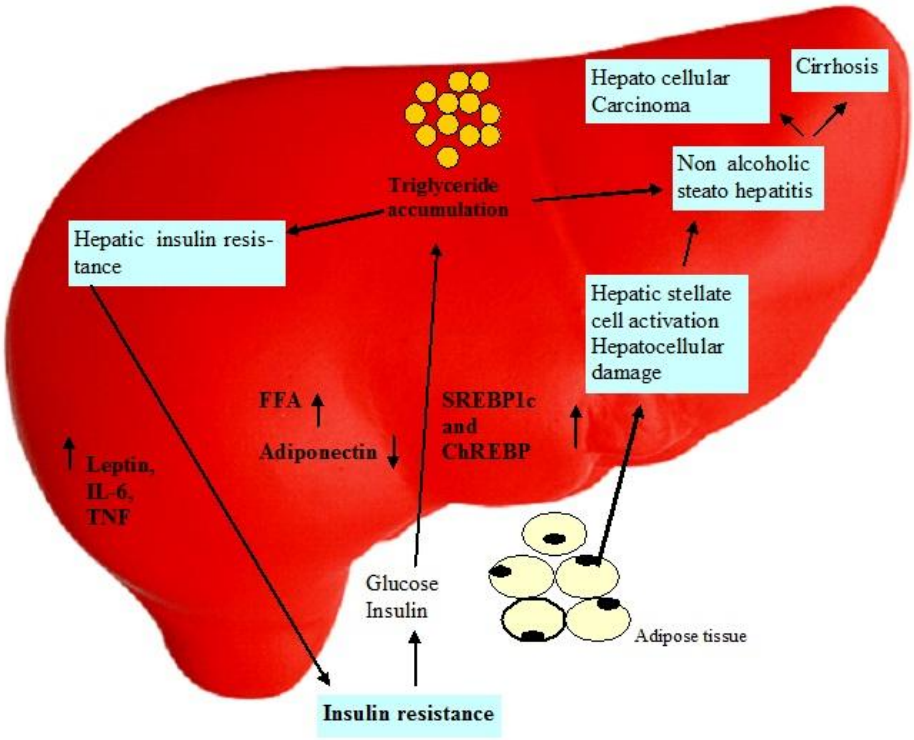


Figure 1.1 Insulin resistance and hepatic triglyceride accumulation, figure adapted from Smith & Adams (2011).

According to Smith & Adams (Smith & Adams, 2011) insulin resistance promotes hepatic triglyceride accumulation by increasing peripheral body fat breakdown and free fatty acid influx as well as increasing levels of liver lipogenic transcription factors sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate response element protein (ChREBP). These are two key factors or regulators of glucose metabolism and lipid synthesis in the liver. An increase in hepatic free fatty acids (FFA) exacerbate hepatic insulin resistance via activation of a number of signalling pathways which have been shown to play an important role in disease processes. This causes a positive feedback loop, which results in a cycle of increased hepatic triglyceride content and hepatic insulin resistance. In a number of patients, an

adipose tissue inflammatory response results in the release of multiple proinflammatory and profibrotic cytokines. Such as tumour necrosis factor (TNF), interleukin 6 (IL-6) which together with liver steatosis induce oxidative stress and activate fibrogenic hepatic stellate cells to cause NASH, liver fibrosis and cirrhosis. (Smith & Adams, 2011).

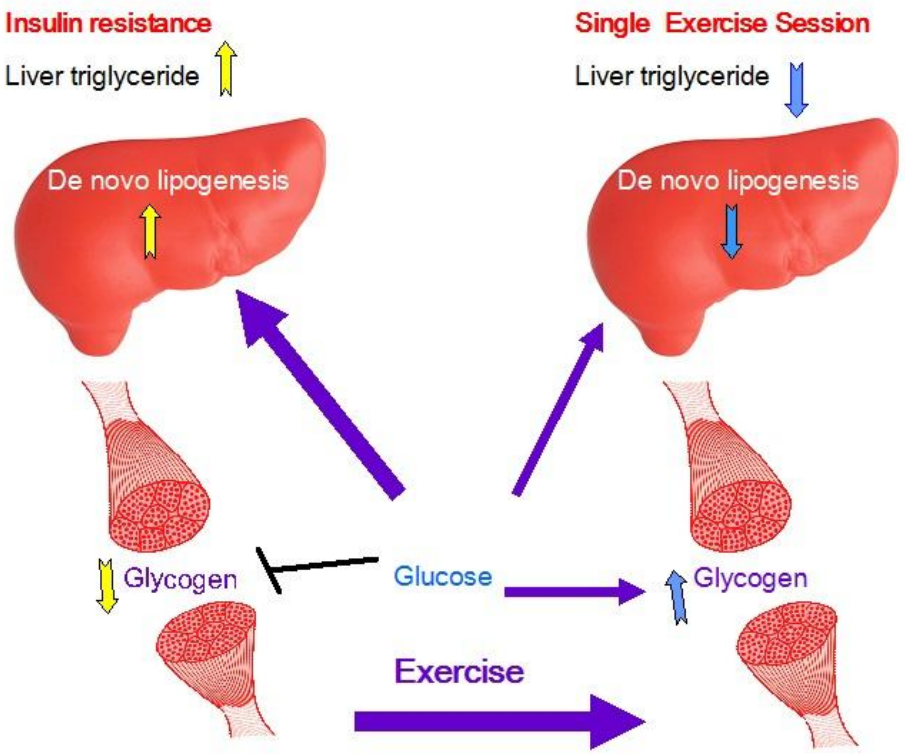


Figure 1.2 Schematic of whole–body energy distribution; adapted from original figure (Rabol *et al.*, 2011).

The dysfunction of adipose tissue also plays a role in the pathogenesis of obesity related Insulin resistance. Both adipose cell enlargement and infiltration of macrophages into adipose tissue result in the release of proinflammatory cytokines and promotes insulin resistance. (Gustafson *et al.*, 2007). Insulin resistance appears

to be the primary mediator of metabolic syndrome (Gustafson *et al.*, 2007). Insulin promotes glucose uptake in muscle, fat, and liver cells and can influence lipolysis and the production of glucose by hepatocytes. Additional contributors to insulin resistance include abnormalities in insulin secretion and insulin receptor signalling, impaired glucose disposal, and proinflammatory cytokines (Gustafson *et al.*, 2007). These abnormalities, in turn, may result from obesity with related increases in free fatty acid levels and changes in insulin distribution (insulin accumulates in fat) (Despres *et al.*, 2008). The condition also has an increased prevalence of T2DM and cardiovascular disease (CVD); this is thought to be mediated by the insulin resistance.

1.6 Relevance of mitochondrial dysfunction in obesity and insulin resistance

Research on mitochondrial dysfunction assisted in the investigation of muscle physiology and energy metabolism and has applications in monitoring degenerative diseases and testing aerobic capacities. Mitochondrial function, oxidative phosphorylation and glycosis account for the most important sources of cellular energy. In addition to producing cellular energy mitochondria are involved in cell signalling, a system that governs cellular activities. They are also involved in cell development where cells develop into more specialised cells, also cellular growth and division of cells and cell death. Mitochondria are known to be involved in a number of diseases, any dysfunction may also play a role in the ageing process (Broskey *et al.*, 2013a).

The Mitochondria account for adenosine triphosphate (ATP) production through oxidative phosphorylation, which as a consequence the performance of Mitochondria

is crucial for organ function, any dysfunction of the mitochondria can therefore play a role in a number of degenerative diseases including T2DM (Larsen *et al.*, 2012).

Impaired oxidative phosphorylation by skeletal muscle mitochondria have been postulated to contribute to age-associated insulin resistance and fat accumulation within skeletal muscle (Short *et al.*, 2005a). This impaired mitochondrial functional capacity in aging has been attributed to a reduced mitochondrial content, as reflected by lower mtDNA content (Petersen *et al.*, 2003). This study was undertaken to assess the role of skeletal muscle mitochondrial dysfunction and its aetiology in subjects with NAFLD versus age and BMI matched controls. As muscle activity increases demand for energy within the myofibrils increases, to provide energy for the muscle contraction the high energy molecule ATP is split into adenosine diphosphate (ADP) and inorganic phosphate as well as (H+) ions (Aliev & Saks, 1993).

The aging process in humans begins around the age of 40 when muscle mass and strength gradually begin to decline at a rate of just under 1% per year. The process is caused by a decreased capacity for oxidative phosphorylation in the muscles (Menshikova *et al.*, 2006). Given the strong evidence linking mitochondria dysfunction with aging, insulin resistance and T2DM, it is important to more precisely define specific loci of these defects, and perhaps more importantly, to determine whether clinical interventions including exercise and diet may help correct these insufficiencies (Menshikova *et al.*, 2006).

1.7 Indirect non invasive assessment of mitochondrial function

Using the rate constant of Phosphocreatine (PCr) recovery as a measure of mitochondrial function in skeletal muscle, PCr recovery from sub maximal exercise has become a reliable and accepted measure of muscle oxidative capacity (Kemp GJ, 1993). As the PCr content decreases transiently during exercise followed by a rapid recovery post exercise in the post state this PCr resynthesis is driven almost purely oxidatively. This resynthesis rate reflects in vivo mitochondrial function. This has been shown to be delayed in various known mitochondrial disorders. The time constants of PCr recovery allow evaluation of mitochondrial oxygen turnover within the muscle. ^{31}P Magnetic Resonance Spectroscopy (MRS) is thereby a unique method for measuring the achievements of the so-called power stations of the cell. Via this approach, the recovery of PCr from exercise in skeletal muscle is a reliable measure of capacity (Kemp GJ, 1993). In muscle physiology, mitochondrial function therefore as determined by the time constant of PCr recovery after a set work load, is of particular interest as this can be measured.

1.8 Physical inactivity in NAFLD

There are factors that have been documented to help improve metabolic flexibility. The first of these is mild-to-moderate exercise. In 2007, Solomon *et al*., described a 12-week program of moderate aerobic exercise in older obese people that improved (decreased) their respiratory quotient (Solomon *et al.*, 2008). Endurance athletes can be seen to show faster regeneration of PCr compared to sprint-trained athletes. Endurance exercise leads to mitochondrial biogenesis and hence to an increased mitochondrial content in the muscle (Pesta *et al.*, 2011). Effective interventions for prevention and treatment of this disease are needed. Increased exercise duration and

intensity has been evaluated as an important therapeutic intervention (Toledo *et al.*, 2008). In a recent study by Broskey, which looked at exercise intervention over a 4 month period it was found that an aerobic exercise program within older age groups can reduce the loss in mitochondrial content within skeletal muscle. This may prevent some comorbidities that can be associated with ageing muscle due to an improvement in mitochondrial content (Broskey *et al.*, 2013a; Broskey *et al.*, 2013b)

Obesity is generally associated with a number of serious complications resulting in significant increases in life threatening diseases resulting in increased morbidity and mortality, relative to lean individuals. Of these complications insulin resistance, T2D and NAFLD are among the most prevalent. Increasing evidence suggests that regular physical activity reduces liver fat, independently of weight loss. Additionally, it may protect NAFLD patients against the development of diabetes and CVD, which are the leading risk factors within this group (Musso *et al.*, 2010). These findings advocate the integration of physical activity as a non-pharmacological treatment of NAFLD however, the question arises which form of exercise would be the most time efficient and effective in reducing liver fat. A number of studies have compared aerobic training with resistance training. One existing study has demonstrated that aerobic training is significantly more effective at improving abdominal fat levels (McLarnon, 2011; Bae *et al.*, 2012), it is also apparent that weight loss and lifestyle modification should also be the primary target for treating NAFLD. A bi-product of exercise is a reduction in weight. As a normal BMI is generally not associated with NAFLD, weight loss even of a modest amount of 5-10% of body weight has been shown to decrease liver fat by 40-80% in non diabetic subjects (Chiang *et al.*, 2011).

In treatment of NAFLD modification of risk factors such as obesity, hyperlipidemia and an improvement in diabetic control are generally recommended, but weight loss appears to be the only available therapy that has some evidence of benefit in the treatment of NAFLD (Chiang *et al.,* 2011). Lack of exercise also increases free fatty acids passing to the liver along with increased de novo lipogenesis, this being the enzymatic pathway for converting dietary carbohydrate (CHO) into fat. Another important source of hepatic tri-glyceride is dietary or exogenous lipids, a high fat diet has been shown to increase liver fat.

In the period following a meal, dietary lipids are transported from the digestive tract into the blood stream in the form of various chylomicrons to be stored in the adipose, cardiac and skeletal muscle tissues. Within these tissues the triglyceride components are broken down and the released free fatty acids are then absorbed. When a large proportion have been broken down or hydrolyzed chlomicron remnants are formed and are then taken up by the liver, thereby transferring dietary fat to the liver. Following this they are processed to form very low density lipoproteins (VLDL). It has been shown that in overweight non-diabetics that a diet high in fat content is able to increase liver fat content by 35% in just a ten day period (Luyckx *et al.,* 1998). In subjects with NAFLD, fat supplied by the diet is said to account for approximately 15% of intrahepatic lipid accumulation (Paschos & Paletas, 2009). The liver is the major organ in the body for lipid distribution; however its ability to store fat is limited and any excess of is oxidised or released as VLDL. When increased this intrahepatic fat content leads to an increase of oxidative mechanisms in NAFLD patients, however non-esterfied fatty acids (NEFA) are oxidized less efficiently because of mitochondrial uncoupling, whereby the processing is uncoupled from the generation of ATP, which results in a reduction in ATP (Day & James, 1998b). In

subjects with NAFLD there are increased rates within the liver of VLDL triglyceride secretion and this circulatory source is the cause of the serum increase that is commonly observed in patients. Therefore it can be postulated that the progression from steatosis to steatohepatitis maybe due to the interaction of a number of mechanisms, with a genetic susceptibility in some individuals to metabolic and liver damage (Day & James, 1998a).

Previous studies have reported beneficial effects of following diet, combined with an increase in physical activity on the progression of NAFLD. Based on available data, NAFLD patients should optimally achieve a 5-10% weight reduction, however a more modest weight loss would still bring a number of health benefits (Bae *et al.*, 2012). However weight gain of 10% by overfeeding of fast food and sedentary lifestyle in a study involving 18 healthy young subjects has been demonstrated to increase liver fat by 2.5 fold over a 4 week period (Kechagias *et al.*, 2008). In other studies, subjects with NAFLD have been shown to have dietary habits that lead them to consume more fat, in particular saturated fat than subjects without NAFLD. The intake of polyunsaturated fat consumed has also been shown to be generally lower in subjects that have been diagnosed with NAFLD than in those without. Within this group they also tend to consume foods with higher glycemic index and drink high calorie drinks when compared with individuals with a normal liver fat of <5% (Chiang *et al.*, 2011).

In small randomised studies in children weight management when used as a treatment have shown improvements in liver histology and aminotransferase activity after weight loss (Dixon *et al.*, 2004). An emphasis on physical activity in the

treatment of NAFLD may be of use in improving insulin sensitivity, as insulin sensitivity appears to be an important link in the pathway for the development of the disease. These changes may be by an underlying mechanism by which improvement in insulin resistance may be brought about by positive changes in fatty acid metabolism within the muscle (Rector *et al.*, 2010).

1.9 Reversibility of mitochondrial dysfunction

There is much evidence to support the hypothesis that mitochondrial function in skeletal muscle may be implicated in the development of insulin resistance and obesity (Kemp, 2006). A challenging issue, methodologically, is how to distinguish between intramyocellular and extramyocellular lipid content. This can be facilitated by ^{1}H spectra analysis as ^{1}H peaks correspond to lipids which are methylene and also methyl protons of the TG acyt chains within the muscle. These two peaks are separated by a shift in frequency from each other by 0.2 parts per million (Ppm) and can be seen to represent two differential compartments of an extramyocellular pool and intramyocellular TG (Kemp GJ, 1993). Also by using this method to non-invasively measure hepatic liver triglyceride (TG) within our study has clear advantages over invasive liver biopsies. This technique was used to assess intramyocellular and intrahepatocellular lipids in the healthy control group versus the group with known NAFLD.

1.10 Decreased Mitochondrial Function

Abdominal obesity and insulin resistance have both been hypothesized to be the primary factors underlying the metabolic syndrome. The exact mechanisms linking these and other risk factors associated with the metabolic syndrome are not fully

understood. However ingested carbohydrates are either oxidized or stored as glycogen in liver and muscles, and to a lesser extent converted to fat in the liver. This storage represents non-oxidative glucose disposal it is suggested that impaired mitochondrial function has a potential link with raised intramyocellular lipids coupled with impaired glucose metabolism and insulin signalling (Sebastian *et al.*, 2012).

The mitochondrion is the major site of fuel oxidation therefore it can be suggested that any decline in mitochondrial function may predispose to muscle fat accumulation. NAFLD, with its associated accumulation of fat within the liver can also possibly be explained by any primary defect in mitochondrial dysfunction within skeletal muscle (Goncalves *et al.*, 2013).

1.11 Methods to measure mitochondrial function

When muscles are used to perform physical activity they must metabolize available fuel to generate energy for contraction, the harder a muscle must work, the more fuel is required. The relation between how hard muscles must work and their need for fuel is an area of interest in this study. In the past, intramuscular energy metabolism has been measured directly by muscle biopsies taken before and after exercise.

1.12 Direct muscle biopsy

This can be facilitated by needle biopsy in order to obtain a small quantity of muscle tissue; however it may not be possible to obtain an adequate sample size particularly if the muscle is atrophic or fatty. This is also an invasive procedure with the risk of infection and pain for the study patient or healthy control. Open biopsy is a more

reliable method in obtaining a sample large enough to do more investigations, however this requires the use of an operating theatre or clean room, causes some degree of post procedural pain and leaves a scar of approximately 5cm.

1.13 Magnetic Resonance

Principles of Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is a non invasive imaging technique which produces multi slice, multi-directional images. It has no known harmful effects and is used for diagnostic and research studies at regular intervals without problems associated with regular exposure as is the case with ionising radiation. The main components of the magnet are the main magnet, gradient coils, radio frequency (RF) transmitter, receiver coils and a computer.

1.14 The main magnet

This was used to produce a strong, uniform external magnetic field i.e. "Bo" that is powerful enough to induce measurable tissue magnetisation. The field of strength being measured in Tesla (T), a 1.5T scanner was used for gaining body composition and a 3T scanner was used to assess mitochondrial function and PCr recovery.

1.15 Gradient Coils

These are electromagnetic coils with magnetic field strengths that are only a fraction of that of the main magnet. These gradient coils produce spatial variation in the Bo field (rotating frame of reference) in a calculated manner with respect to their slope

direction and timing. They are switched on and off very rapidly during image acquisition and are used to provide spatial localisation (While & Forbes, 2004).

1.16 Radio frequency (RF) coils

Radiofrequency coils transmit an RF pulse into the subject to excite sensitive nuclei and also receive back signals from the patient. These coils can be both transmitters as well as receivers; in this case they are called transceivers. Energy was transmitted in bursts of radiofrequency pulses this caused phase coherence and changed or flipped protons from low to high energy states. From this a signal was detected by the receiver coil. A body coil was used as this is a volume coil which covers a large area giving a signal to noise ratio (SNR) that is uniform over the whole imaging volume (Redpath & Wiggins, 2000). The computer manipulated and stored this data to produce images.

1.17 Basic MR Physics

All matter is composed of atoms, which consist of a central nucleus with electrons orbiting this nucleus at distinct energy levels. The atomic nuclei consist of protons and neutrons which have a positive and neutral charge, a nucleus is said to be observable by MR or "MR active" when it has an odd number of protons or neutrons. These unbalanced nuclei possess an angular momentum and as a consequence this makes them behave like a spinning top or gyroscope. According to the laws of electromagnetic induction a moving electronic charge induces a magnetic moment pointing along the axis of rotation of the nucleus, thus the nuclei become tiny bar magnets having two poles of equal and opposite polarity (Pooley, 2004). The most abundant unbalanced nucleus within the body is the Hydrogen nucleus which

consists of a single proton, it also possesses the strongest magnetic moment of any element, and as a consequence it is the most widely used nucleus in MR procedures (Pagel-Langenickel *et al.*, 2010).

1.18 Nuclei and their alignment

The measure of the Earth's magnetic field is estimated at about 5.0×10^{-5} tesla [T = kg/Cs = 1 N/Am], standardized result 50 Ut, the hydrogen nuclei are randomly orientated in biological tissues and possesses a minimal magnetisation (Pooley, 2004). Hydrogen nuclei when subjected to an external magnetic field as when a patient is placed in a 1.5T scanner. These nuclei align themselves either parallel or anti parallel in the lower or higher energy states to Bo respectively, as demonstrated in 1.2 the nuclei spin around an axis.

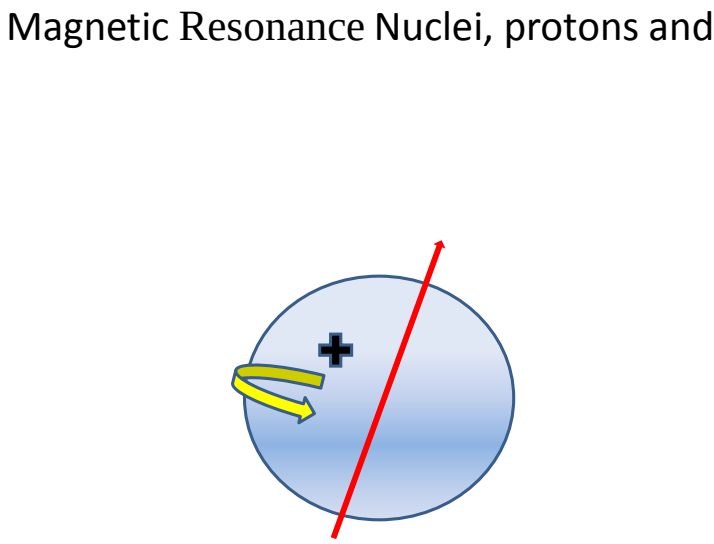


Figure 1.3 Spinning nuclei, behave like spinning tops around bo.

The energy states are almost equally populated as there is such a small energy differential between the two. The population ratio is according to Keller 100,000 to

100,006 (Keller, 1988). The nuclei are in fact tilted at an angle and behave like spinning tops, precessing around Bo as demonstrated in figure 1.3.

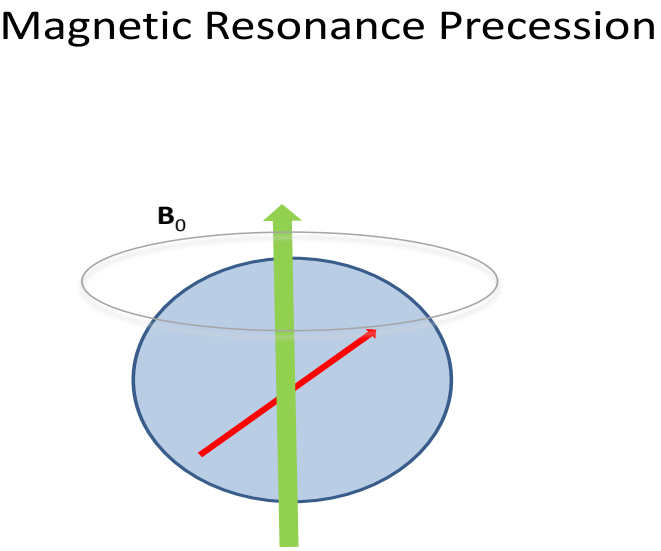
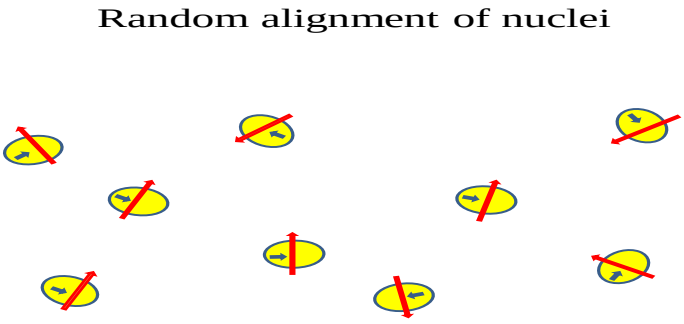


Figure 1.4 Nuclei precessing around Bo as demonstrated above.

As can be seen in figure 1.4 there is a random alignment of nuclei when in the absence of an external magnetic field.



.

Figure 1.5 Random alignment of nuclei when in the absence of an external magnetic field.

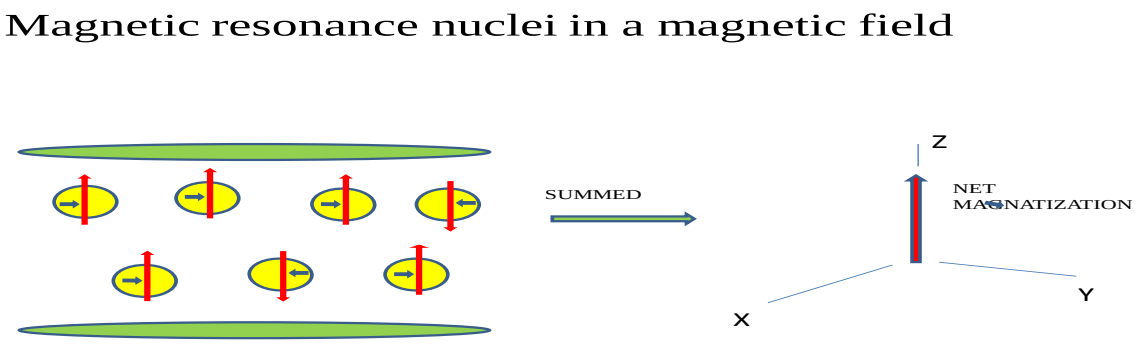


Figure 1.6 Magnetic resonance nuclei in magnetic field align parallel and anti-parallel

However following the application of an external magnetic field (Bo), as in a 1.5T scanner nuclei align parallel and anti-parallel, as can be seen in figure 1.5. Because of the influence of Bo this causes the hydrogen nucleus to wobble or precess, which can be likened to a spinning top as it comes to rest, the axis of the nuclei forms a path around Bo which is known as the precessional path. This precessional frequency is termed the Larmor frequency as expressed in the following equation (Keller, 1988) which shows the relationship between the previously mentioned factors.

Larmor frequency = gyromagnetic ratio x external field

(mHz) (mHz/Tesla) (Tesla)

This equation indicates that if the strength of the magnetic field changes this affects the speed at which hydrogen precesses dependant on the strength of Bo and is termed the precessional frequency. The precessional frequency of hydrogen in a 1.5T magnetic field is =63.86MHz. The precessional paths of hydrogen nuclei on an individual basis are random or out of phase, however they need to be in phase to

resonate. Resonance occurs when an object is exposed to an oscillating perturbation with a frequency which is close to its own inherent frequency of oscillation. This frequency in a 1.5T magnetic field can be found in the RF band of energy in the electromagnetic spectrum. Application of a radiofrequency pulse produces radiofrequency energy which following the law of electromagnetism, charged particles in motion will generate a magnetic field which is known as B_1. Which when applied as a pulse during MR sequences this causes B_1 to flip, the degree of which this net magnetization is deflected is termed the flip angle (Keller, 1988).

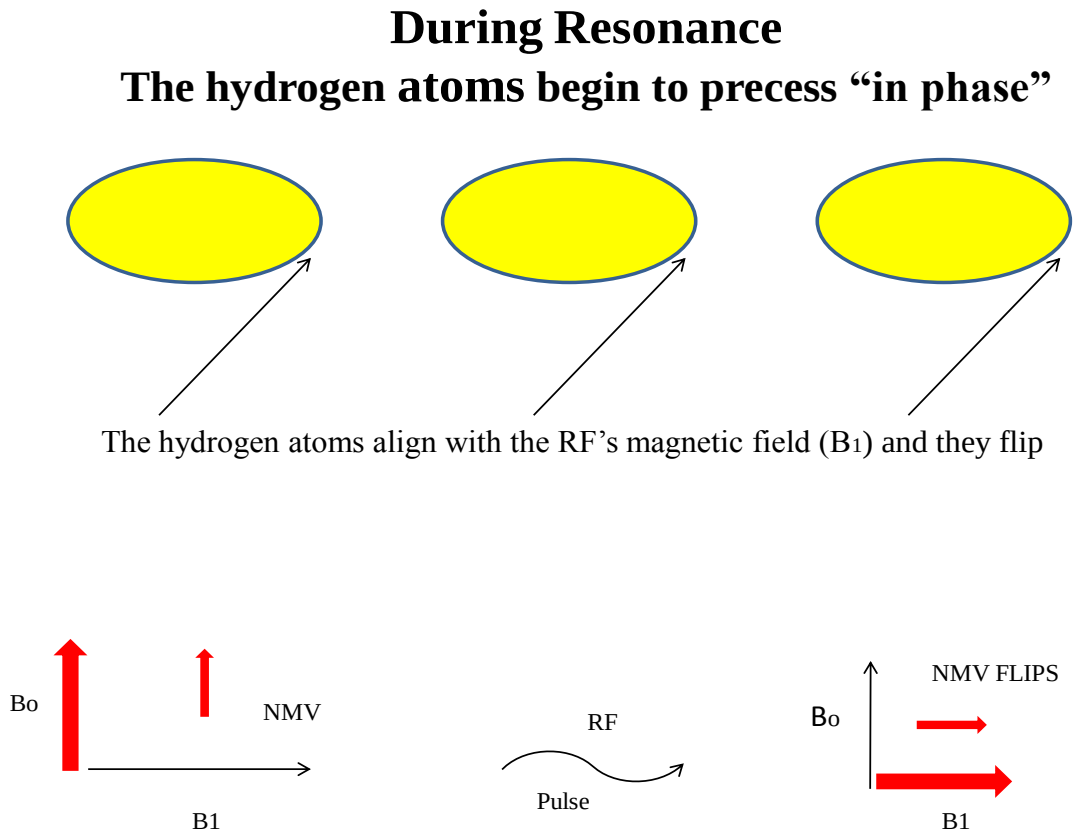


Figure 1.7 As can be seen in figure 1.6 the nuclei precess in phase in the B_1 plane this creates a changing magnetic field, the flip angle is proportional to the duration and amplitude of the RF pulse (Keller, 1988). This information is collected by the coil being used and is then encoded through mathematical calculations, the resultant data is stored; it is this data which formulates the image.

1.19 Magnetic Resonance Spectroscopy (MRS)

Magnetic resonance Spectroscopy can be used in order to assess microscopic chemical and physical information about molecules. It can identify various metabolites and biochemical components within body tissues non-invasively (Bachert & Schroder, 2003). MRI and MRS utilise atoms that have positively charged nuclei and possess a property called spin where they precess around their axis. A spinning charge generates a magnetic field that results in a magnetic moment proportional to the spin. Within their natural environment the nuclei exist randomly, when subjected to an external magnetic field as in an MR scanner, the nuclei act as tiny dipole magnets and will align either parallel or anti parallel to the external magnetic field. The speed at which the nuclei precess is dependent upon their inherent properties, the media in which they exist and the strength of the external magnetic field it is subjected to and is termed the precessional frequency. This difference causes a chemical shift i.e. small frequency differences between signals from protons therefore the intensity of the metabolic signal is directly related to its concentration (Bachert & Schroder, 2003).

The principles of MRS are broadly similar to Magnetic Resonance Imaging (MRI) however a number of differences do exist. The images obtained by MR use the whole proton signal from tissues dominated by fat and water protons. However other metabolites do not contribute to this imaging due to the small concentration of protons. MRS is designed to detect these small metabolites which resonate between the frequencies of fat and water. In order to do this the large water proton signal needs to be suppressed, smaller tissue metabolites are then detected from chemical

shifts, whereas in MRI this causes artefacts. This chemical shift provides information about the structure of the molecule.

MRS measures the chemical content of MR-visible nuclei including the metabolically relevant elements, for the purpose of this study Phosphorous was used (^{31}P). This is advantageous when assessing metabolism as the chemical properties of the nuclei of interest are expressed at certain frequencies within the MR spectrum, giving rise to peaks correspondent to these Phosphorus metabolites (Jeneson *et al.*, 2008). Repeated measurements of these metabolites and associated metabolic changes make MRS ideally suited in monitoring responses to the exercise intervention within this study, as it allows a real time dynamic monitoring of in vivo muscle metabolism and ATP turnover. Rate constant of PCR recovery can be monitored following the two periods of exercise from analysis of the 128 spectra taken during the MRS exercise protocol (Kemp & Radda, 1994). Within this study a ^{31}P radiofrequency (RF) coil was used as this has the sensitivity to assess the nuclei of interest, which within this study are gamma, alpha, delta ATP and Phospocreatine.

The magnetic fields need to be at least 1.5T to facilitate satisfactory MRS signals, a 3T scanner was used within this study as Phosphorous (^{31}P) has a lower Larmor frequency than hydrogen and a higher field strength is required in order to achieve good spectral resolution. Phosphorus is a major component in adenosine triphosphate (ATP) this being consumed and renewed in the conversion of sugars to energy and can therefore be used to study muscle metabolism at the mitochondria. Although MRS cannot completely replace the direct biochemical measurements obtained from muscle biopsy samples, it has distinct advantages that are not available with biopsies.

It provides a non-invasive direct measurement of muscle energy metabolite concentrations (glycogen, creatine phosphate, glucose 6-phosphate, inorganic phosphate, and lactate) with better time resolution, repeatability, and better precision (Kemp & Radda, 1994).

Exercise causes a decrease in the in the PCr signal and an increase in the Pi signal, changes in muscle PH can be detected by this shift in the Pi peak frequency. The spectral peaks can be converted into meaningful metabolic measures from the physiological concentrations. Using the rate constant of PCr recovery as a measure of mitochondrial function in skeletal muscle, phosphocreatine (PCr) recovery from sub maximal exercise has become a reliable and accepted measure of muscle oxidative capacity (Kemp & Radda, 1994).

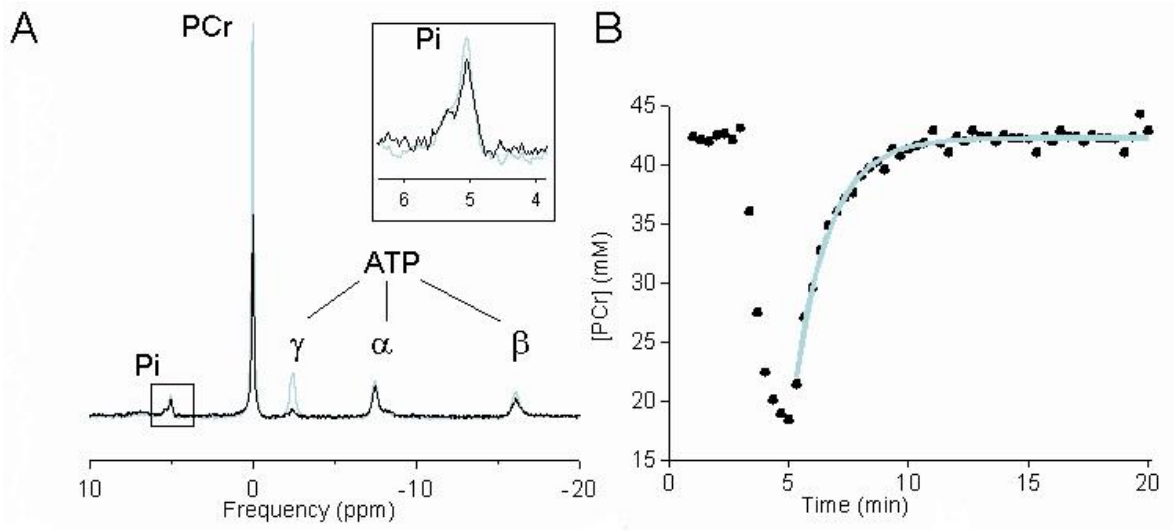


Figure 1.8 Rate constant of PCr recovery as a measure of mitochondrial function in skeletal muscle.

Figure 1.8A saturated transfer spectra of PCr and γATP in blue before depletion and highlighted in black post depletion, figure 1.8B shows Mono-Expodential fit of PCr

recovery following exercise. This has a place in investigating muscle physiology and energy metabolism and has applications in monitoring degenerative diseases and testing aerobic capacities. Mitochondrial function and Oxidative phosphorylation and glycosis account for the most important sources of cellular energy. The Mitochondria account for ATP production through oxidative phosphorylation, which as a consequence performance of Mitochondria is crucial for organ function, any dysfunction of the mitochondria can therefore play a role in a number of degenerative diseases including type II diabetes (Petersen *et al.*, 2003).

As muscle activity increases demand for energy within the myofibrils increases, to provide energy for the muscle contraction the high energy molecule adenosine triphosphate (ATP) is split into adenosine diphosphate (ADP) and inorganic phosphate as well as (H+) ions. In studying oxidative metabolism this is dependent on the relationship between oxidative turnover of ATP and the concentrations of ADP, phosphcreatine (PCr) and inorganic phosphate. Initially as muscle is exercised ATP use temporarily runs ahead of ATP synthesis, due to the workings of creatinine kinase equilibrium which ensures that this shortfall initially is made up but this is at the expense of a decrease in the concentration of PCr , this is associated with an increase in concentration of ADP, this plays a role in the feedback stimulation of oxidative ATP synthesis (Kemp, 2006).

In studying oxidative metabolism this can be approached by measurements of steady state and also recovery kinetics. As exercise increases there is a fall in PCr and a rise in Pi such that their sum remains constant ATP being buffered at a constant value (Kemp & Radda, 1994). At higher levels of exercise the pH falls moving the Pi peak

to the right as the chemical shift difference from PCr decreases. The time constants of PCr recovery allow evaluation of mitochondrial oxygen turnover within the muscle. ^{31}P MRS spectroscopy is thereby a unique method for measuring the achievements of the so-called power stations of the cell. Via this approach, the recovery of PCr from exercise in skeletal muscle is measurable (Kemp, 2006). For this reason in muscle physiology, mitochondrial function determined by the time constant of PCr recovery after a tiring load, is of particular interest as it is measureable.

1.20 Study Aims

NAFLD, which is characterized by lipid deposition effecting greater than 5% of hepatocytes, has an association with the metabolic syndrome and insulin resistance. It has been suggested that an impairment within the skeletal muscle in mitochondrial function may be a contributory factor to ectopic fat accumulation within the liver (Johannsen *et al.*, 2012). Therefore, the aim of this study is to use rate constant PCr recovery data to look at mitochondrial function in age and BMI matched controls versus subjects with Non Alcoholic Fatty Liver Disease.

1.21 Objectives

The objectives of this study is to recruit 15 controls and 15 subjects with known NAFLD age, BMI, and fitness matched, to investigate any differences in mitochondrial function. As evidence suggests that mitochondrial function within skeletal muscle may have an involvement in the development of obesity and insulin resistance (Menshikova *et al.*, 2006). We will determine body composition allowing

quantification of total fat, total sub-cutaneous, abdominal sub-cutaneous adipose tissue, total visceral, and abdominal visceral fat by carrying out whole body MRI. Liver fat will also be determined and quantified, this will be determined by Magnetic Resonance Spectroscopy, and results will be expressed as a percentage ratio of the CH2 lipid peak relative to the water peak from the spectra obtained.

We hypothesise that this investigation may enable us to determine if mitochondrial dysfunction within skeletal muscle has a link to ectopic fat accumulation within the liver, and the associated metabolic syndrome by altering energy storage. We will also obtain within this study other measurements including aerobic fitness, via VO_{2peak} testing, anthropometrics and various blood tests on all individuals, to look at any associations that may be apparent between the two groups.

CHAPTER 2

SUBJECTS AND METHODS

2.1 Inclusion & Exclusion criteria

Inclusion criteria

All subjects were over 18 years of age with no history of regular structured exercise or highly physical employment. Male participants consumed <21 units of alcohol per week and females <14 units, recorded by a screening questionnaire. A clinical diagnosis of NAFLD was based upon the following criteria;

(i) Exclusion of other causes of liver disease such as Hepatitis B and C serology, a negative auto-immune profile and normal caeruloplasmin concentrations;

(ii) Ultrasound appearances suggestive of fatty liver with no evidence of cirrhosis, in some cases the diagnosis was confirmed histologically by liver biopsy and tissue analysis.

Exclusion Criteria

Participants were excluded if they reported alcohol consumption that exceeded the recommended guidelines of >21 units a week for men and >14 units for females. Consumption of certain prescription medications known to cause secondary steatohepatitis, such as amioderone, tamoxifen or methotrexate were grounds for exclusion. Persons with a contraindication to attaining the various positions within the scanner due to shoulder mobility issues, as the prone position is required for two of the scans. Patients with type 2 diabetes were excluded as we are examining the involvement of insulin resistance when it is at a reversible stage and before beta cell failure has occurred. Individuals with contra indications for MRI scanning, for example those with metal implants for safety reasons and those who suffer from claustrophobia were also excluded.

In total 17 subjects with NAFLD (mean±SD) age 47±11yrs; BMI 31.6 ± 3.4 kg/m^2 and 18 controls matched for age (47±17 yrs) and BMI (30.5±5.2 kg/m^2). None of the controls were taking any prescribed medication and all had liver transaminases within normal range. Allocation of subjects to either the NAFLD or control group was only confirmed following confirmation that liver triglyceride content was either <5.5% for the controls or ≥5.5% for the NAFLD group. Analysis of data following MRS of the liver was assessed and quantified; some subjects originally joining the study as a control were allocated to the NAFLD group and one subject thought to have NAFLD was allocated to the control group.

2.2 Subject recruitment

Patients were recruited via the Liver clinics at Royal Liverpool University Hospital and University Aintree Hospital. Patients attending the Hepatology clinics who had been diagnosed with NAFLD and who on initial assessment met the inclusion criteria were invited to participate in the study. They were approached by their physicians or affiliated research nurses and the research study was verbally discussed. Follow up and initiation onto the study occured after participants had read the patient information sheet. The content and requirements of the study were discussed by me, as the facilitating research nurse, and any questions answered prior to obtaining signed informed consent, a copy of which was given to each participant for their records. Age and BMI matched controls were recruited following their response to local and internal adverts within the University of Liverpool and Aintree University Hospital.

All participants attended the laboratory on two different occasions. Measurements were taken following an overnight fast, they were also required to abstain from caffeine for a 12 hour period and not undertake strenuous exercise or drink alcohol for a 24 hour period prior to attending for their scan and blood samples.

2.3 Ethical considerations

The study conformed to the Declaration of Helsinki and was approved by the local research ethics committee. All participants were informed of the methods verbally and in writing before providing informed written consent prior to any tests being performed.

2.4 Safety of magnetic resonance imaging (MRI)

All individuals were initially screened either in person or over the phone prior to their attendance at the MRI centre; a number of individuals were excluded at this stage. On arrival, the subject was required to fill in a safety questionnaire. Whilst MRI is a relatively safe imaging technique, as it does not involve ionizing radiation, there are a number of hazards to both patients and staff working in the department. The MRI system and the surrounding environment are potentially hazardous. Primarily if a ferromagnetic object, for example one containing iron or steel, is close to the magnet it will experience a force known as ferromagnetic attraction. If this is close to the magnet the object can be turned into a projectile and therefore items such as scissors could therefore be fatal to the occupant of the scanner or staff in attendance. The larger the object the greater the force, even in the absence of field changes any ferromagnetic object will twist with force that is much greater than its

mass in an attempt to align along its axis with static field lines of force; this twisting is known as torque (Vonsmekal *et al.*, 1995).

This ability to twist objects and the magnets ability to turn objects into projectiles is a major risk facture; this ability extends beyond the immediate area around the scanner and is referred to as the fringe field, in order to ensure safety a controlled area is clearly defined. As well as completing a safety questionnaire, participants were also screened in person to ensure that they were safe to enter the scanning area by a member of staff who has the authority to sign the form passing the person as 'safe to scan'. Participants with any history of internal metal, pacemakers, or ferromagnetic metallic implants, intraocular foreign bodies or cerebral aneurysm clips were denied access to the scanning area. A number of volunteers were excluded from the study for safety reasons due to the presence of ferromagnetic objects. Some subjects had a history of eye injury due to industrial accidents and although they gave a history of the foreign object having been removed they were excluded as small fragments could remain. The scanner is noisy during operation this is caused by vibrations of gradient coils; levels of this can exceed safety guidelines as the noise increases with heavy duty cycles and sharper pulse transition. For this reason, all volunteers for the study were issued with ear-plugs. In addition to this during magnetic resonance spectroscopy of the liver, ear defenders were also used as this scan sequence was particularly loud.

2.5 Bio-effects.

A major safety consideration was the length of exposure time that volunteers were in the scanner, when both body composition and mitochondrial scans were both

performed on the same day. The body composition scan required four separate scans with positional changes for each data acquisition, total scan time of approximately one hour in a 1.5 Telsa (T) MR scanner. This was followed by a scan were the volunteer performed exercises in a 3T MR scanner lasting ~45 minutes. This required the Specific Absorption Rate (SAR) to be calculated, as the doubling of field strength from 1.5 T to 3T leads to a quadrupling of SAR. RF Pulse can be defined as a burst of energy; this energy rotates the magnetisation away from the axis of the magnet to create a transverse magnetisation. A 90 degree pulse will rotate the magnetisation by 90 degrees.

Radio frequency (RF) magnetic fields can result in energy deposition and produce tissue heating, SAR is a measure of tissue energy deposition, its unit of measure is Watt/Kg the limit for clinical examination is SAR<0.4W/Kg, in order to prevent soft tissue heating of >1 degree Celsius (Vonsmekal *et al.*, 1995). Ensuring the safety of participants in this instance involved entering each participant's weight into the computer during the scanning protocols, which gave a calculated SAR value and a scan time limit before the limit was reached.

2.6 Biochemical measurements

Plasma samples were analysed using the Olympus AU2700 analyser (Beckham Coulter, High Wycombe, UK). Fasting blood samples were taken for fasting glucose, also a further spun sample was used for insulin levels from which HOMA-IR as an index of insulin resistance was calculated, this is a method used to quantify insulin resistance and beta cell function using the equation (fasting glucose x fasting insulin / 22.5). Fasting Lipid profiles were also taken including total cholesterol Triglycerides,

low density lipoproteins (LDL), high density lipoproteins and cholesterol: HDL ratio was calculated. The intra- and inter-assay coefficient of variation was ≤10%. Low-density lipoprotein (LDL) was calculated according to the Freidwald formula. Serum liver enzymes were taken for aspirate aminotransferase (AST), Alanine aminotransferase (ALT) and Gamma-glutamyltransferase (GGT) with International Federation of Clinical Chemistry (IFCC) kinetic UV (without pyridoxal phosphate activation). A full blood count to gain platelet counts was also obtained, along with a further spun sample which was used to obtain free fatty acid levels. Caeruloplasmin levels were also taken to exclude Wilson's disease. All laboratory assays were performed in the clinical laboratory at the University Hospital Aintree, Liverpool.

2.7 Anthropometric Measurements

After a full medical history was obtained and a physical examination, a single observer performed all the anthropometric assessments to ensure continuity of measurements. Height was measured in all individuals using a Seca 216 wall mounted device rounded to the nearest 0.5cm, as height differed from the expressed height in patients and controls. Waist measurements were taken at the umbilicus and the hip at the greater trochanter to facilitate calculation of waist hip ratios in all participants. This measurement was recorded during the study as waist circumference is typically used in clinical practice to both quantify abdominal obesity and to make the diagnosis of metabolic syndrome. Whereas the waist hip ratio can be used to assess risk of fat related diseases, subjects may be at a healthy weight based on their BMI but waist hip ratios above 0.8 for females and 1.0 for males are associated with obesity and can be linked to increased risk of health complications and diseases including NAFLD and heart disease (Czernichow *et al.*, 2011). Assessment of body

composition was taken on all subjects using Tanita bio-impedance SC- 331S scales; this facilitated a print out of total body composition readings including: weight, fat percentage, fat mass, total body water, muscle mass, basal metabolic rate, bone mass, visceral fat indicator, and body mass index. Blood pressure was taken using a manual Reister Big Ben desk top sphygmomanometer from all subjects at the point of their attendance for their body composition scan.

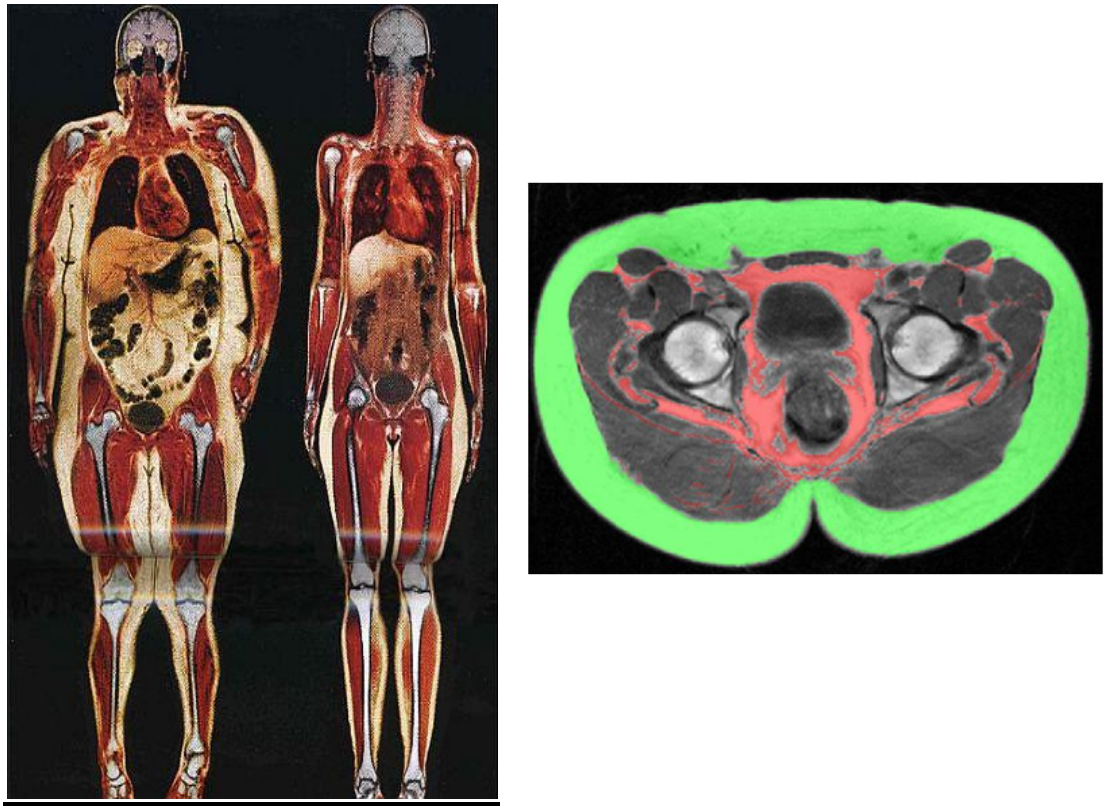


Figure 2 The Coronal view above left follows front to back, as though cutting through a corona, in medical terms anterior means the front and posterior means the back of the body. A transverse view, or Axial as left, is formed by a series of circumferential slices these transverse sections run from the top of the body (superior) to inferior or lower down the body. In the Coronal view in figure 2 the body fat can be seen as yellow/white in colour and in the axial view the fat mass is stained green in order to differentiate it from other tissues.

2.8 Maximal Oxygen Consumption Test ($\dot{V}O_{2peak}$)

All subjects were required to carry out a fitness test at the start of the study in order to assess their aerobic capacity. This was facilitated by using a treadmill Ergometer (H/P/ Cosmos, Pulsar 4.0, Nussdorf-Traunstein, Germany) within an air conditioned environment. The Modified Bruce treadmill protocol (Sotelo-Peryea *et al.*, 2010) was used which was designed in 1963 by Robert. A. Bruce, MD, as a non invasive method for estimating $\dot{V}O_{2peak}$ or maximal oxygen uptake. This was used to determine each of the participant's capacity to perform sustained exercise which is linked to aerobic endurance capability. $\dot{V}O_{2peak}$ refers to the maximum amount of oxygen that an individual can utilize during intense or maximal exercise. It is measured as "millilitres of oxygen used in one minute per kilogram of body weight" (ml/kg/min).

The treadmill speed and incline was progressively increased after an initial 2-min warm up which was at 2.2 $Km.h^{-1}$ on a flat gradient, thereafter step-wise increments in speed and grade were employed every minute. Their VO_{2peak} was calculated from minute to minute ventilation, this was measured using a pneumotach and simultaneous breath-by-breath analysis of expired gas fractions (Oxycon Pro, Jaegar, Germany). All machines used were calibrated before each test to ensure validity of results. All results were expressed relative to body weight, that is ($ml \cdot kg^{-1} \cdot min^{-1}$). Peak oxygen consumption was calculated as the highest consecutive 15 second period of gas exchange data which occurred in the last minute before exhaustion, which usually occurred due to leg fatigue or breathlessness. A respiratory exchange ratio (RER) of 1.15 combined with a maximal heart rate of 90% of age predicted maximal heart rate, which was calculated as 220 minus the participant's age. During

this $V0_{2peak}$ their heart rate was continuously measured (Polar Electro Oy, Finland). Participants condition was also monitored using the BORG scale this scale allowed participants to point to the level of perceived difficulty they felt at periodic intervals. 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 a 5% gradient.

2.9 MRI scanning and determination of body composition

In order to obtain data on body composition a Siemens Symphony 1.5 Tesla scanner (Siemens Medical Solutions, Erlangen Germany) was used to acquire imaging data. All scanning was carried out at the University of Liverpool Magnetic Resonance and Image Analysis Research Centre, a single experienced radiographer (VA) carrying out all scans (Gardner *et al.*, 2011). Quantification of total fat, total sub-cutaneous, abdominal sub-cutaneous adipose tissue, total visceral, and abdominal visceral fat was facilitated, by carrying out a whole body Magnetic Resonance scan. Whole body axial T1-images were obtained from a set of fast spin echo scans, with a slice thickness of 10mm followed by a gap of 10mm with a TR of 705ms and a TE 12ms using the integral body coil. This was acquired in 10 slice, contiguous blocks with table movement between to minimise in homogeneity. The resultant data being analysed by Vardis (Vardis Group Inc., London UK) using SliceOMatic (Tomovision, Montreal, Canada).

In this study whole body scanning was firstly used to produce quantification of total body fat for each participant, also intrahepatocellular and intramyocellular lipids were measured. On a number of occasions controls were found to have greater than 5% liver fat so they were reclassified as NAFLD and included within this group for

the study. Their GP was informed for their information and future treatment prescribed as required. There were four basic steps involved in obtaining an MR image; firstly the research volunteer was placed in the magnet where radio frequency pulses were sent by a radiofrequency (RF) coil. These signals were received by the coil and then sent to the computer for processing in order to get an image from which total body fat could be assessed and computed.

2.10 Liver MRS

MRS provides biochemical information by tuning the MR system to identify signals from different chemical nuclei such as Hydrogen (^{1}H) and Lipids (Ch^{2}), which are indicative of intrahepatocellular water and fat content (Reeder *et al.*, 2011). In the same imaging session as body composition scans quantification of intra hepatic fat was also gained by using Magnetic Resonance Spectroscopy (MRS) protocols, T2 weighted true fisp images through the liver were used for optimum positioning of the spectroscopy voxels. This was facilitated by placement of three voxel's within the liver to gain data on intrahepatocellular lipids avoiding ducts and vasculature within the liver structure. Voxel size was 20^{3}mm with an echo time TE of 135ms, TR 1500ms, with 64 acquisitions. An image segmentation software program was used to analyse the images, in which liver contours were manually drawn for each slice (Saeed *et al.*, 1998). Liver volumes were calculated from this by multiplying the liver tissue volume of each slice by slice thickness (6mm).

^{1}H MR spectra were acquired at 1.5T from the liver using a PRESS sequence, (TR 1500ms/ TE 135ms) without water saturation and with 64 signal averages, transverse images of the liver were used to ensure accurate positioning of the voxel in the liver.

The AMARES algorithm included in the MRUI software package jMRUI-3.0 was used to analyse ^{1}H MR Spectra in the time domain (Vanhamme *et al.*, 1997; Naressi *et al.*, 2001). After correcting for T1 and T2, peak areas for all resonances were obtained and lipid resonances were quantified with reference to water resonance (Thomas *et al.*, 2005). These T1 and T2 values for IHCL were obtained by a single experienced Radiographer for all subjects within the study to avoid any variance of operation.

Results are expressed as percentage ratio of the CH2 lipid peak area relative to the water peak area from the spectra obtained (Zhong *et al.*, 2009). To assess the reproducibility of the ^{1}H MRS results, analyses were repeated in triplicate and the coefficient of variation measured between the three voxels. Quantification of fat using ^{1}H MRS has been validated against gold standard biochemical measurements (Szczepaniak *et al.*, 1999). As previously described Magnetic Resonance Spectroscopy (MRS) protocols were used to gain data on the intrahepatic fat, using 3 different voxel positions figure 2.1 within the liver, taking care to avoid inclusion of intra hepatic ducts, vessels and any overtly fatty areas which appear as white areas as seen in figure 2.1.

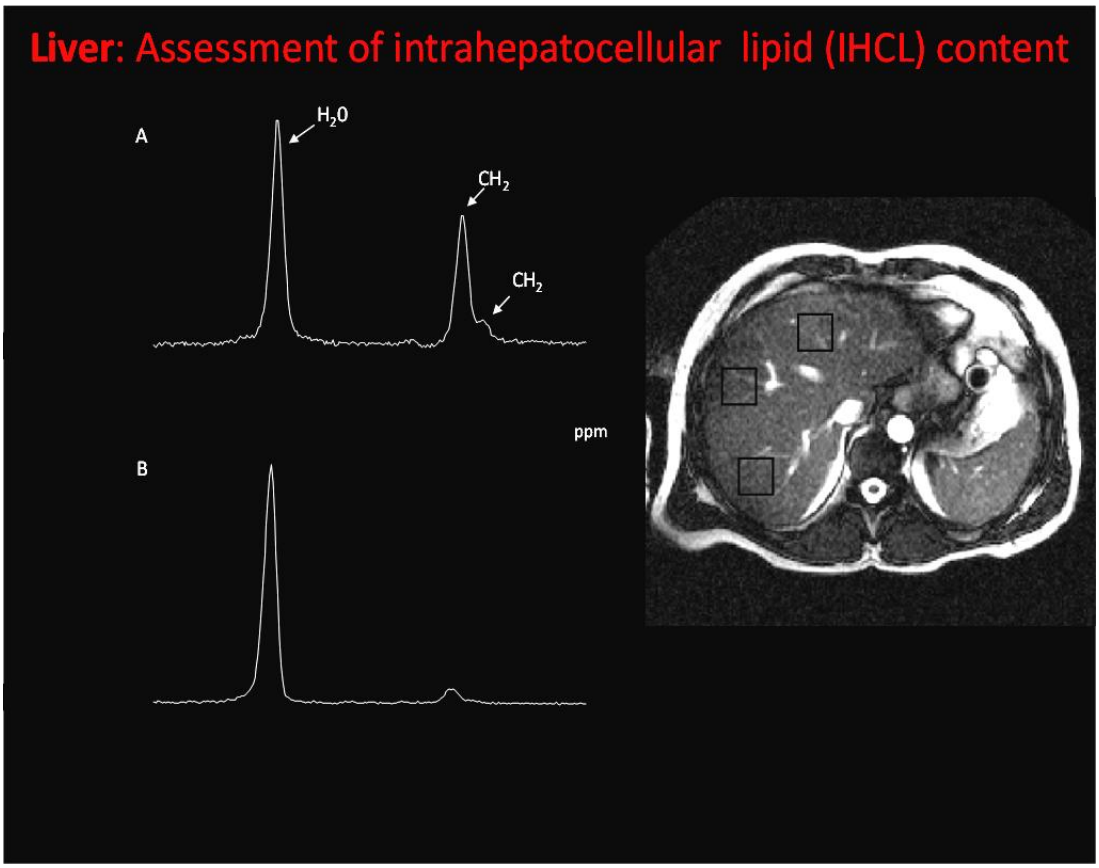


Figure 2.1 Voxel placements for liver spectroscopy.

Above can be seen an axial image of a liver with three voxel positions for liver spectroscopy, these can been seen as the black outlined box's within the liver structure. The positioning was facilitated by using different planes to allow three dimensional positioning. The associated hepatic lipid and water peak that are obtained for analysis and quantification can be seen in figure 2.2, this allowed an initial visual assessment prior to in depth analysis.

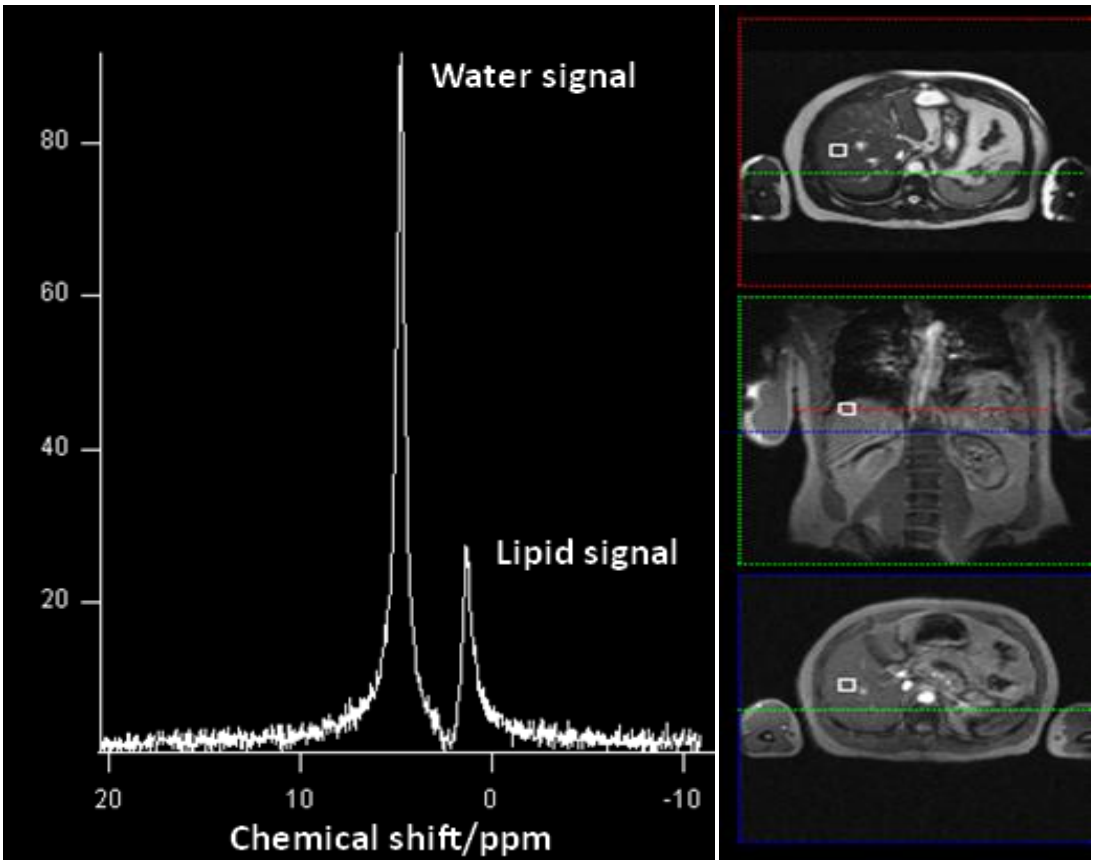


Figure 2.2 Liver spectroscopy peaks above left showing water and lipid signals, coronal middle right and axial scanning views above and bottom right

These coronal and axial views helps facilitate a three dimensional view of voxel placement as can be seen on the right in figure 2.2, the peaks on the left represent intrahepatecellular water spectrum whilst the lipid signal peak represent interhepatecellular lipids, measured in chemical shift in parts per million.

2.11 Magnetic resonance spectroscopy

This was carried out in both groups, to obtain a quantification of intrahepatocellular and intramyocellular lipids.

2.12 Liver spectroscopy NAFLD group

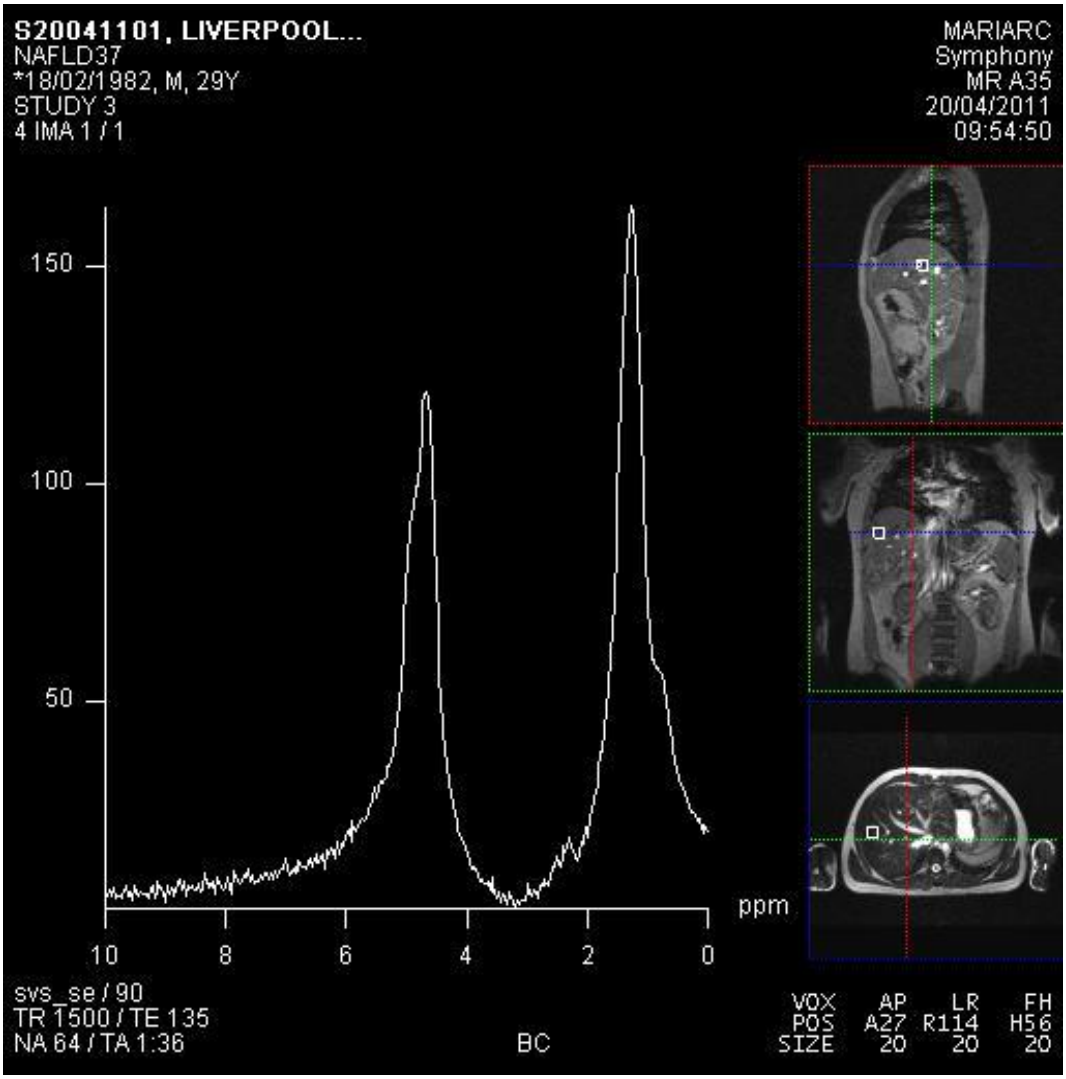


Figure 2.3 NAFLD group liver spectroscopy peaks, coronal middle right and axial scanning views above and bottom right

As in figure 2.3, liver spectroscopy from a NAFLD subject in the study, the peaks on the left represent the intrahepatecellular water spectrum whilst the peak on the right represents interhepatecellular lipids spectrum. The level of liver fat after quantification in this subject was 32.1%.

2.13 Liver spectroscopy Control group

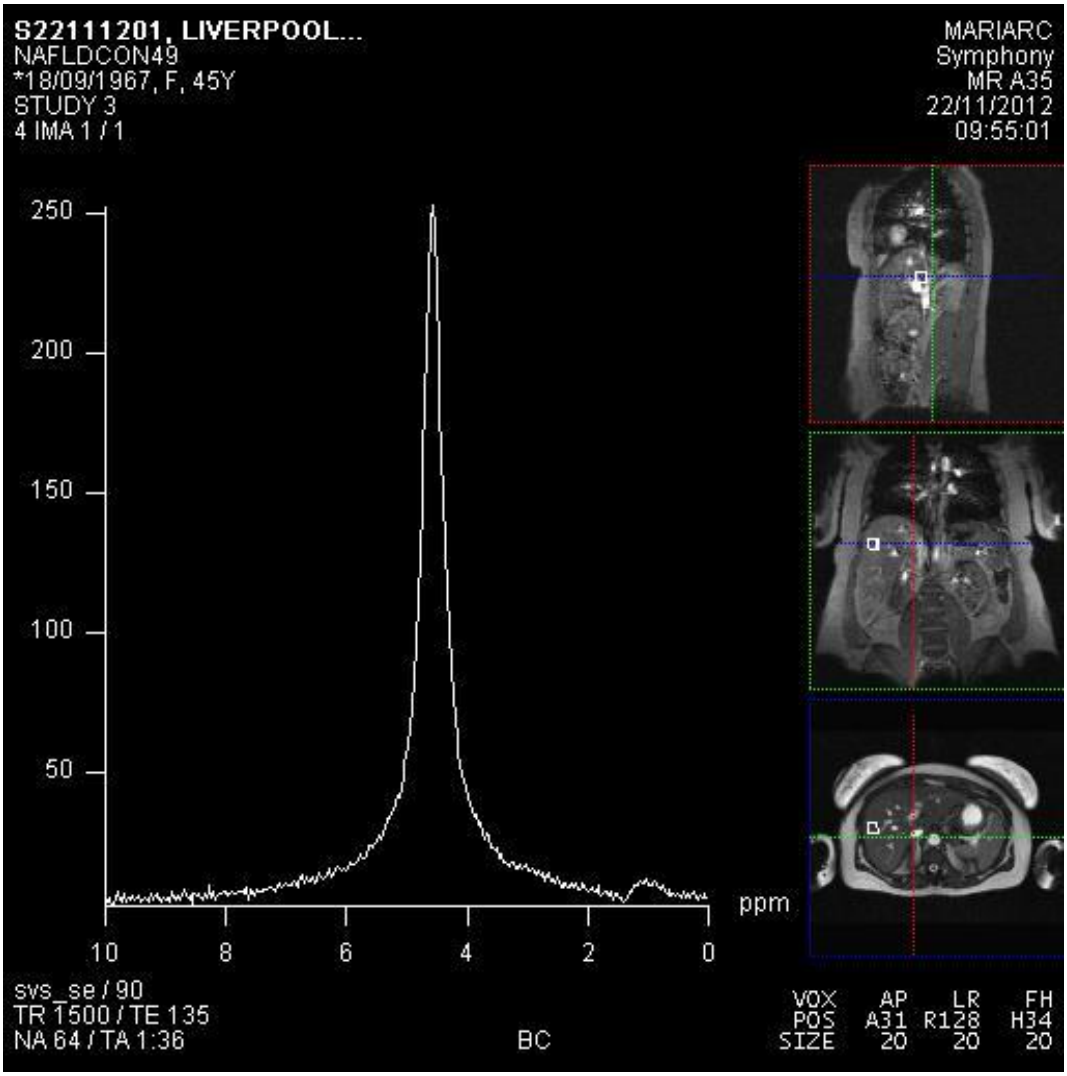


Figure 2.4 Control group, liver spectroscopy peaks, coronal middle right and axial scanning views above and bottom right.

Figure 2.4 shows the control group the peak on the right representing the intrahepatocellular lipid content was visibly lower and was 1.1% in this subject. This was carried out by referencing the voxel position in three planes, namely axial, sagittal and coronal, facilitating a near three dimensional guided placement. One interesting finding was that there was a variance in the levels of liver fat in some individuals within the three areas that were assessed, whilst in others it was relatively the same. The average of the intrahepatocellular lipid content from all three data

acquisitions was therefore used.

2.14 Intramyocellular lipid assessment

Image-guided, localised proton magnetic resonance spectra of the left soleus and tibialis anterior muscles were generated on a Siemens Symphony 1.5T scanner (Siemens Medical Solutions, Erlangen Germany). For this the subject lay supine within the MR scanner with a circularly polarized extremity coil around their left calf. Spectra were collected from a voxel similar to that used for the liver spectroscopy with the exception of when the musculature was too small requiring a smaller voxel size (15 x 15 x 20) in this case the number of acquisitions increased (to 200) to maintain the signal to noise ratio (SNR).

IMCL peaks were represented as CH_2 lipid amplitude in relation to total creatine amplitude following correction for T_1 and T_2 (Gardner *et al.*, 2011). In total four positional changes were required, total scan time was approximately one hour for the body composition scan. As illustrated in figure 2.5 the tibialis anterior and the soleus muscles were used to obtain quantification of intramyocellular lipids for both NAFLD and control groups within the study. Anatomy and voxel placement varied when detemining lipid content, the placements being made to avoid blood vessels and overtly fatty areas of muscle tissue.

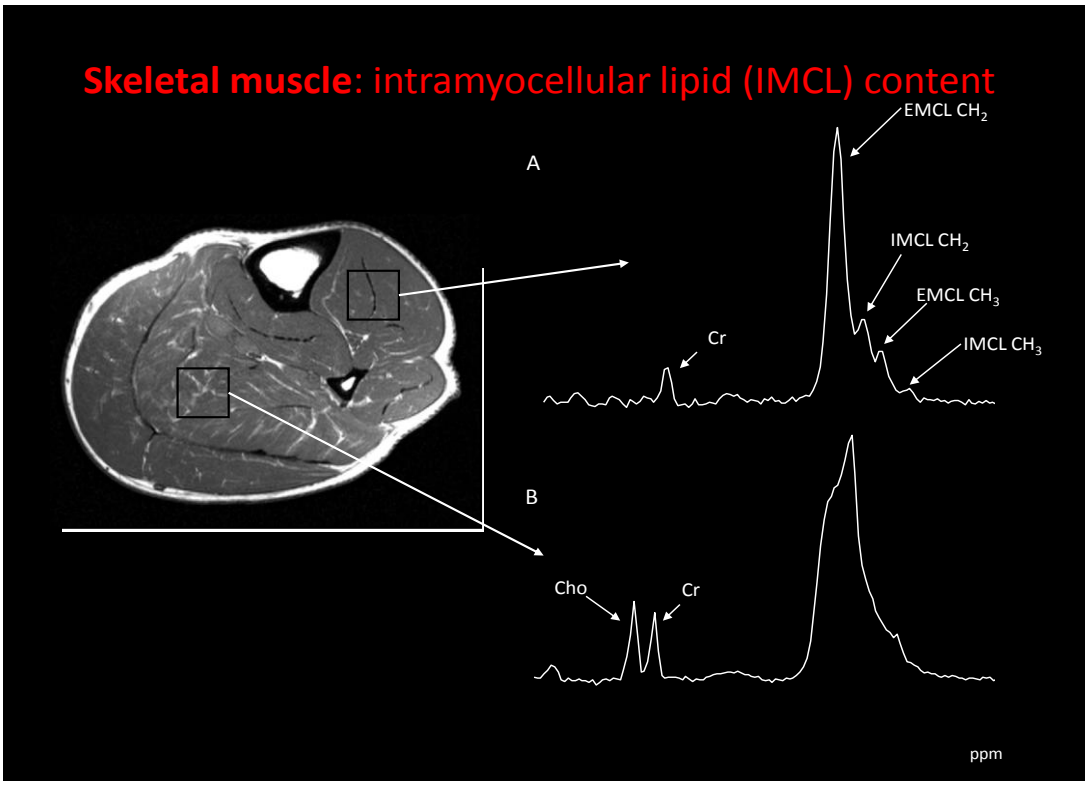


Figure 2.5 Axial views of the Soleus and Tibialis Anterior muscles showing voxel placement and intramyocellular lipid data above right

Intramyocellular fat was also assessed by using Magnetic Resonance Spectroscopy (MRS) by placement of two voxels within the tibialis anterior and the soleus muscle (Figure 2.5). In all total fat, total subcutaneous, abdominal subcutaneous, total visceral, abdominal visceral, liver fat %, and Intra-myocellular fat in the tibialis and soleus muscles were measured in all subjects for the study.

2.15 Skeletal muscle Intramyocellular Lipid assessment

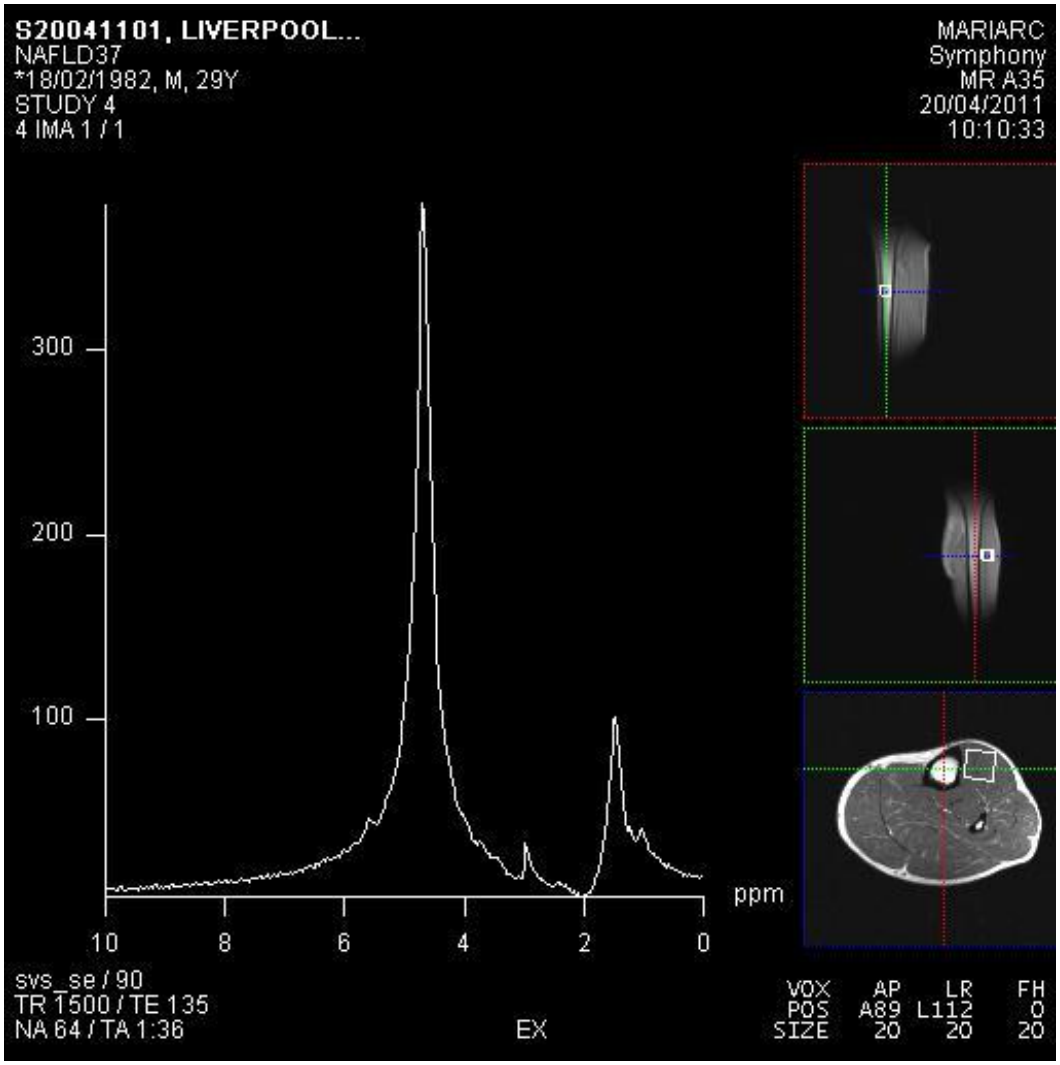


Figure 2.6 MRS of the Tibialis anterior muscle spectroscopy peaks the larger peak on the left being intramyocellular water whilst the peak on the right is intramyocellular lipids, coronal and axial scanning views above right.

The views in figure 2.6 are representative of voxel placement in the tibialis anterior muscle, whilst in figure 2.7, can be seen voxel placement in the soleus muscle.

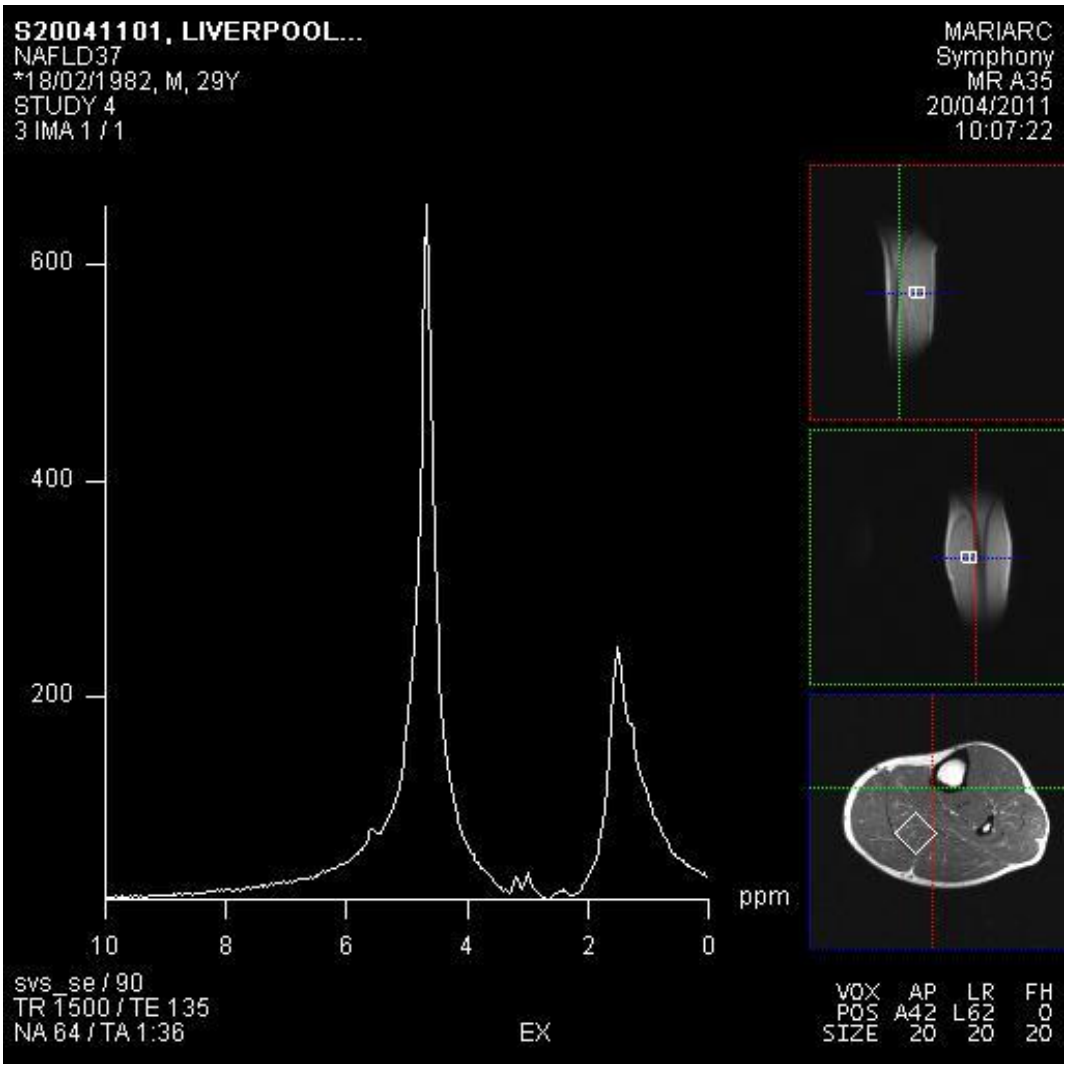


Figure 2.7 MRS of the Soleus muscle spectroscopy peaks the larger peak on the left being intramyocellular water whilst the peak on the right is intramyocellular lipids, coronal and axial scanning views above right.

The intramyocellular lipid content was 3.6% in the tibialis and 11.7% in the soleus muscle within this subject from the NAFLD group.

2.16 Background to methods

The focus of this study is to use rate constant PCr recovery data to look at mitochondrial function in normal controls versus subjects with NAFLD, taking into consideration other measurement modalities of aerobic fitness. VO_2 peak is

expressed either as an absolute rate in litres of oxygen per minute (l/min) or as a relative rate in millilitres of oxygen per kilogram of bodyweight per minute (ml/kg/min). This last modality, VO_2 Peak ml/kg/min has been used in testing on all subjects within this study.

MRS provides biochemical information by tuning the MR system to identify signals from different chemical nuclei such as Hydrogen (^{1}H) & Phosphorous (^{31}P), which are indicative of intracellular muscle fat and high energy phosphates within the body respectively. The use of non-invasive ^{31}P MRS in vivo protocols were used to measure inorganic phosphate (Pi), phosphocreatine (PCr), and Adenosine Triphosphate (ATP) within the muscle tissue to track changes in cell recovery.

2.17 MRS Quadriceps Muscle

For both the NAFLD and control groups ^{31}P MRS assessments of muscle mitochondrial function in the quadriceps muscle were carried out using a Siemens 3T Trio MR scanner (Siemens AG, Erlangen, Germany) using an isometric knee extension exercise protocol as developed by the Magnetic Resonance and Image Analysis Research Centre facility at Liverpool University (Kemp GJ, 1993). Subjects lay supine (secured with a Velcro strap across the hips) with the right knee flexed by c. 30° over a rigid foam support in a custom-built rig permitting isometric knee extension exercise against a strap across the anterior lower shin/ankle connected to an aluminium bar fitted with a strain gauge. ^{31}P MRS data were acquired from right quadriceps muscle using a dual-tuned 15cm/18cm diameter $^{31}P/^{1}H$ surface coil (RAPID Biomedical, Rimpar, Germany), Velcro-strapped to the anterior thigh (midway between anterior superior iliac spine and patella). After automated set-up

and manual shimming using tissue water, an 8-scan fully relaxed (TR=10s) and a 32-scan partially saturated (TR=2s) resting spectra were collected. The exercise protocol consisted of 1.1 min rest followed by 2 bouts of isometric exercise each followed by 5.2 min recovery periods, while spectra were collected (TR=2) every 8 s paced at 0.25 Hz (2 s on, 2 s off) by an audible cue, exercise force being feedback visual via an LED display visible at the end of the bore through a head mirror. Two exercise intensities were used, corresponding to 70% and 90% of maximal voluntary contraction established in 3 brief trials prior to MRS acquisition (in pilot experiments these intensities were found to give acceptable PCr depletion with minimal acidification in typical subjects (Kemp, 2006). All spectra were acquired with Nuclear Overhauser enhancement (NOE), using 1H spins were saturated by 10 radiofrequency pulses (900 flip angles), 5 ms pulse duration, and 10 ms interpulse delay. Block MRS data output files from exercise-recovery acquisitions were converted to text using a specially-written Matlab routine (courtesy of Dr Damian Tyler, University of Oxford). All ^{31}P MRS data were processed using the java-based Magnetic Resonance User Interface (jMRUI v.3.0), using the AMARES time-domain fitting algorithm. Data were fitted assuming Lorentzian line shapes for PCr, Pi, PDE, PME, NADP and ATP (β-ATP a 1:2:1 triplet, α-ATP and γ-ATP both 1:1 doublets), using prior knowledge files kindly supplied by Dr Damian Tyler, University of Oxford. The chemical shift of the inorganic phosphate (Pi) peak relative to phosphocreatine (PCr) (σ parts per million) was used to determine intracellular pH. PCr recovery time courses were fitted to a monoexponential function to determine the recovery rate constant (k min^{-1}). In the absence of appreciable changes in pH (as here), this is accepted as a measure of effective muscle mitochondrial function, the latter being a system property which reflects

cardiovascular oxygen supply and mitochondrial oxygen usage (Johannsen *et al.*, 2012).

The purpose of this study was to prospectively evaluate subjects using this method by the noninvasive assessment of muscle metabolites during exercise. Hydrogen (^{1}H), inorganic phosphate (Pi), phosphocreatine (PCr), and Adenosine Triphosphate (ATP) within the muscle were measured by magnetic resonance (MR) spectroscopy. Peaks were measured during muscle contraction allowing the kinetics of PCr rate constant to be assessed continuously before, during, and after a period of exercise and quantified post scan for statistical analyses. Using this PCr rate constant muscle oxidative capacity was therefore measured as it allowed mitochondrial oxygen turnover within the quadriceps muscle to be obtained using this unique method of 31P Magnetic Resonance Spectroscopy.

From this data differences in PCr rate constant between the NAFLD group and age and BMI matched controls were assessed, the two groups being well matched with no significant differences in age, total body weight, body mass index or percentage body fat mass (table 3.1).

2.18 Mitochondrial function assessment protocol

The use of non-invasive ^{31}P MRS in vivo protocols were used to measure inorganic phosphate (Pi), phosphocreatine (PCr), and Adenosine Triphosphate (ATP) within the muscle tissue to track changes in cell recovery during the programme. Mitochondrial function was assessed during the study by measuring Phosphocreatine (PCr) recovery kinetics.

MRS provides biochemical information by tuning the MR system to identify signals from different chemical nuclei such as Hydrogen (^{1}H) & Phosphorous (^{31}P), which are indicative of intracellular muscle fat and high energy phosphates within the body respectively (Kemp & Radda, 1994). Changes in cell energy expenditure and recovery were measured during a scan in which subjects performed leg extension exercises for a set number of repetitions. These were at two points of intensity 25 repetitions at 90% followed by 15 at 70%. This exercise resistance methodology to facilitate assessment of the leg muscle within the scanning protocols was developed by the Magnetic Resonance and Image Analysis Research Centre facility at Liverpool University. The quadriceps muscle was used for this assessment, for practical reasons, as not many muscles are accessible to ^{31}P MRS from an anatomical or practical perspective, also the quadriceps is probably less vulnerable to subclinical microvascular disease than the calf muscle. Due to the set up of the exercise equipment the right leg was used in all but one subject during the study.

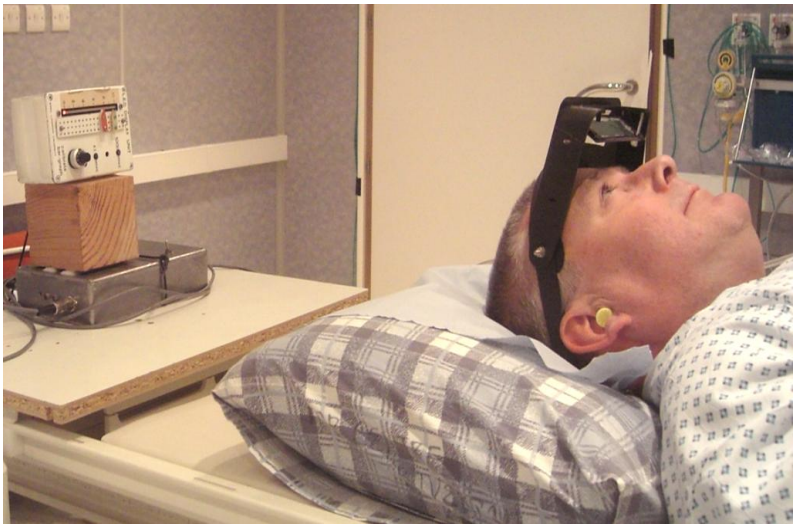


Figure 2.8 LED bar graph readout showing arrows at 70% and 90% of MVC to ensure accuracy of exercise effort at the timed intervals of repetition.

Figure 2.9 Exercise rig positioning and ^{31}P coil placement over the quadriceps muscle to enable data acquisition.

Figure 2.10 final positions in the scanner for data acquisition all but the head being enclosed.

The subject received feedback on how much force they generated from viewing an LED bar graph above their head via a head band mounted mirror figure 2.8. Their maximum ability was firstly assessed by performing a maximum voluntary contraction (MVC) necessitating achieving a 100% contraction as verified by the LED display and also this was recorded on the graph master in the scanner control room for reference purposes. Two points were then used during the exercise phase which were staged at 70% and 90% of their (MVC) and highlighted by red and green

arrow markers on the display. Detailed instructions were given and a test period was used for familiarisation purposes to ensure all participants were aware of the protocol and the exercise requirements, which were in time with a beeper at pre set intervals for the duration of the scan. This ensured that each participant performed all the required exercises to the same exertion levels of 70% and 90% of their individually assessed MVC, and for the same number of repetitions in order to ensure the validity of the results obtained.

The exercise rig structure in figure 2.9, used for the quadriceps muscle studies with a curved phosphorus ^{31}P RF coil mounted on the anterior quadriceps muscle and the NIRS light guides again mounted laterally. A strap across the abdomen prevents abdominal muscles contributing to the force generated, as there is a tendency for subjects to tilt their pelvis in order to assist in generating additional force as muscle tiredness increases towards the end of the exercise periods.

A voice intercom was used to instruct all volunteers to ensure safety and comfort, and an alarm was also hand held so that each participant could alert the scanner operator should they wish to stop the study. A visual display was also available in the control room to observe participants during the exercise element of the study. Figure 2.10 shows the final position in the scanner this is with the volunteer fully enclosed with the head only protruding; individuals who became claustrophobic could not tolerate this and were unable to proceed with the scan. Individuals with known claustrophobia were differed at the initial screening stage to avoid this happening at the scanning stage as much as possible.

2.19 Points used for measurement

Magnetic resonance spectroscopy (MRS) offers a non-invasive window on cellular metabolism in vivo. The main techniques used in vivo are phosphorus ^{31}P MRS, which yields information about energy turnover and proton handling, combined with near-infrared spectroscopy (NIRS), an optical technique which reports muscle oxygenation (Kemp & Radda, 1994).

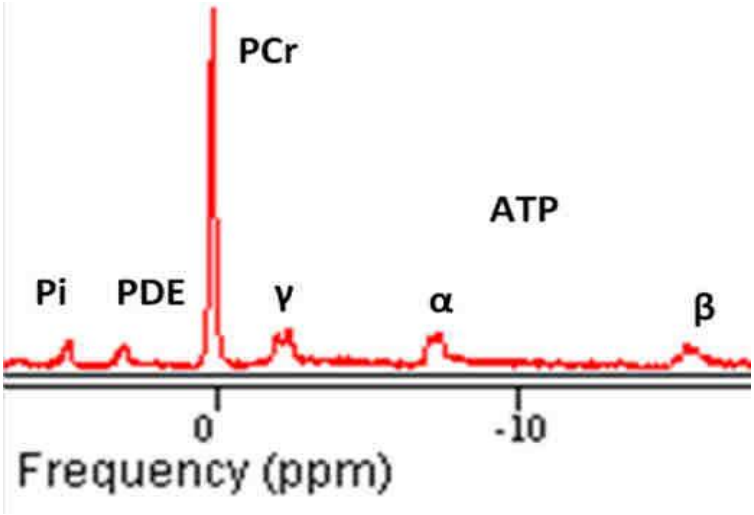


Figure 2.11 Points for assessment diagram, showing peaks representing inorganic phosphate, phosphocreatine, gamma, alpha and beta ATP.

Resting ^{31}P MRS spectrum from human quadriceps muscle, showing the fit generated by jMRUI, the software used to quantify the peaks. As previously stated initially the subject was asked to do a maximum voluntary contraction (MVC) in order to ascertain their 100% value, following which they exercised for a number of repetitions in time with a timed beeper at 90% of their maximal contractile force followed by a rest period after which the process was repeated at 70% of MVC.

Initially after a series of scans to shim the magnet and obtain the best spectra form, 8 resting spectra were obtained this scan lasting 1.1 minute followed by the acquisition

of 33 spectra over 3.1 minutes at 90% MVC. This was followed by 39 spectra being obtained during 5.2 minutes of recovery. During the second phase of exercise at 70% MVC 15 spectra were obtained over a two minute period, after which during the second recovery phase 39 spectra were then obtained over a 5.2 minute period.

Figure 2.12 Monitor showing two wave forms of exercise repetition which represent leg extension effort and timing, this allowed for a visual check to be made at the data acquisition stage.

As in figure 2.12, a graph was viewed and recorded in the control room to ensure the subjects accuracy and timing of the exercise repetitions in time with an electronic beep. Each exercise effort being initiated following an audible sound and held for the prescribed time. PCr rate constant was used to assess mitochondrial degradation and recovery kinetics; this was calculated manually by assessment of the spectra acquired following the scan. The spectra obtained at base line during the initial resting period and after each exercise period were checked on the monitor following the scan to

check on data acquisition and subject compliance with the protocol.

Figure 2.13 Monitor showing PCr baseline top left of the screen and depletion after exercise at 70% top right and lastly bottom left after exercise at 90% of MVC.

Figure 2.13 shows the top left hand box represents PCr at rest, the top right hand box represents data acquired immediately after exercise at 70% of maximum voluntary contraction (MVC) and the bottom left after exercise at 90% of MVC, the level of depletion can be clearly seen. In all 128 spectra were recorded with two recovery periods between the two exercise efforts. This was to allow wash out periods at the end of the two data acquisitions. This also allowed for a recording of recovery time and monitoring of the return period to the initial base line measurements, total scan time was 17.1 minutes.

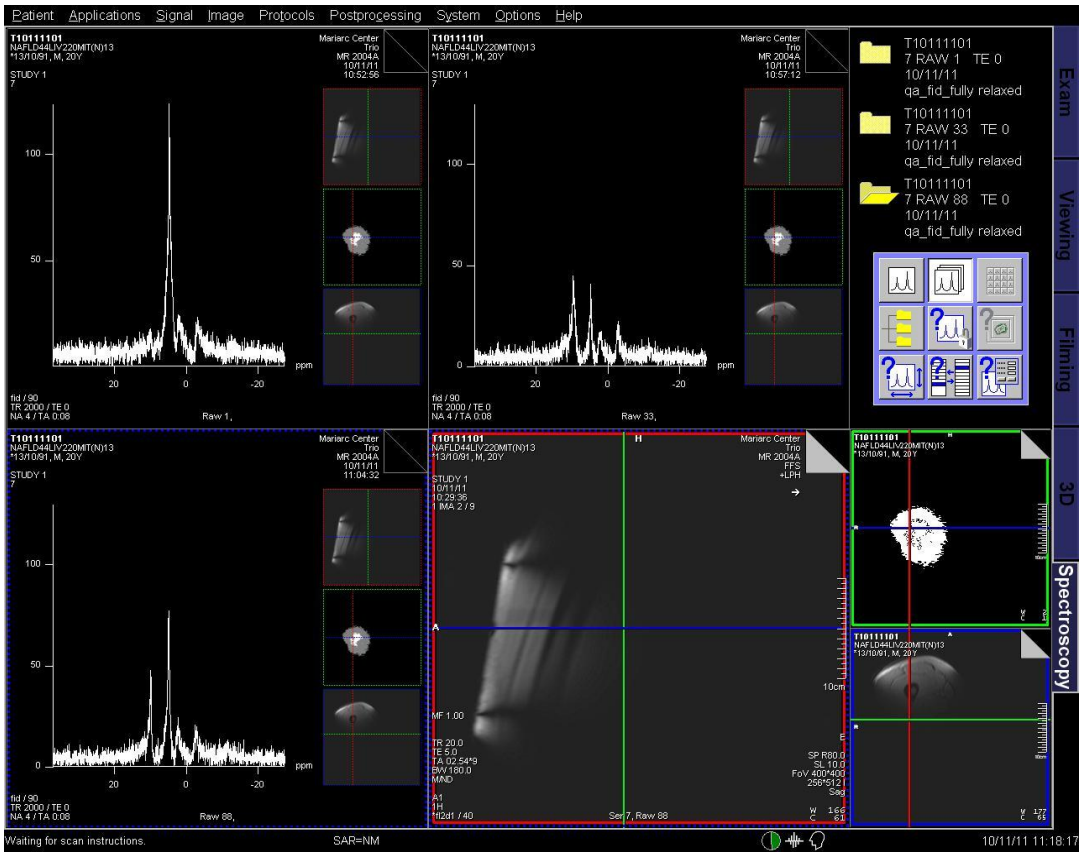


Figure 2.14 PCr depletion and recovery peaks in this post scan check differ in screen placement from the previous figure 2.13, with the base line peaks being top left, after exercise at 70% of MVC the peaks can be now be seen bottom left, top right the peaks are representative of PCr depletion after exercise at 90% of MVC

2.20 Patients

Seventeen patients with known NAFLD and 18 age and BMI matched controls were studied using these protocols. Within the NAFLD group there was 11 males and 6 females, in the control group we had 7 males and 11 females.

2.21 Statistical analysis

The primary outcome variable for the present study was the PCr recovery rate constant *k*. Groups were compared using the two-sample Student's *t* test or Mann–

Whitney *U* tests if distributional assumptions were not met following logarithmic or Box–Cox transformation. To assess the sensitivity of some of the results to the gender imbalance between groups, we also compared groups using linear regression/analysis of covariance (ANCOVA), adjusting for sex. Poisson's regression was used to compare the number of metabolic components between groups. Spearman's correlation coefficient (*rs*) was used to describe the strength of association between continuous variables. Statistical significance was delimited as $P<0.05$. Data are presented as means [95%confidence intervals (CIs)], unless stated otherwise, and exact *P* values are cited (values of *P* of '0.000' provided by the statistics package are reported as '<0.001'). All analyses were conducted using Stata 13.

CHAPTER 3

RESULTS

3.1 Results

The two groups (patients with NAFLD and control subjects) were well matched with no significant differences in age, total body weight, body mass index or percentage body fat mass (*Table 3.1*). Despite this matching of weight, BMI and total adiposity, the patients with NAFLD had a significantly greater waist circumference 108.6± (9) versus controls 100.8±(11.8) indicating increased central obesity. There were a greater proportion of males in the NAFLD group while there were a greater proportion of females within the control group (*Table 3.1*). Cardio respiratory fitness $\dot{V}O_{2peak}$ measured as (ml/kg/min), even when normalized to total body weight did not differ significantly between the two groups.

3.2 MRI scanning and determination of body composition

In total, 35 individuals were studied this included 17 individuals with known NAFLD 11 males and 7 females and 18 controls, 8 males and 10 females.

3.3 Magnetic resonance spectroscopy

Table 1 demonstrates a marked difference in liver fat between the two groups with the NAFLD group having a liver fat of 24.8±2% *vs*. 2.2±1.5% in the control group. However IMCL in both the soleus and tibialis anterior was not significantly different between NAFLD and control individuals (Figure 3).

3.4 Biochemical characteristics and components of the metabolic syndrome

Patients with NAFLD had a significantly higher ALT but AST and GGT were not significantly different (Table 1). Although total cholesterol was not significantly different, patients with NAFLD had a significantly higher triglyceride and lower

HDL cholesterol. Fasting glucose concentrations did not differ significantly between the two groups. The prevalence of metabolic syndrome was greater in the NAFLD group (*n*=13, 76 % *vs.* *n*=5, 28%) and the patients with NAFLD had a higher mean number of components of the metabolic syndrome (Table 1).

3.5 MR-derived measures of body composition

Total abdominal fat did not differ between NAFLD patients and controls although when this depot was subdivided into subcutaneous and visceral fat, there was clearly a greater proportion of visceral fat in patients with NAFLD (Table 1). As expected, liver fat was higher in NAFLD patients but IMCL fat in both soleus and tibialis anterior was not significantly different between the two groups (Figure 3).

3.6 Assessment of muscle mitochondrial function

There were no significant differences in resting muscle intracellular pH (pH_i) or phosphate metabolite ratios between the two groups. There was no significant cytosolic acidification during exercise in either group. The post exercise PCr recovery rate constant (a measure of muscle mitochondrial function *in vivo*) did not differ significantly between the two groups.

3.7 Skeletal muscle pH and phosphorus metabolites

Resting muscle intracellular pH between the two groups showed no significant difference and also there was no significant acidification during exercise in either the NAFLD or control group. Resting phosphate metabolic ratios of inorganic phosphate: ATP and PCr: ATP also did not differ between the two groups and there was no significant difference in the amount of PCr depletion during the exercise

protocol at either 70% or 90% of MVC.

3.8 Relationship of muscle mitochondrial function with the metabolic syndrome (MS) and the number of MS components

Comparing the two groups according to the presence (*n*=18) or absence (*n*=17) of the metabolic syndrome, we were not able to detect any significant difference in the PCr recovery rate constant with a difference of 0.04 (k min^{-1}). Similarly, when we examine the number of components of the metabolic syndrome in both controls and NAFLD patients we found no correlation (*r*=-0.03, *P*=0.83).

3.9 Correlation of muscle mitochondrial function with biochemical and body composition measurements

There were no significant correlations between PCr recovery constant and age, weight, BMI, fat mass, VO_2 peak, any biochemical measure or any MR-derived measure of body composition.

Table 3.1 Baseline characteristics of participants (mean +/- SD).

	NAFLD (*n*=17)	**Controls (*n*=18)**	***P***
Age (y)≠	47 (41,52)	47 (34,51)	**0.68**
Gender (M,F)	11 (65%), 6 (35%)	7(39%), 11(61%)	**0.13**
Weight (kg)	96.1 (14.8)	85.6 (15.7)	**0.05**
BMI (kg/m^2)	31.5(3.4)	30.53(5.24)	**0.5**
% fat mass≠	40.5 (30.1,44.1)	34.2 (29.2,44.8)	**0.69**
Waist circumference (cm)≠	108.6 (9)	100.8 (11.8)	**0.03**
Systolic blood pressure≠	125(120,140)	123.5(120,132)	**0.62**
Diastolic blood pressure≠	82(78,88)	75(70,80)	**0.03**
Biochemical			
AST (mU/l)≠	24(9.5,42.5)	21(16,25)	**0.37**
ALT (mU/l)≠	61 (30,83)	21 (16,32)	**0.001**
GGT (mU/l)≠	48(42,79)	28(23,40)	**0.03**
Cholesterol (mmol/l)≠	5.9(5.1,6.1)	5.2(4.7,5.7)	**0.54**
HDL≠	1(.9,1.2)	1.2(1.1,1.5)	**0.007**
Triglyceride (mU/l)≠	2.3(1.6,3.4)	1.3(1,2.3)	**0.007**
Fasting glucose (mmol/l)≠	5(4.7,5.3)	4.85(4.4,5.1)	**0.18**
Metabolic syndrome (MS)			
Number with MS	13/17 (76 %)	5/18 (28 %)	**0.004**
Components of MS†	2.9 ± 1.0	1.8 ± 1.2	**0.05**
Cardio respiratory fitness			
$V0_2$ peak (ml/kg/min)≠	23.5(20,31.3)	25.75(22.9,30)	**0.69**
$V0_2$ peak/weight (ml/min)≠	0.3(0.2, 0.3)	0.3(0.2, 0.4)	**0.14**
Muscle ^{31}P MRS*			
Resting Pi/ATP≠	0.56 (0.49, 0.63)	0.44 (0.38, 0.58)	**0.09**
Resting pH†	7.08 (7.06, 7.09)	7.08 (7.06, 7.10)	**0.39**
End-exercise pH change	0.14±0.12/ -0.06±0.09	0.12±0.13/ -0.10±0.09	**0.07/0.62**
Resting PCr/ATP	4.25±0.68	4.07±0.57	**0.4**
End-exercise PCr depletion (%)*≠	0.19 (0.14/, 0.26)/0.35 (0.24, 0.50)	0.20 (0.08, 0.29)/0.32 (0.12, 0.44)	**0.82/0.75**
PCr recovery rate constant (k.min^{-1})≠	1.62 (0.34, 1.86)	1.50 (1.18, 1.77)	**0.81**

Values are means±S.D, medians (lower quartile, upper quartile) or frequency (%).
*Pairs of results represent 70%/90% MVC (maximal voluntary contraction) separately. ≠Mann–Whitney *U* test; †Poisson regression.

3.10 Body composition data

Body composition data demonstrating total abdominal fat, abdominal subcutaneous (SC) fat, visceral fat, intramyocellular fat (IMCL) of soleus and tibialis anterior muscles, liver fat and the number of components of the metabolic syndrome in patients with NAFLD (*n*=17) and age and BMI-matched controls (*n*=18).

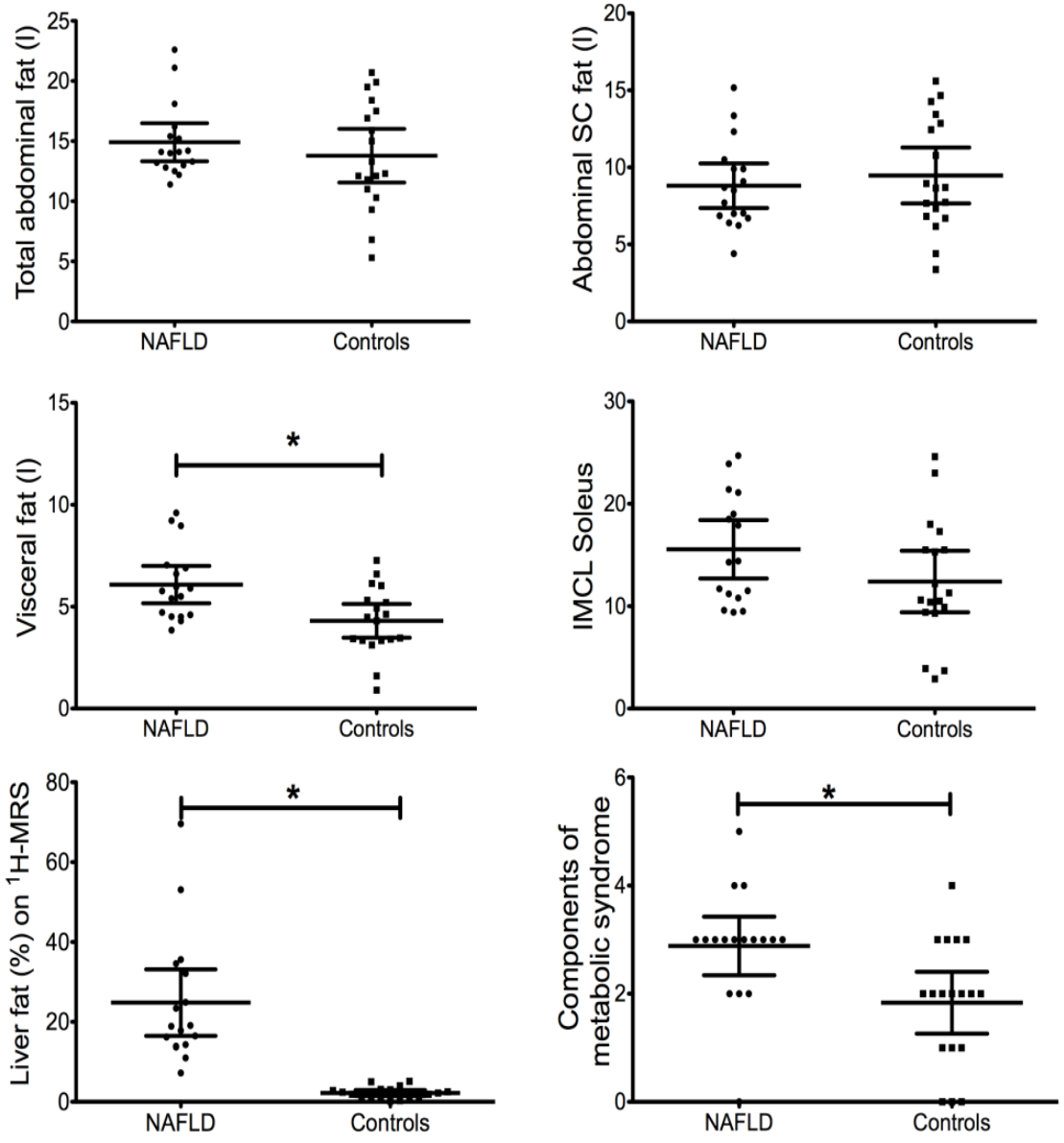


Figure 3 Body composition data above figures represent results of body fat, intraomyocellular and intrahepatocellular lipids and components of the metabolic syndrome

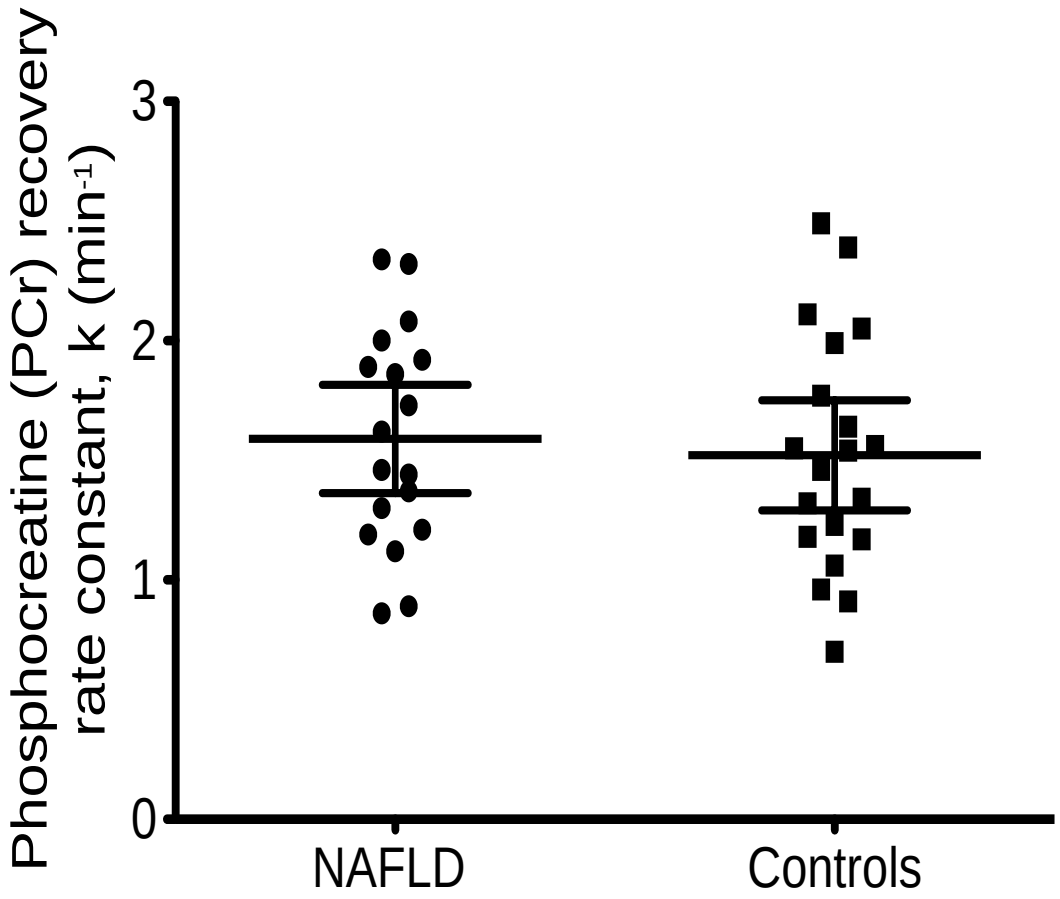


Figure 3.1 Phosphocreatine (PCr) recovery rate constant results, k (min^{-1}) in patients with NAFLD (*n*=17) and age and BMI-matched controls (*n*=18).

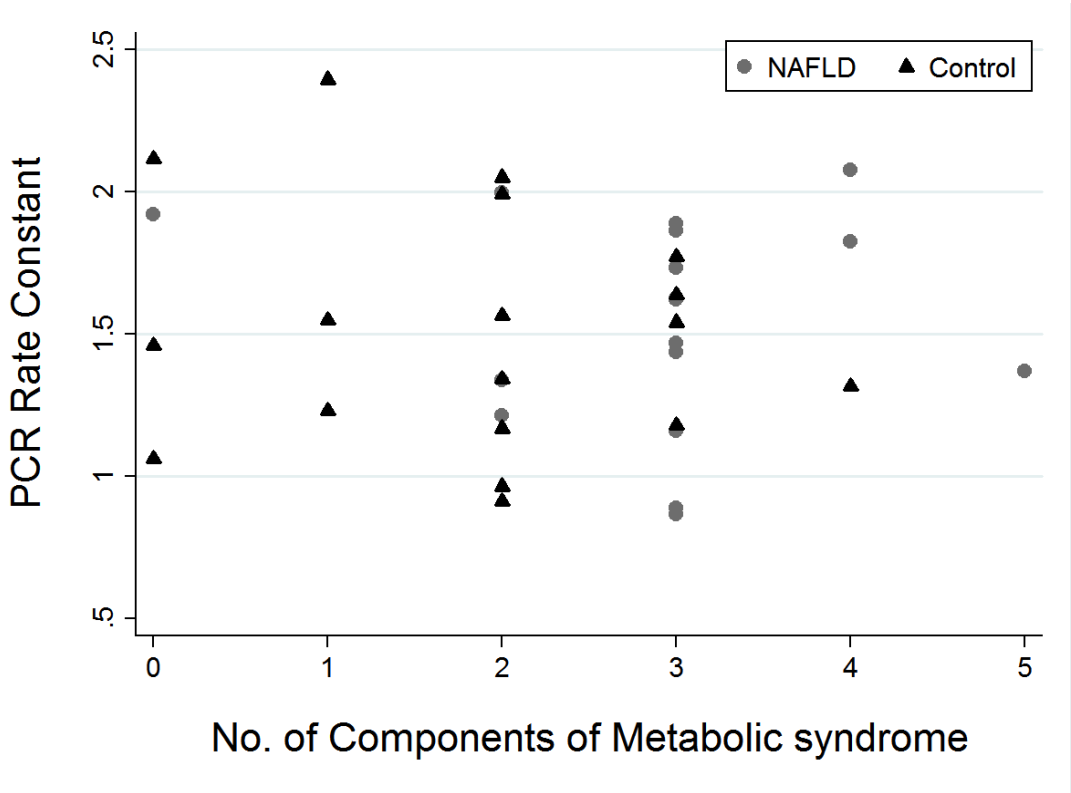


Figure 3.2 Relationship between PCr rate constant, k (min^{-1}) and the number of components of the metabolic syndrome within the controls (▲) and patients with NAFLD (o).

3.11 Results overview

We have provided evidence that mitochondrial function in skeletal muscle is maintained in patients with NAFLD, in comparison to controls that are age, BMI and fitness matched, and cannot account for high disposition of fat or the increased prevalence of the metabolic syndrome. As a consequence of the findings of this study it would appear that there are additional factors other than skeletal muscle dysfunction that have a role in the phenotype of NAFLD. Available data suggests that skeletal muscle and adipose tissue insulin resistance may have a role (Bloem & Chang, 2008).

CHAPTER 4

DISCUSSION

4.1 Discussion

In this study we set out to determine the oxidative capacity of skeletal muscle by investigating PCr recovery kinetics in the quadriceps muscle of NAFLD patients and age, BMI and cardio respiratory fitness matched controls. Although the phenotype of the NAFLD patients was found to be characterized by a disproportionately greater accumulation of liver and visceral fat coupled with increased components of the metabolic syndrome, compared to the controls, the rate of PCr recovery was similar between the control and NAFLD groups. The NAFLD group within this study from our results was not associated with any increase in intramyocellular lipid (IMCL), which has been linked with insulin resistance and muscle mitochondrial dysfunction in other studies (Toledo *et al.*, 2008; Smith & Adams, 2011). Similarly, we did not observe any relationship between the components of the metabolic syndrome and the PCr rate constant, there also was not any observable difference in the PCr rate constant between those with and without the metabolic syndrome as found within this study.

4.2 Role of skeletal muscle

The data reported in this study suggests that defects in muscle mitochondrial function may occur later perhaps as a consequence rather than a cause of increased lipid deposition and associated metabolic syndrome, which has been supported in a number of other studies (Pessayre, 2008). Impaired oxidative phosphorylation by skeletal muscle mitochondria have been postulated to contribute to age-associated insulin resistance and fat accumulation within skeletal muscle (Short *et al.*, 2005a). This impaired mitochondrial functional capacity in ageing has also been attributed to

a reduced mitochondrial content, as reflected by lower mtDNA content (Petersen *et al.*, 2003).

The aging process in humans begins around the age of 40 when muscle mass (sarcopenia) and strength gradually begin to decline at a rate of just under 1% per year. The process is caused by a decreased capacity for oxidative phosphorylation in the muscles (Menshikova *et al.*, 2006). Given the strong evidence linking mitochondria dysfunction with aging, insulin resistance and T2DM, it is important to more precisely define specific loci of these defects. The focus of this study was to explore the possibility that deficiencies in muscle mitochondrial function plays a role in the aetiology of NAFLD, but we found no association with any increase in intramyocellular lipid (IMCL), and the rate of PCr recovery was similar between the control and NAFLD groups.

Perhaps more importantly further research should aim to determine whether clinical interventions including exercise, diet and lifestyle changes may help correct these insufficiencies (Menshikova *et al.*, 2006). Physical activity is reported to protect against sarcopenia and preserve mitochondrial function the origins of sarcopenia although common is poorly understood, oxidative stress and reduced mitochondrial function are thought to play a part (Short *et al.*, 2005b). To what extent mitochondria are involved in the ageing process and reduction in muscle mass is controversial, a lot of the decline in the mitochondrial oxidative capacity may be more linked to a lack of physical activity and disease rather than age in itself (Waters *et al.*, 2009).

As PCr recovery (k min^{-1}) was similar between the control 1.50 (1.18, 1.77) and NAFLD 1.62 (0.34, 1.86) groups, the defects that later cause a reduction in mitochondrial function may well be due to elements of the metabolic syndrome and insulin resistance, as well as due to ageing in general. As a consequence of these other metabolic defects, dysfunction of muscle mitochondria does not appear to be a major contributor to muscle fat accumulation liver disease and insulin resistance as explored within this study. This may be associated with an insulin resistance in muscle which occurs at a later point within the disease process were unused energy is stored as fat in the liver. It suggests that this may be linked to the insulin signalling cascade in association with increased intra myocellular lipid accumulation at a later time in the disease process and may also be dependent on the severity of NAFLD and intra-hepatocellular lipid accumulation (Young & Leighton, 1998).

As obese people have a reduced capacity to oxidize fatty acids as fuel compared with lean people during exercise, and because fatty acids are oxidized in the mitochondria, research into other mitochondrial defects may well be of value in the search for an explanation for this problem. In other animal studies it has been found that there is evidence that hepatic mitochondrial dysfunction precedes the development of NAFLD and insulin resistance in the hyperphagic, obese OLETF rat and in this study it was also documented that a progressive loss of mitochondrial function in conjunction with the transition from insulin resistance to T2D potentially contributes to NAFLD progression (Rector *et al.,* 2010). These researchers also looked at the relationship between hepatic mitochondrial dysfunction and the link with hepatic steatosis and resistance to insulin and the potential role in any progression of NAFLD using OLETF rats. The results of this study raised the possibility that

mitochondrial dysfunction can be a cause, effect or a concurrent feature in the development of NAFLD (Rector *et al.*, 2010). Our study set out to explore if there is any involvement of skeletal muscle mitochondrial function in the development of NAFLD. However our results indicate that mitochondrial function, which was determined via ^{31}P-MRS measurements of the quadriceps muscle were similar after exercise in both groups. Moreover this similarity in the function of the mitochondria was apparent despite higher levels of liver fat and more elements of the metabolic syndrome. There was also no correlation with aerobic fitness as measured by $V0_2$ peak (ml/kg/min), the NAFLD group results being 23.5 (20,31.3) versus the controls 25.75 (22.9,30). However this could be due to a relatively small range of cardiorespiratory fitness that we observed in both groups within the study.

In other studies it has been found that skeletal muscle mitochondria dysfunction has been linked as one of the main features of T2DM and that exercise enhances mitochondria electron transport chain activity in older human skeletal muscle, particularly in subsarcolemmal mitochondria, which is likely related to the concomitant increases in mitochondrial biogenesis (Menshikova *et al.*, 2006). However the mechanisms of the pathogenesis of resistance to insulin and its link with diabetes have as yet to be clarified and therapeutic approaches like the effects of exercise and lifestyle changes are of general interest as potentially useful treatment modalities for NAFLD, and are worthy of further investigation.

4.3 Role of adipose tissue

In looking at treatments for metabolic diseases that are linked to obesity, a greater understanding of the origin and development of adipose tissue is therefore relevant to

any analysis. It was previously thought that adipocytes were inert cells with a single function of lipid storage, however it is now known that they have a number of other functions including sensitivity to insulin, they also have an ability to secrete adipocyte specific endocrine hormones which have a regulatory effect on energy homeostasis within other tissues within the body (Stephens, 2012). Leptin is one such example which has inspired investigation among obesity researchers. Studies have shown that the hormone can rewire part of the brain that regulates appetite, future studies may shed new light on how metabolism goes awry in obesity and the importance of gaining more data on adipose tissue and its actions.

Adipocytes are therefore known to be involved in the development of a number of metabolic diseases including the metabolic syndrome. Some studies have looked at the development of adipocytes from precursor cells and the proteins associated with this, with the possibility of the development of adipocyte development inhibitors.

In a recent study by Rosen which revealed a new zinc finger protein Zfp 521 in the process of predipocyte differentiation and the ability of this to mediate its inhibitory effects on adipocyte development, future work within this area may be of value in obesity management (Stephens, 2012).

4.4 Role of liver

The mitochondria are important in hepatocyte metabolism, playing a role in the oxidation of fatty acids and oxidative process of phosphorylation (Hagopian *et al.*, 2005). Other mitochondrial dysfunctions within the body causes a decreased capacity to oxidise fatty acids, increases the transport and delivery of free fatty acids into the

liver and augmented hepatic fatty acid syntheses are thought to play a role in the pathogenesis of NAFLD. This raises the point that mitochondrial abnormalities have such a part in the development of the disease that NAFLD is in fact a mitochondrial disease (Parsons & Green, 2011).

4.5 Assessment of PCr depletion and recovery

For our study we used a gold standard method to investigate muscle mitochondrial function that was safe and non-invasive facilitated by the use of MRS to assess PCr depletion and recovery, as was collecting data on total body fat using whole body MRI and intramyocellular and hepatocellular lipids by MRS. A positive element of our study has been that we did not find that skeletal muscle mitochondrial function was part of the aetiology of NAFLD which we initially suspected would be the case. More generalised mitochondrial disease caused by dysfunctional mitochondria within other systems of the body may be the link in the pathogenesis of the development of the metabolic syndrome and NAFLD.

4.6 Study limitations

A limitation of this study was that we focused only on the role of skeletal muscle mitochondrial dysfunction in the aetiology of NAFLD. We could also have looked at mitochondrial function within other tissues e.g. adipose tissue and liver using ^{31}P MRS or other methods, such as metabolite analysis of blood and urine or muscle biopsy allowing biochemical examination within our study subjects. Areas of weakness within this study have been the gender imbalance as within the NAFLD group (n=17) there were 11 males versus 6 females so a 65% male bias, whilst in the control group (n=18) there was 7 males versus 11 female a female bias of 61%. This

may have weakened the data on the prevalence of central adiposity and its link with the metabolic syndrome within this study.

It was intended to include data on measures of insulin resistance e.g. HOMA-IR, but unfortunately this data set was incomplete at the time of writing. This or even data from oral glucose tolerance tests, would have been of use in determining differences in insulin sensitivity between the two groups within the study. The gold standard for investigating and quantifying insulin resistance is the hyperinsulinemic euglycemic clamp. This could have also been used within the study, but would have posed issues for volunteers time and added greatly to their commitment to an already busy study protocol.

4.7 Conclusions and realisation of aims

NAFLD, which is characterized by lipid deposition effecting greater than 5% of hepatocytes, has an association with the metabolic syndrome. This we found to be the case within our study as our results indicated that participants with NAFLD had thirteen out of seventeen (76%) 2.9 ± 1.0 more components of the metabolic syndrome compared to controls who had five out of eighteen (28%) 1.8 ± 1.2. It has been suggested that an impairment within the skeletal muscle in mitochondrial function may be a contributory factor to ectopic fat accumulation within the liver (Johannsen *et al.*, 2012).

The outcomes from this study indicates that the PCr rate constant k (min^{-1}) is broadly similar between the controls and the subjects with NAFLD, which suggests that

skeletal muscle mitochondrial function is preserved in subjects with NAFLD. We also found that IMCL were not elevated in the NAFLD group versus controls.

There was also no link between the subjects with the metabolic syndrome and their PCr rate constants, k (min^{-1}) results or the number of components of the metabolic syndrome within the age and BMI matched controls and patients with NAFLD. We aimed to determine total body fat, which as was expected was higher in the NAFLD group who had a % fat mass of 40.5 (30.1,44.1) compared to controls 34.2 (29.2,44.8). Liver fat which was determined by MRS, this in the NAFLD group was 24.8±2% were as in the controls it was 2.2±1.5% , but as stated previously we did not find any link to mitochondrial dysfunction that could account for this.

CHAPTER 5

FUTURE DIRECTIONS

5.1 Future directions

The results of this study in investigating the role of skeletal muscle mitochondrial function in the aetiology of NAFLD, suggests that the problem does not arise in skeletal muscle. However there could be other defects in muscle that were not detected by our methods. Biochemical measures of mitochondrial proteins, enzyme activities and lipids are often used as markers of mitochondrial content and muscle oxidative capacity, whereas we used ^{31}P MRS spectrum from human quadriceps muscle to assess mitochondrial function. Therefore, future directions for this study may focus upon sites other than skeletal muscle e.g. liver or adipose tissue mitochondrial dysfunction in the aetiology of NAFLD.

Progress in understanding the pathogenesis of NAFLD has lead to a greater understanding of the disease, however the primary metabolic issues that lead to lipid accumulation within the hepatocytes remains poorly understood. Due to the fact that mitochondria are critical metabolic organelles and that there is evidence indicating that any hepatic mitochondrial dysfunction is critically linked to the pathogenesis of NAFLD, this is therefore an area worthy of further investigation. In order to further our study, an investigation of hepatic mitochondrial function and its association with NAFLD and insulin resistance would be of value, as there are a number of methodologies to facilitate this. It would be of interest to investigate hepatic mitochondrial function by obtaining ^{1}H MR spectra from the liver. Other relevant tests would be to investigate insulin resistance by hyperinsulinemic euglycemic clamp and see if there are any improvements in individuals along with reductions in liver fat in conjunction with an exercise and diet restriction regime, as a potential treatment modality.

Although all participants were age and BMI matched the control group in this study had a BMI of 30.53 (SD 15.7) versus the NAFLD group BMI 31.5 (3.4). A non diseased control group would therefore have been a positive addition to this study, as both groups studied were in the obese category. A further group with normal BMI of <25 would have been a useful addition.

5.2 Factors relating to adipose tissue

Adipocytes are involved in the maintenance and balance of energy storage and expenditure, therefore any impairment of mitochondrial activity could predispose individuals to obesity, a reduction in mitochondria biogenesis has also been linked. This perhaps highlights the important role of mitochondria and its associated complications with disease and obesity in any dysfunctional state. To further this study adipose tissue could therefore be an area of investigation into the effects of mitochondrial dysfunction within this tissue in the aetiology of NAFLD. Target areas in looking at obesity and its associated complications could involve looking at white and brown adipose tissue mitochondria and exploring any relationship between dysfunction and obesity and other associated complications, such as insulin resistance. This could facilitate more understanding on what potential treatments targeting adipose tissue and their mitochondria that may have benefits to increase oxidative capacity within these tissues.

Obesity is a condition in which an excess of adipose tissue results in a positive energy balance, a metabolic disorder which is increasing in most societies. There are a number of clinical problems that can be associated with this which include cancers, atherosclerosis and an increased risk of T2DM. In looking at treatments for

metabolic diseases that are linked to obesity, a greater understanding of the origin and development of adipose tissue is therefore relevant to any analysis. It was previously thought that adipocytes were inert cells with a single function of lipid storage, however it is now known that they have a number of other functions including sensitivity to insulin, they also have an ability to secrete adipocyte specific endocrine hormones which have a regulatory effect on energy homeostasis within other tissues within the body (Stephens, 2012).

5.3 Vitamin D

Vitamin D has been shown to have an involvement in muscle contraction and energy levels, recent research has shown that muscle function can be improved with supplements of this vitamin (Sinha *et al.*, 2013). In a study conducted at Newcastle University, Sinha used non-invasive MR scans in a cohort of 12 patients with muscle fatigue and severe deficiency of vitamin D in order to measure their response to exercise. Apart from the well documented effects on bone health, fatigue within muscle is a symptom of vitamin D deficiency which was postulated within this study to be due to a reduced efficiency of the muscle mitochondria and to explore any link with vitamin D. In line with our methodologies, this research team used ^{31}P MRS of muscle, but using the calf muscle instead of the quadriceps to measure phosphcreatine recovery times as a measure of mitochondrial function, shorter recovery times being linked with improved mitochondrial function.

The results of this small n=12 study found that there was an improvement after the group took oral supplements of vitamin D for a period of just 10-12 weeks. It was reported that the PCr recovery half time improved from the base line figure of 34.4

seconds to 27.8 seconds with a concomitant improvement in fatigue symptoms. Using a parallel study it was demonstrated that there is a link between vitamin D and mitochondrial function. According to Sinha this data suggests that vitamin D may modulate mitochondrial energy transduction. This may represent the bioenergetic basis for the fatigue experienced by vitamin D deficient adolescents and adults, although further studies are required to establish causality.

As understanding of the many functions of vitamin D has grown the presence of vitamin D deficiency has become more evident within Western populations, as has NAFLD as the most common cause of chronic liver disease. NAFLD and vitamin D deficiency are often found together although there are similar associations with sedentary lifestyle and obesity, there is also evidence to a closely linked and potentially causative relationship between deficiency of vitamin D and NAFLD (Kwok *et al.*, 2013). We could have added this to our study and obtained vitamin D levels from blood samples, to see if there was any link with low levels and skeletal muscle mitochondrial dysfunction and NAFLD.

5.4 Ageing and Sarcopenia

Physical activity is reported to protect against sarcopenia and preserve mitochondrial function the origins of sarcopenia although common is poorly understood; oxidative stress and reduced mitochondrial function are thought to play a part. To what extent mitochondria are involved in the ageing process and reduction in muscle mass is controversial, a lot of the decline in the mitochondrial oxidative capacity may be more linked to a lack of physical activity and disease rather than age in itself (Waters *et al.*, 2009). It is likely that a decline in muscle mass and strength with a decline in

endurance capacity causes this reduction in physical activity which is then followed by this loss of muscle. This reduction in muscle mass which is responsible according to Waters for 30% of resting energy expenditure and protein turnover, as well as 70% body cell mass. Physical activity levels that can be attributed to this account for a decrease in energy expenditure and increase the prevalence of obesity in older age groups, in particular abdominal fat accumulation. Alterations such as these in body composition can be linked to insulin resistance and the development of T2D and other metabolic abnormalities such as hyperlipidemia (Waters *et al.*, 2009).

Muscle weakness can lead to reduced muscle mass, but a reduction in ATP production may according to Waters also decrease endurance and muscle strength. This may be linked to mitochondrial DNA oxidative damage due to ageing, which with any cumulative effect may explain any overall reduction in mitochondrial DNA (Waters *et al.*, 2009) and have a further effect muscle strength.

5.5 Nutritional issues and NAFLD

Certain natural nutrients are also known to have strong anti-inflammatory properties and have been shown to be of value in the treatment of NAFLD. These include Vitamin E (Tocopherol) and omega 3 fatty acids. Vitamin C is also known to react with reactive oxygen species (ROS) blocking the propagation of radical reactions in a wide range of oxidative stress situations, but the potential therapeutic efficiency of the use of antioxidants in NAFLD is not fully understood. Most human studies have been limited, as they have been retrospective and therefore relied on diet recall within the groups studied. This being self reported could have weakened these

studies, therefore larger investigations may be of value in establishing causal relationships between certain nutrients and NAFLD.

In a study that induced fatty liver disease in Wistar rats it was found that vitamin C reduced oxidative stress and inhibited the development of liver steatosis. Whilst in the other group, vitamin E did not prevent development of fatty liver disease nor did it reduce oxidative stress as observed within this study (Oliveira *et al.*, 2003). There is therefore evidence to suggest a role for dietary antioxidants, oxidative stress which is said to be caused by an imbalance between the harmful oxidants and protective antioxidants, but further research is warranted. These being diet derived or endogenous, and occurs in patients with NAFLD due to increased lipid oxidation (Browning & Horton, 2004).

As Hippocrates said, "let food be thy medicine and medicine be thy food", this area maybe worthy of further investigation as to casual impacts of diet and the potential benefits of nutrients in patients with the metabolic syndrome and NAFLD.

Certain diets may have a positive effect in the prevention and treatment of certain characteristic traits of NAFLD. Low fat high fiber vegetarian diets have had positive effects on cardiovascular disease and diabetes in certain clinical trials, but this diet has not been evaluated in NAFLD. These diets do typically cause weight loss and can lower the blood lipids that contribute to NAFLD, similar diets have also been associated with a reduction in insulin resistance which is a known symptom of NAFLD and greater antioxidant protection in comparison with omnivorus diets (Kuo *et al.*, 2004; Videla *et al.*, 2006). An addition to this, it has been demonstrated that

accumulation of iron aggravates insulin resistance and associated oxidative stress. As plant based diets have somewhat less bioavailability of iron vegetarians therefore have lower body iron stores (Hua *et al.*, 2001).

CHAPTER 6

REFERENCES

References

Aliev MK & Saks VA. (1993). Quantitative analysis of the 'phosphocreatine shuttle': I. A probability approach to the description of phosphocreatine production in the coupled creatine kinase-ATP/ADP translocase-oxidative phosphorylation reactions in heart mitochondria. *Biochimica et biophysica acta* **1143,** 291-300.

Bachert P & Schroder L. (2003). Magnetic resonance imaging spectroscopy. Part 1: Basics. *Der Radiologe* **43,** 1113-1126.

Bae JC, Suh S, Park SE, Rhee EJ, Park CY, Oh KW, Park SW, Kim SW, Hur KY, Kim JH, Lee MS, Lee MK, Kim KW & Lee WY. (2012). Regular exercise is associated with a reduction in the risk of NAFLD and decreased liver enzymes in individuals with NAFLD independent of obesity in Korean adults. *PloS one* **7,** e46819.

Bhaskaran K, Forbes HJ, Douglas I, Leon DA & Smeeth L. (2013). Representativeness and optimal use of body mass index (BMI) in the UK Clinical Practice Research Datalink (CPRD). *BMJ open* **3,** e003389.

Bloem CJ & Chang AM. (2008). Short-term exercise improves beta-cell function and insulin resistance in older people with impaired glucose tolerance. *The Journal of clinical endocrinology and metabolism* **93,** 387-392.

Broskey NT, Boss A, Fares EJ, Kreis R, Gremion G, Boesch C & Amati F. (2013a). Exercise Capacity In Older Adults: A Matter Of Mitochondrial Function, Content Or Efficiency? *Med Sci Sport Exer* **45,** 517-518.

Broskey NT, Fares EJ, Greggio C & Amati F. (2013b). The effect of exercise training on skeletal muscle mitochondrial content in older adults correlates with exercise efficiency and fat oxidation. *Diabetologia* **56,** S32-S32.

Browning JD & Horton JD. (2004). Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest* **114,** 147-152.

Burkhalter N. (1996). Evaluation of Borg's perceived exertion scale in cardiac rehabilitation. *Rev Lat Am Enfermagem* **4,** 65-73.

Campbell IW. (2000). Antidiabetic drugs present and future: will improving insulin resistance benefit cardiovascular risk in type 2 diabetes mellitus? *Drugs* **60,** 1017-1028.

Chiang DJ, Pritchard MT & Nagy LE. (2011). Obesity, diabetes mellitus, and liver fibrosis. *American journal of physiology Gastrointestinal and liver physiology* **300,** G697-702.

Cortez-Pinto H & Camilo ME. (2004). Non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH): diagnosis and clinical course. *Best practice & research Clinical gastroenterology* **18,** 1089-1104.

Czernichow S, Kengne AP, Huxley RR, Batty GD, de Galan B, Grobbee D, Pillai A, Zoungas S, Marre M, Woodward M, Neal B, Chalmers J & Grp AC. (2011). Comparison of waist-to-hip ratio and other obesity indices as predictors of cardiovascular disease risk in people with type-2 diabetes: a prospective cohort study from ADVANCE. *Eur J Cardiov Prev R* **18,** 312-319.

Day CP & James OF. (1998a). Hepatic steatosis: innocent bystander or guilty party? *Hepatology* **27,** 1463-1466.

Day CP & James OF. (1998b). Steatohepatitis: a tale of two "hits"? *Gastroenterology* **114,** 842-845.

Despres JP, Lemieux I, Bergeron J, Pibarot P, Mathieu P, Larose E, Rodes-Cabau J, Bertrand OF & Poirier P. (2008). Abdominal obesity and the metabolic syndrome: contribution to global cardiometabolic risk. *Arteriosclerosis, thrombosis, and vascular biology* **28,** 1039-1049.

Dixon JB, Bhathal PS, Hughes NR & O'Brien PE. (2004). Nonalcoholic fatty liver disease: Improvement in liver histological analysis with weight loss. *Hepatology* **39,** 1647-1654.

Evans B & Colls R. (2009). Measuring fatness, governing bodies: the spatialities of the Body Mass Index (BMI) in anti-obesity politics. *Antipode* **41,** 1051-1083.

Flegal KM, Carroll MD, Kit BK & Ogden CL. (2012). Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA : the journal of the American Medical Association* **307,** 491-497.

Gaggini M, Morelli M, Buzzigoli E, DeFronzo RA, Bugianesi E & Gastaldelli A. (2013). Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease. *Nutrients* **5,** 1544-1560.

Gallagher EJ, LeRoith D & Karnieli E. (2010). Insulin resistance in obesity as the underlying cause for the metabolic syndrome. *Mt Sinai J Med* **77,** 511-523.

Gardner CJ, Richardson P, Wong C, Polavarapu N, Kemp GJ & Cuthbertson DJ. (2011). Lesson of the week: Hypothyroidism in a patient with non-alcoholic fatty liver disease. *Brit Med J* **342,** c7199.

Giovannucci E. (2007). Metabolic syndrome, hyperinsulinemia, and colon cancer: a review. *The American journal of clinical nutrition* **86,** s836-842.

Goncalves IO, Passos E, Rocha-Rodrigues S, Diogo CV, Santos-Alves E, Oliveira PJ, Ascensao A & Magalhaes J. (2013). The preventive role of voluntary physical activity against high-fat diet-induced liver mitochondrial dysfunction. *Eur J Clin Invest* **43,** 47-47.

Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Jr., Spertus JA, Costa F, American Heart A, National Heart L & Blood I. (2005). Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* **112,** 2735-2752.

Gustafson B, Hammarstedt A, Andersson CX & Smith U. (2007). Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* **27,** 2276-2283.

Hagopian K, Harper ME, Ram JJ, Humble SJ, Weindruch R & Ramsey JJ. (2005). Long-term calorie restriction reduces proton leak and hydrogen peroxide production in liver mitochondria. *Am J Physiol-Endoc M* **288,** E674-E684.

Hua NW, Stoohs RA & Facchini FS. (2001). Low iron status and enhanced insulin sensitivity in lacto-ovo vegetarians. *Brit J Nutr* **86,** 515-519.

Jeneson J, Schmitz J & Nicolay K. (2008). In-magnet bicycling exercise: a novel 31P MRS window on the energetics of human locomotion. *Faseb J* **22,** 1176.

Johannsen DL, Conley KE, Bajpeyi S, Punyanitya M, Gallagher D, Zhang Z, Covington J, Smith SR & Ravussin E. (2012). Ectopic lipid accumulation and reduced glucose tolerance in elderly adults are accompanied by altered skeletal muscle mitochondrial activity. *The Journal of clinical endocrinology and metabolism* **97,** 242-250.

Kechagias S, Ernersson A, Dahlqvist O, Lundberg P, Lindstrom T, Nystrom FH & Fast Food Study G. (2008). Fast-food-based hyper-alimentation can induce rapid and profound elevation of serum alanine aminotransferase in healthy subjects. *Gut* **57,** 649-654.

Keller DP. (1988). Basic principles of magnetic resonance imaging. *GE Medical Systems*.

Kemp GJ. (2006). Altered creatine dependence of muscle mitochondrial respiration in vitro: what are the likely effects in vivo? *J Appl Physiol (1985)* **101,** 1814-1815.

Kemp GJ & Radda GK. (1994). Quantitative interpretation of bioenergetic data from ^{31}P and ^{1}H magnetic resonance spectroscopic studies of skeletal muscle: an analytical review. *Magnetic resonance quarterly* **10,** 43-63.

Kemp GJ & Radda GK. (1993). Control of phosphocreatine resynthesis during recovery from exercise in human skeletal muscle. *NMR in Biomed* **6,** 66-72.

Kuo CS, Lai NS, Ho LT & Lin CL. (2004). Insulin sensitivity in Chinese ovo-lactovegetarians compared with omnivores. *Eur J Clin Nutr* **58,** 312-316.

Kwok RM, Torres DM & Harrison SA. (2013). Vitamin D and nonalcoholic fatty liver disease (NAFLD): is it more than just an association? *Hepatology* **58,** 1166-1174.

Larsen S, Hey-Mogensen M, Rabol R, Stride N, Helge JW & Dela F. (2012). The influence of age and aerobic fitness: effects on mitochondrial respiration in skeletal muscle. *Acta Physiol* **205,** 423-432.

Lavine JE & Schwimmer JB. (2004). Nonalcoholic fatty liver disease in the pediatric population. *Clinics in liver disease* **8,** 549-558.

Luyckx FH, Desaive C, Thiry A, Dewe W, Scheen AJ, Gielen JE & Lefebvre PJ. (1998). Liver abnormalities in severely obese subjects: effect of drastic weight loss after gastroplasty. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* **22,** 222-226.

Malavolti M, Battistini NC, Miglioli L, Bagni I, Borelli L, Marino M, Scaglioni F & Bellentani S. (2012). Influence of lifestyle habits, nutritional status and insulin resistance in NAFLD. *Frontiers in bioscience* **4,** 1015-1023.

McLarnon A. (2011). Liver: potential of resistance exercise as a lipid-lowering treatment for NAFLD that is independent of weight loss. *Nature reviews Gastroenterology & hepatology* **8,** 476.

Menshikova EV, Ritov VB, Fairfull L, Ferrell RE, Kelley DE & Goodpaster BH. (2006). Effects of exercise on mitochondrial content and function in aging human skeletal muscle. *J Gerontol a-Biol* **61,** 534-540.

Mirza MS. (2011). Obesity, Visceral Fat, and NAFLD: Querying the Role of Adipokines in the Progression of Nonalcoholic Fatty Liver Disease. *ISRN gastroenterology* **2011,** 592404.

Musso G, Gambino R, Cassader M & Pagano G. (2010). A meta-analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology* **52,** 79-104.

Naressi A, Couturier C, Devos JM, Janssen M, Mangeat C, de Beer R & Graveron-Demilly D. (2001). Java-based graphical user interface for the MRUI quantitation package. *Magma* **12,** 141-152.

Niaz A, Ali Z, Nayyar S & Fatima N. (2011). Prevalence of NAFLD in Healthy and Young Male Individuals. *ISRN gastroenterology* **2011,** 363546.

Oliveira CP, Gayotto LC, Tatai C, Della Nina BI, Lima ES, Abdalla DS, Lopasso FP, Laurindo FR & Carrilho FJ. (2003). Vitamin C and vitamin E in prevention of Nonalcoholic Fatty Liver Disease (NAFLD) in choline deficient diet fed rats. *Nutrition journal* **2,** 9.

Pagel-Langenickel I, Bao J, Pang L & Sack MN. (2010). The role of mitochondria in the pathophysiology of skeletal muscle insulin resistance. *Endocrine reviews* **31,** 25-51.

Parsons MJ & Green DR. (2011). Death Receptors and Mitochondria: Life Depends on the Liver. *Hepatology* **54,** 13-15.

Paschos P & Paletas K. (2009). Non alcoholic fatty liver disease and metabolic syndrome. *Hippokratia* **13,** 9-19.

Pessayre D. (2008). Role of mitochondria in non-alcoholic fatty liver disease (vol 22, pg S20, 2007). *J Gastroen Hepatol* **23,** 501-501.

Pesta D, Hoppel F, Macek C, Messner H, Faulhaber M, Kobel C, Parson W, Burtscher M, Schocke M & Gnaiger E. (2011). Similar qualitative and quantitative changes of mitochondrial respiration following strength and endurance training in normoxia and hypoxia in sedentary humans. *Am J Physiol-Reg I* **301,** R1078-R1087.

Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW & Shulman GI. (2003). Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science* **300,** 1140-1142.

Pooley RA. (2004). AAPM/RSNA physics tutorial at the 2004 RSNA annual meeting. *AAPM/RSNA*.

Rabol R, Petersen KF, Dufour S, Flannery C & Shulman GI. (2011). Reversal of muscle insulin resistance with exercise reduces postprandial hepatic de novo lipogenesis in insulin resistant individuals. *P Natl Acad Sci USA* **108,** 13705-13709.

Rector RS, Thyfault JP, Uptergrove GM, Morris EM, Naples SP, Borengasser SJ, Mikus CR, Laye MJ, Laughlin MH, Booth FW & Ibdah JA. (2010). Mitochondrial dysfunction precedes insulin resistance and hepatic steatosis and contributes to the natural history of non-alcoholic fatty liver disease in an obese rodent model. *J Hepatol* **52,** 727-736.

Redpath TW & Wiggins CJ. (2000). Estimating achievable signal-to-noise ratios of MRI transmit-receive coils from radiofrequency power measurements: applications in quality control. *Physics in medicine and biology* **45,** 217-227.

Reeder SB, Cruite I, Hamilton G & Sirlin CB. (2011). Quantitative Assessment of Liver Fat with Magnetic Resonance Imaging and Spectroscopy. *J Magn Reson Imaging* **34,** 729-749.

Rofsky NM & Fleishaker H. (1995). CT and MRI of diffuse liver disease. *Seminars in ultrasound, CT, and MR* **16,** 16-33.

Saeed N, Hajnal JV, Oatridge A & Young IR. (1998). Regions of interest tracking in temporal scans based on statistical analysis of gray scale and edge properties and registration of images. *Jmri-J Magn Reson Im* **8,** 182-187.

Schwenzer NF, Springer F, Schraml C, Stefan N, Machann J & Schick F. (2009). Non-invasive assessment and quantification of liver steatosis by ultrasound, computed tomography and magnetic resonance. *Journal of hepatology* **51,** 433-445.

Sebastian D, Hernandez-Alvarez MI, Segales J, Sorianello E, Munoz JP, Sala D, Waget A, Liesa M, Paz JC, Gopalacharyulu P, Oresic M, Pich S, Burcelin R, Palacin M & Zorzano A. (2012). Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis. *P Natl Acad Sci USA* **109,** 5523-5528.

Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S & Nair KS. (2005a). Decline in skeletal muscle mitochondrial function with aging in humans. *P Natl Acad Sci USA* **102,** 5618-5623.

Short KR, Vittone JL, Bigelow ML, Proctor DN, Coenen-Schimke JM, Rys P & Nair KS. (2005b). Changes in myosin heavy chain mRNA and protein expression in human skeletal muscle with age and endurance exercise training. *J Appl Physiol* **99,** 95-102.

Sinha A, Hollingsworth KG, Ball S & Cheetham T. (2013). Improving the vitamin D status of vitamin D deficient adults is associated with improved mitochondrial oxidative function in skeletal muscle. *The Journal of clinical endocrinology and metabolism* **98,** E509-513.

Smith BW & Adams LA. (2011). Nonalcoholic fatty liver disease and diabetes mellitus: pathogenesis and treatment. *Nature reviews Endocrinology* **7,** 456-465.

Solomon TPJ, Sistrun SN, Krishnan RK, Del Aguila LF, Marchetti CM, O'Carroll SM, O'Leary VB & Kirwan JP. (2008). Exercise and diet enhance fat oxidation and reduce insulin resistance in older obese adults. *J Appl Physiol* **104,** 1313-1319.

Sorbi D, Boynton J & Lindor KD. (1999). The ratio of aspartate aminotransferase to alanine aminotransferase: potential value in differentiating nonalcoholic steatohepatitis from alcoholic liver disease. *The American journal of gastroenterology* **94,** 1018-1022.

Sotelo-Peryea PJ, DeVinney-Boymel LA & Kozlowski KF. (2010). Validity Of A Sub-maximal Branched Treadmill Exercise Test Compared With A Modified Bruce Protocol. *Med Sci Sport Exer* **42,** 601-601.

Stephens JM. (2012). The Fat Controller: Adipocyte Development. *Plos Biol* **10,** e1001436.

Szczepaniak LS, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD & Stein DT. (1999). Measurement of intracellular triglyceride stores by H-1 spectroscopy: validation in vivo. *Am J Physiol-Endoc M* **276,** E977-E989.

Thomas EL, Hamilton G, Patel N, O'Dwyer R, Dore CJ, Goldin RD, Bell JD & Taylor-Robinson SD. (2005). Hepatic triglyceride content and its relation to body adiposity: a magnetic resonance imaging and proton magnetic resonance spectroscopy study. *Gut* **54,** 122-127.

Toledo FGS, Menshikova EV, Azuma K, Radikovi Z, Kelley CA, Ritov VB & Kelley DE. (2008). Mitochondrial capacity in skeletal muscle is not stimulated by weight loss despite increases in insulin action and decreases in intramyocellular lipid content. *Diabetes* **57,** 987-994.

Turkoglu C, Duman BS, Gunay D, Cagatay P, Ozcan R & Buyukdevrim AS. (2003). Effect of abdominal obesity on insulin resistance and the components of the metabolic syndrome: Evidence supporting obesity as the central feature. *Obes Surg* **13,** 699-705.

Vanhamme L, van den Boogaart A & Van Huffel S. (1997). Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. *J Magn Reson* **129,** 35-43.

Videla LA, Rodrigo R, Araya J & Poniachik J. (2006). Insulin resistance and oxidative stress interdependency in non-alcoholic fatty liver disease. *Trends Mol Med* **12,** 555-558.

Vonsmekal A, Seelos KC, Kuper CR & Reiser M. (1995). Patient Monitoring and Safety during Mri Examinations. *Eur Radiol* **5,** 302-305.

Waters DL, Mullins PG, Qualls CR, Raj DS, Gasparovic C & Baumgartner RN. (2009). Mitochondrial function in physically active elders with sarcopenia. *Mechanisms of ageing and development* **130,** 315-319.

While PT & Forbes LK. (2004). Electromagnetic fields in the human body due to switched transverse gradient coils in MRI. *Physics in medicine and biology* **49,** 2779-2798.

Yilmaz Y. (2012). NAFLD in the absence of metabolic syndrome: different epidemiology, pathogenetic mechanisms, risk factors for disease progression? *Seminars in liver disease* **32,** 14-21.

Young ME & Leighton B. (1998). Evidence for altered sensitivity of the nitric oxide/cGMP signalling cascade in insulin-resistant skeletal muscle. *Biochem J* **329,** 73-79.

Zhong L, Chen JJ, Chen J, Li L, Lin ZQ, Wang WJ & Xu JR. (2009). Nonalcoholic fatty liver disease: Quantitative assessment of liver fat content by computed tomography, magnetic resonance imaging and proton magnetic resonance spectroscopy. *J Dig Dis* **10,** 315-320.

CHAPTER 7

APPENDICIES

Appendix 1:

7.1 Publications arising from the thesis, with abstracts

Published manuscript

- DJ Cuthbertson, **A Irwin**, CJA Pugh, H Jones, VS Sprung, C Daousi, VL Adams, WE Bimson, F Shojaee-Moradie, AM Umpleby, JP Wilding & GJ Kemp (2014). Ectopic lipid storage in Non-Alcoholic Fatty Liver Disease is not mediated by impaired mitochondrial oxidative phosphorylation in skeletal muscle. *Clinical Science,* 127(12), 655-663.
-

Published Abstracts

- Pugh CJA, Jones H, Sprung VS, Kemp GJ, **Irwin A**, Adams VL *et al*. (2011). Exercise as a treatment strategy for non-alcoholic fatty liver disease: impact on vascular health and hepatic steatosis. *Diabetologia, 54,* S247-S247.

- Pugh CJA, Jones H, Sprung VS, Kemp GJ, **Irwin A**, Adams VL *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.*

- Pugh CJA, Jones H, Sprung VS, Kemp GJ, **Irwin A**, Adams VL *et al*. (2010). Exercise-induced reduction in liver fat is accompanied by improvements in vascular function in non-alcoholic fatty liver disease. *Diabetologia, 53,* S30.

Conference Communications

- Cuthbertson DJ, **Irwin A**, Jones H, Daosui C, Adams VL, Bimson WE, Shoajee-MoradieF,Umpleby M, Wilding JP, & Kemp GJ. Skeletal muscle mitochondrial function is not impaired in patients with nafld compared with age and bmi-matched healthy controls. 5th International Congress on Prediabetes and the Metabolic Syndrome. Vienna, Austria, April 2013.
  Poster Presentation

- Pugh CJA, Jones H, Sprung VS, Kemp GJ, **Irwin A**, Adams VL, Cable NT, Richardson P, Green DJ & Cuthbertson DJ. Exercise for fatty liver disease: Impact on vascular health and hepatic fat. European College of Sports Science. Liverpool, UK, July 2011.
  Oral presentation

- Pugh CJA, Jones H, Sprung VS, Kemp GJ, **Irwin A**, Adams VL, Cable NT, Umpleby AM, Richardson P & Cuthbertson DJ. A 16-week moderate intensity exercise intervention reduces body mass, hepatic triglyceride and

abdominal subcutaneous fat in NAFLD patients. Prediabetes and the Metabolic Syndrome. Madrid, Spain, April 2011.
Poster presentation

- Pugh CJA, Jones H, Sprung VS, Kemp GJ, **Irwin A**, Adams VL, Bimson WE, Cable NT, Green DJ, Richardson P & Cuthbertson DJ. Exercise-induced reduction in liver fat is accompanied by improvements in vascular function in non-alcoholic fatty liver disease. European Association for the Study of Diabetes. Stockholm, Sweden, September 2010.
Oral presentation

Appendix 2:

7.2 MRS methods Shimming 31P Protocol

Press view make sure adjust volume on is ticked. Move green grid box over area of interest, click options- Adjustments, click go Click Options-Adjustments-Frequency-Press go until difference =0'HZ then Click apply.

Go to 3D Shim tab at bottom of screen, click standard, then click measure , calculate then apply (bottom of 2 options) after shim has stopped

Then into interactive shim then click measure, if you can't see left hand edge of peak click A1to move peak to the right. Full Width half maximum, lowest number possible to be achieved by adjusting X, Y & Z axis using (+) or (-) icons. Press stop then apply.

Go back into Frequency then press go until number difference is =0'HZ, press apply then close.

Back to main screen click apply to start.

When Spectra available click the fid icon then right click in spectrum window then go to parameters, enter 0 to 10 for width then check for any splits. Back to exam then drag last series down to copy then in dialogue box click Manual, change parameters to 2000 and averages to 32 then click apply.

Set up *Meas. System* by CTRL Escape to access advanced user, then type meduser1 then click CTRL escape, then click run, then select *MstartMate*. Follow instructions on sheet then close. Windows Explorer. Go to 192.168.2.3 (Q), click on *Sims-meas*-data right side look for meas.asc and *meas.out*, view data Patient and browser.

7.3 Data save

Click on Spectroscopy and on main screen to highlight box in blue go to options export raw data. File C/temp, Andrew store x2 spectroscopy label as fully relaxed (FR) and 32 Averages (32avg). For 128 exercise need to follow *meas.out* and meas.asc protocol. Press Control, escape, Windows Explorer, My computer, Med system C, Temp File-Andrew.

To archive to disc, enter disc, then click transfer to DVD in patient browser, check bottom of screen accepted, open DVD to check when completed (in patient browser). To transfer to memory sticks go into My Computer click on192.168.2.3(Q)Then click on Temp, then Andrew then rename *meas* files with patient code, then copy and paste into Mitochondrial file on memory stick.

7.4 Exercise Protocol, 128 MRS Spectra

90%of MVC

- Start instructions at spectra 6, to ensure exercise starts at spectra 9.
- Begin instructions at spectra 30, to ensure exercise stops at end of spectra 32.

70% of MVC

- Start instructions at spectra 70, to ensure exercise starts at spectra 73.
- Begin instructions at spectra 86, to ensure exercise stops at end of spectra 88.

- Recovery spectra, automatically stops at spectra 128.

Appendix 3:

7.5 Extract from participant Information Sheet

Fatty Liver Disease and Blood Vessel Function

(Endothelial function and structure in patients with Non-Alcoholic Fatty Liver Disease)

Study 1: Observational Study (NAFLD Patient/**Healthy Control**).

Invitation to participate

You have been invited to participate in a research project. However, before you decide whether or not you wish to participate, it is important that you understand why you are doing it and what it will involve. Please take time to read this information sheet carefully and be sure to ask any questions you have, and if you wish, discuss it with friends, relatives or your GP. We will do our best to explain and to provide any further information you may ask for now or later. You do not have to make an immediate decision with regards to whether you wish to take part in the study.

Background to research area of interest

Non-alcoholic fatty liver disease (fatty liver disease) results in an inflamed fatty liver and, *if left untreated*, over many years can sometimes progress to cirrhosis (the replacement of liver tissue by scar tissue) and very occasionally liver failure. Fatty liver disease is thought to be largely explained by the additional weight carried in certain individuals and the associated insulin resistance.

Insulin resistance occurs when the response to the hormone insulin is blocked in different tissues in the body (skeletal muscle, fat tissue and liver). As a result of this, higher than normal amounts of insulin are required to exert its effects. Insulin resistance is a main contributor in causing type 2 diabetes.

Insulin is involved in regulating blood flow. The flow of blood to our limbs, organs and skin is altered during various circumstances (e.g. exercise) to maintain vital functions in the body. The response of the body is a marked widening of the arteries; this response increases the flow of blood to the limbs, organs and skin. A reduction in the ability of the arteries to widen is one of the contributory factors of cardiovascular disease such as high blood pressure, stroke and heart attack. We are interested in how insulin resistance alters the ability of blood vessels to widen. We will focus on the function of both the large and small blood vessels.

Why have I been chosen?
You have been invited to participate in this study as a patient that has been diagnosed with fatty liver disease, or as a healthy individual (control). The object of the study is to get a better understanding of the role of insulin resistance in causing fatty liver disease and its effect on heart function.

Do I have to take part?
Your participation in the study is entirely voluntary. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You can withdraw from the study at any point without having to give a reason and without affecting your future medical care or relationship with the medical staff looking after you.

What does the study entail?
Now that you have expressed interest in this study we have provided the following information. Following this, if you agree to participate in the study, you will be invited to attend the University Hospital Aintree (UHA) to undergo some physiological tests. The tests we propose are for research purposes and will take approximately 2 hours of your time at the University Hospital Aintree. This was previously known as Fazakerley Hospital.

The researcher will meet you at a predetermined time at the Clinical Sciences Building, at the University Hospital Aintree. This is the large white building located between the Accident and Emergency Department and the Walton Centre for Neurology and Neurosurgery (green building).

We will ask you to undergo the following tests:

(A) Blood Sample: (10 minutes)
10 mls of blood will be taken for liver function and lipid profile tests.

(B) MRI scan: (1 - 2 hours)
This will take place at the MARIARC (Magnetic Resonance and Image Analysis Research Centre) Centre on Pembroke Place immediately behind the Royal Liverpool University Hospital in the city centre at a pre-determined time (see map).
You will be asked to attend the Unit having fasted from the night before and not performed strenuous physical activity for 2 days beforehand. You will be weighed and the fat distribution in your body will be measured using magnetic resonance scanning. Your body dimensions will also be measured.
Firstly, the staff at the MARIARC will ask you to fill in a short safety screening form to make sure there are no reasons why you would not be suitable for magnetic resonance scanning. You will be asked to wear a gown (changing rooms are provided) and remove items which are affected by the magnetic field (e.g. hearing aids, mobile phones, keys, coins, pens, credit cards (secure lockers are provided).

While inside the scanner, you will be asked to wear earplugs to protect your hearing from the noise of the scanner. This visit involves you having a scan of your whole body to determine how much fat is present and specifically how much is present within your liver and muscle. During your scans you will be asked to carry out some leg exercises, this will allow us to assess your leg muscle and its recovery following a period of rest.

(C) Echocardiography Examination (ECHO) *(1 hour)*
Heart size, structure and function will be assessed using echocardiography. This will involve an ultrasound scan (on the chest) and the attachment of ECG electrodes to measure heart rate.

(D) Fitness Test (30 minutes)
The researcher will assess your fitness level. You will be asked to bring with you a pair of trainers and suitable clothing to exercise on a treadmill. Briefly, you will be required to walk on a motorised treadmill where the speed and treadmill increment will be increased every 2 min until you choose to end the test. This will measure the amount of work you perform at increasing grades of difficulty. Oxygen uptake will be measured breath−by−breath using an online gas analyser. This procedure will require you to wear a facemask connected to the gas analyser. Heart rate will also be measured so you will be required to wear a chest strap, which will transmit heart rate data to a monitor which is similar to a watch worn around the wrist.

What are the possible disadvantages of taking part?
Magnetic Resonance Scanning. The scan is very safe involving no x-ray exposure and without side effects. We will ask a radiologist (a specialist doctor in reading scan images) to review the scan images to ensure there are no abnormalities. Occasionally we may find abnormalities within the abdomen. Almost always these are of no medical or clinical significance but sometimes will require further inspection or evaluation. If we find any such abnormalities we will write to your GP and inform them of the abnormalities and ask them to refer to an appropriate specialty for further investigation.

What are the benefits of taking part?
Although by taking part in this study you will gain no direct benefit, the information gained from these measurements will help us to gain a greater understanding of fatty liver disease, its effect on the heart, and assist us to design more effective treatment strategies in the future for all patients with this condition. Additionally, if we do identify a health problem that requires further medical attention, for example, high blood pressure, we will explain this to you, and inform your GP for further management.

What expenses will I receive?
You will receive all your travelling expenses if you participate.

You will be given a copy of the information sheet and a signed consent form to keep.

Thank you for reading this information sheet and considering taking part in the study.

Appendix 4:

7.6 Participant consent form

UNIVERSITY OF LIVERPOOL

Patient Identification Number for this trial:

Title of Project: **Exercise and fatty liver disease**
(Endothelial Function and Structure in Patients with Non-Alcoholic Fatty Liver Disease)

Name of Researcher: Dr Daniel Cuthbertson

Please initial the boxes below

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study and that any results of scanning are reported to my GP for he/she to action as appropriate. ☐

5. I agree to take part in the above study.

Name of Patient	Date	Signature
Name of Researcher	Date	Signature

Appendix 5:

7.7 Ethical approval

Liverpool (Adult) Research Ethics Committee
Bishop Goss Complex
Victoria Building
Rose Place
Liverpool
L3 3AN

Telephone: 0151 330 2077
Facsimile: 0151 330 2075

23 March 2009

Dr Daniel Cuthbertson
Clinical Senior Lecturer and Honorary Consultant Physician
University of Liverpool
Clinical Sciences Centre,
University Hospital Aintree
Liverpool
L9 7AL

Dear Dr Cuthbertson

Full title of study:	**Endothelial function and structure in patients with Non-Alcoholic Fatty Liver Disease (NAFLD).**
REC reference number:	**09/H1005/7**

Thank you for your letter of 28 February 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to complete Part C of the application form or to inform Local Research Ethics Committees (LRECs) about the research. The favourable opinion for the study applies to all sites involved in the research

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Response to Request for Further Information		
	28 February 2009	
C.V. for Supervisor		
	30 September 2008	
Participant Consent Form		
2	28 February 2009	
Participant Information Sheets for study 1 and study 2		
2 -	28 February 2009	
Advertisement		
2	28 February 2009	
Questionnaire: Validated		
Statistician Comments		
1	17 December 2008	
Protocol		
2	28 February	

	2009	
Investigator CV		
Application		
2.0	09 January 2009	

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review –guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1005/7	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Professor S Vinjamuri
Chair

Email: Ronald.Wall@liverpoolpct.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mr Neil Whalley, R & D< UHA NHS Trust*

Appendix 6:


Cox & Forkum
©2007
WHAT DO YOU MEAN I HAVE TO PEDAL?
www.CoxAndForkum.com

...